CLINICAL ASPECTS OF IMMUNOLOGY

EDITED BY

P.G.H.GELL

AND

R.R.A.COOMBS

SECOND EDITION

CLINICAL ASPECTS OF IMMUNOLOGY

CLINICAL ASPECTS OF IMMUNOLOGY

EDITED BY

P. G. H. GELL
M.B., M.R.C.S.
Professor of Experimental Pathology
University of Birmingham

AND

R. R. A. COOMBS
Ph.D., Sc.D., M.R.C.V.S., F.R.S.
Quick Professor of Biology
Immunology Division
Department of Pathology
University of Cambridge

SECOND EDITION

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD AND EDINBURGH

SBN 632 01800 3

FIRST PUBLISHED 1963
REVISED REPRINT 1964
SECOND EDITION 1968

SPANISH EDITION 1965
ASIAN EDITION 1967

Printed in Great Britain by
ALDEN & MOWBRAY LTD
AT THE ALDEN PRESS, OXFORD
and bound at
KEMP HALL BINDERY

CONTENTS

SECTION IV
THE ALLERGIC STATE AS RESPONSIBLE FOR CLINICAL HYPERSENSITIVITY AND DISEASE

A. ALLERGIC DISEASES WITH GENERAL MANIFESTATIONS

LIST OF CONTRIBUTORS

J. F. ACKROYD D.SC. M.B. M.R.C.P.
Senior Lecturer in Medicine, St Mary's Hospital Medical School, London W2

P. ALEXANDER PH.D. D.I.C. D.SC.
Professor of Radiobiology, Chester Beatty Research Institute, Royal Cancer Hospital, London SW3

JOHN R. ANDERSON B.SC. M.D.(ST AND.) F.R.C.P.G. M.R.C.P. F.C.PATH.
Professor of Pathology, The University and Western Infirmary, Glasgow

IRENE BATTY B.SC.
The Wellcome Research Laboratories, Langley Park, Beckenham, Kent

W. E. BROCKLEHURST B.PHARM. B.SC. PH.D.
Department of Pharmacology, Lilly Research Centre Limited, Erl Wood Manor, Windlesham, Surrey

C. D. CALNAN M.A. M.B. B.CHIR. F.R.C.P.
Professor of Dermatology, St John's Hospital for Diseases of the Skin, Leicester Square, London WC2

I. CHANARIN M.D. D.C.P. M.C.PATH
Reader in Haematology, St Mary's Hospital Medical School, London W2

R. R. A. COOMBS PH.D. SC.D. M.R.C.V.S. F.R.S.
Quick Professor of Biology, Immunology Division, Department of Pathology, University of Cambridge

J. V. DACIE M.D. F.R.C.P.
Professor of Haematology, Royal Postgraduate Medical School of London, Ducane Road, London W12

JAMES G. DEVLIN M.D. MSC.. M.R.C.P.I.
Visiting Physician (Endocrinology), St Lawrence's Hospital; Consultant, Endocrine Immunology, St Vincent's Hospital; Wellcome Trust Research Fellow, Department of Medicine, University College, Dublin

BARBARA E. DODD M.SC. PH.D.
Senior Lecturer in Forensic Medicine, London Hospital Medical College, London E1

DEBORAH DONIACH M.D. B.S. M.R.C.S. L.R.C.P.
Senior Lecturer, Middlesex Hospital Medical School, London W1

D. G. EVANS PH.D. D.SC. F.C.PATH. F.R.S.
Professor of Bacteriology and Immunology, London School of Hygiene and Tropical Medicine, London WC1

G. HAMILTON FAIRLEY D.M. M.R.C.P.
Chester Beatty Research Institute, Clifton Avenue, Belmont, Sutton, Surrey

E. J. FIELD M.D. M.S. PH.D. M.R.C.P.
Professor of Experimental Neuropathology, University of Newcastle upon Tyne; Honorary Director, M.R.C. Demyelinating Diseases Research Unit

A. W. FRANKLAND M.A. D.M.
Director, Allergy Department, Wright-Fleming Institute, St Mary's Hospital, London W2

J. D. FULTON M.A. PH.D. M.B. CH.B. D.T.M.
Supported by grant from Medical Research Council, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1

P. G. H. GELL M.B. M.R.C.S.
Professor of Experimental Pathology, University of Birmingham, Birmingham 15

L. E. GLYNN B.SC. M.D. F.R.C.P.
Director, Department of Pathology, Canadian Red Cross Memorial Hospital, Taplow, Bucks; Member of Scientific Staff, Medical Research Council; Honorary Consultant Pathologist, Metropolitan Regional Hospitals Board, N.W

K. L. G. GOLDSMITH M.B. B.S. PH.D. M.C.PATH.
Director, M.R.C. Blood Group Reference Laboratory, Gatliff Road, London SW1

C. N. HALES M.A. M.B. B.CHIR. PH.D.
Lecturer in Biochemistry, Department of Biochemistry, Fellow of Downing College, University of Cambridge

J. HARDWICKE B.SC. M.D. M.C.PATH.
Reader in Experimental Pathology, Birmingham University, Birmingham 15

E.J.HOLBOROW M.A. M.D.
Honorary Consultant Bacteriologist, N.W. Metropolitan Regional Hospital Board; Member of Scientific Staff, M.R.C. Rheumatism Research Unit, Canadian Red Cross Memorial Hospital, Taplow, Bucks.

JAMES HOUSLEY M.B. CH.B. M.R.C.P.
Medical Research Council Clinical Research Fellow, Department of Experimental Pathology, University of Birmingham, Birmingham 15; Honorary Senior Registrar in Immunology, United Birmingham Hospitals

C.KAPLAN M.SC. M.B. CH.D. DIP. BACT.
Virus Vaccine Department, Lister Institute of Preventive Medicine, Elstree, Herts.

R.KNOX M.A. M.D. F.R.C.P. F.C.PATH.
Professor of Bacteriology, Guy's Hospital Medical School, London SE1

P.J.LACHMANN PH.D. M.B. M.R.C.P.
Assistant Director of Research. Immunology Division, Department of Pathology, University of Cambridge; Fellow of Christ's College

J.L.LONGBOTTOM PH.D.
Senior Lecturer in Immunochemistry, Department of Clinical Immunology, Institute of Diseases of the Chest, University of London, Brompton, London SW3

F.O.MacCALLUM B.SC. M.A. M.D. M.R.C.P. F.C.PATH
Consultant Virologist, United Oxford Hospitals; University Lecturer in Virology, Radcliffe Infirmary, Oxford

P.E.C.MANSON-BAHR M.D. F.R.C.P. D.T.M. & H.
Professor of Tropical Medicine, School of Tropical Medicine and Public Health, Tulane University, New Orleans, USA

J.F.A.P.MILLER B.SC(MED) M.B. B.S. PH.D. D.SC.
Head of Experimental Pathology Unit, Walter and Eliza Hall Institute of Medical Research, Melbourne, 3050, Australia

I.G.MURRAY M.B. CH.B. M.C.PATH. D.T.M. & H.
Director of Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1

C.L.OAKLEY D.SC. M.D. F.C.PATH. F.R.S.
Brotherton Professor of Bacteriology, Medical School, University of Leeds

W.E.PARISH PH.D. M.A. B.V.SC. M.R.C.V.S. M.C.PATH.
Head of Serum Department, Lister Institute of Preventive Medicine, Elstree, Hertfordshire

J.PEPYS M.B. CH.B. M.R.C.P. F.R.C.P.E.
Professor in Clinical Immunology; Director, Department of Clinical Immunology, Institute of Diseases of the Chest, University of London, Brompton, London SW3

F.T.PERKINS M.SC. PH.D.
Director, Division of Immunological Products Control, M.R.C. Laboratories, Hampstead, London NW3

I.M.ROITT M.A. D.PHIL.
Professor of Immunology, Middlesex Hospital Medical School, London W1

ARTHUR ROOK M.A. M.D. F.R.C.P.
Consultant Dermatologist, Addenbrooke's Hospital, Cambridge

D.S.ROWE B.SC. M.B. CH.B. M.D.
Director, WHO International Reference Centre for Immunoglobulins, 21 rue de Bugnon, 1000 Lausanne, Switzerland

Ph. RÜMKE M.D.
The Netherlands Cancer Institute, Sarphati Straat 108, Amsterdam

ARTHUR M.SILVERSTEIN PH.D.
Professor of Ophthalmic Immunology, The Wilmer Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland, U.S.A.

H.SMITH PH.D. D.SC.
Professor of Microbiology, University of Birmingham, Birmingham 15

J.MORRISON SMITH M.D. F.R.C.P.ED.
Consultant Physician, Birmingham Chest Clinic; Physician, Asthma Clinic, Birmingham School of Medicine, Birmingham 15.

J.F.SOOTHILL M.A. M.D. M.R.C.P. F.C.PATH.
Head, Department of Immunology, Institute of Child Health, London WC1

E.J.L.SOULSBY M.A. PH.D. M.R.C.V.S.
Professor of Parasitology, Department of Pathobiology, University of Pennsylvania, U.S.A.

M. STERNE D.V.SC. DIP. BACT. M.R.C.V.S.
Wellcome Research Laboratories, Langley Court, Beckenham, Kent

S. C. TRUELOVE M.A. M.D. F.R.C.P.
Nuffield Department of Clinical Medicine, University of Oxford

W. WEINER M.D.
Late Director, Regional Blood Transfusion Service, 15 Ampton Road, Birmingham 15

B. WEITZ D.SC. M.R.C.V.S.
Director, National Institute for Research in Dairying, University of Reading, formerly of the Lister Institute of Preventive Medicine, Elstree, Herts

R. G. WHITE M.A. D.M. B.CH.
Professor of Bacteriology, University of Glasgow and Western Infirmary, Glasgow W1

M. F. A. WOODRUFF D.SC. M.D. M.S. F.R.C.S. F.R.S.
Professor of Surgical Science, University of Edinburgh, Surgeon, Edinburgh Royal Infirmary, University of Edinburgh

SHEILA M. WORLLEDGE M.D. B.S.
Lecturer in Blood Transfusion, Royal Postgraduate Medical School of London, Ducane Road, London W12

G. PAYLING WRIGHT D.M. F.R.C.P.
Late Professor of Pathology, University of London, Guy's Hospital Medical School, London SE1

R. WRIGHT D.PHIL. M.D. M.R.C.P.
Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford

PREFACE

It is five years since the first edition of *Clinical Aspects of Immunology* appeared and it is sad that we no longer have with us Carl Prausnitz, who cracked the bottle of champagne over our bows as the book left the press. He died a few days before the book appeared. We have also been greatly saddened by the deaths of three of our contributors: George Payling Wright, John Squire and W. Weiner. Payling Wright's chapter (16) has been reprinted from the first edition: Squire's (19) has been revised and brought up to date by his collaborator John Soothill: while the fully revised Chapter 28, which includes so much important recent work, was the last piece of writing which Dr Weiner completed before his death. The other chapters from previous contributors have without exception been either entirely re-written or very extensively revised. The subject of Bruce Cruickshank's chapter has been divided up into three new ones: his manifold responsibilities in the Chair of Pathology at Salisbury, Rhodesia, unfortunately made it impossible for him to contribute to this edition.

In the period since the first edition there has been no evidence of a slackening of impetus or loss of vitality or significance of immunology in either clinical medicine or general biological research. This is reflected to some extent in the need to introduce fourteen new chapters into the present edition: there could well have been more.

A very comprehensive chapter (3) on immunodiagnostic aids in fungal diseases has been contributed by Longbottom, Murray and Pepys; much of this information is difficult to find elsewhere. The immuno-assay of hormones has now reached the importance of requiring a chapter (10) to itself; Hales has made many contributions to this field and it is certain that these methods will become increasingly refined and with wider application.

The immunoglobulins (Chapter 13) also have now graduated to a chapter for themselves and have been dealt with by Rowe, an expert from a leading school in this field. The difficult task of writing on the biology of the allergic response (Chapter 11) has been undertaken by Miller.

Another chapter (18) has been introduced on the allergic response in malignant disease written as a collaborative effort by a leading experimental laboratory worker, Alexander, and a physician, Hamilton-Fairley, who deals with patients afflicted with these diseases. Such a combination ensures a clear and practical

exposition of the present position. There is no doubt now about the existence of tumour specific antigens and of there being an allergic response mounted against them; the problem is how best to exploit it so that it may be effective in destroying cancerous cells *in situ*.

Hormone resistance (Chapter 25) as met in clinical medicine, has been dealt with by Devlin, and a new chapter (30) on rheumatic fever written by Glynn.

Many of the auto-allergic diseases have been dealt with in new chapters devoted to particular body organs: e.g. the kidney (Chapter 34) by Hardwicke: muscle (Chapter 37) by Housley: stomach (Chapter 38) by Chanarin: ileum and colon (Chapter 39) by Truelove and Wright: brain and the nervous system (Chapter 40) by Field: liver, pancreas, adrenal and prostrate (Chapter 41) by Anderson: and the testes (Chapter 42) by Rümke. In addition Worlledge now collaborates with Dacie (Chapter 29): Morrison Smith with Gell (Chapter 24): Kaplan with Perkins (Chapter 45) and Weitz with Manson-Bahr (Chapter 46). All these contributors are acknowledged specialists in their fields.

A general introductory chapter (21) on auto-allergy, giving overall concepts for its genesis, has been written by Lachmann. Another general chapter (15) formulating the interplay of non-specific factors and specific allergic reactants in mechanisms of immunity is presented by Coombs and Smith. Finally, to enforce our intention that the book should be equally useful to those dealing with problems of veterinary medicine a chapter (48) by Sterne and Batty dealing with immuno-prophylaxis and -therapy in veterinary medicine has also been added. Many of the other chapters are equally relevant to problems of diseases of domestic animals.

Besides mentioning these new chapters and thanking these new contributors, we must make abundantly clear the debt of gratitude we owe to old friends—those authors who have contributed to both editions and who have been ready to undertake the burden of re-writing or revising their chapters for this second edition.

For this edition, the indexing was undertaken by Harry Amos, Robin Hastie and Ian Tizard with the assistance of Jennifer Biggar, who has also done much of the proof correcting, and we are most grateful to them; also to B.W.Gurner for many of the old and some of the new illustrative diagrams.

Once again we are delighted to say that there have been no clouds to overshadow the happy relationship with our publishers.

P.G.H.G.

1968 R.R.A.C.

FOREWORD TO FIRST EDITION

It is a great privilege to write a foreword to this remarkable book, and I am very grateful to Professor Gell and Dr Coombs for entrusting me with this task. The past decades have been so rich in valuable research and new ideas on immunity and allergy that there is a great and growing need for a comprehensive and authoritative survey of present-day knowledge of this vast and complex field. How spectacular the advance in this branch of medical science has been might perhaps be best envisaged if we imagine the plight of an experienced bacteriologist of the 'nineties' of last century, who had followed and participated in the fundamental work of the Pasteur–Koch–Ehrlich era, but at the turn of the century was overcome by profound sleep, only to awaken—a modern Rip van Winkle—in these days. He had been familiar with the bacterial agents of disease, with vaccine prophylaxis, antitoxins, bacteriolysins, cytolysins, agglutinins, precipitins, and, in the tuberculin test, with a single facet of the yet uncharted domain of allergy. But that was his limit. What faced him in his new life must be overwhelming.

The electron microscope had revealed within the formless bacterial bodies nuclear matter and other structural details. The viruses had been discovered as important causes of infection, they had been made visible and the study of their life cycle had added fresh mysteries. The antibodies, which in the past had been regarded as somewhat enigmatic substances demonstrable only by their biological effects, were now being studied by a very different group of scientists using all the newly discovered methods of physical and biological chemistry.

The ultracentrifuge, chromatography, gel diffusion, electrophoresis, immuno-electrophoresis, the fluorescent antibody technique and the isotopes had given a deep insight into the constitution of the antigens and antibodies, on the role of the gamma-globulins and the cells producing them. New theories had been evolved on the production of the antibodies. Almost one could hope that in not too distant a future analysis of the antibodies might lead to their artificial synthesis. New domains had been opened up by the discoveries in the fields of allergy, immune tolerance and auto-immunity, or, as it is better named, auto-allergy.

This development has not always been easy to follow in all its ramifications by scientists who had lived with, followed up and taken part in investigating these manifold and complex problems, so diverse and yet so closely inter-locked.

To collect this knowledge and to present it clearly and critically, in a form intelligible to the beginner, informative and helpful to the experienced investigator, is the object of this book. The editors, Professor Gell and Dr Coombs, together with their several contributors whose names are well known for their important researches on these subjects, are to be congratulated on the fruit of their labours. They have earned a great debt of gratitude from all who work in this field.

What emerges is a comprehensive and detailed picture of the close connexion between Immunity and Allergy. The first Section of the book deals with the methods of diagnosis and includes a chapter on blood transfusion serology. In the second Section the physiological systems operating in the allergic state are discussed. This leads to the third Section devoted to the relations between the allergic state and immunity, and thence to the important fourth Section on the allergic state as responsible for hypersensitivity and clinical disease. The opening chapter by Dr Coombs and Professor Gell is a masterly presentation of the different forms and aspects of the allergic state and thus forms the foundation for the subsequent chapters. In this Section much work of the highest importance is discussed on that most mysterious group of diseases due to auto-allergy. The fifth Section deals with the practical application of present knowledge to prophylaxis.

It may confidently be hoped that this excellent book will prove of great and lasting value to the research worker and the clinician, to the student and the experienced investigator. Its clear formulation and critical presentation will be very helpful to the reader. By taking every opportunity of pointing out the many unsolved problems it will stimulate research in this exciting field of medical study.

May it prosper, succeed and find the many friends it so well deserves!

CARL PRAUSNITZ

1963

INTRODUCTORY CHAPTER

P.G.H. Gell & R.R.A. Coombs

We use this introductory chapter, as in the first edition of this book, to refer to some developing but still incompletely defined aspects of Immunology. Most of the lines of work discussed then have now been elaborated into chapters of their own; for this and for other reasons this second edition is longer by some 60% than the first and is now quite unsuitable for reading in the bath. Reluctant though we be to add to the Information Explosion, this has been forced upon us through our aim of covering the whole subject in its clinical aspects and of giving some prospective indication at least of its future lines of growth. Immunology has certainly been booming and is now an O.K. subject second only to the study of The Code. Nowadays one is hardly surprised to see advertisements for Professors of Byzantine History 'with an interest in Molecular Biology and/or Immunology'; while even Scoutmasters or Town Clerks are thought ill-equipped for their jobs without the capacity to discuss those magic Triplets or to deprecate the prevalence of Forbidden Clones. In-groups spawn Inner Groups, while the Out-Groups howl like wolves in the wilderness: pity the poor Ishmael who still confuses Antibody with Anti-matter.

Terminology

This adolescent science of Immunology, however—like a school-boy burdened with a surname too readily adaptable to humorous or obscene variants—remains shackled to a terminology which though seldom actually improper is apt to

confuse rather than define thought. Though the editors have no ambition—

> . . . to bear through snow and ice
> A banner with this strange device
> 'Allergology!'

—yet we feel all the more convinced of the rationality of and the need for the terminology we advocated in the first edition, in spite of the reluctance of many distinguished workers to adopt it. Some comments on this are made in Chapter 20, but we may add here a little glossary of some usages we like to hate, and our modifications of them.

Immunology: too late to change this word: we shall have to learn to think straight in spite of it.

Immunized: MEANING: 'made immune' 'protected against'. USAGE: subjected to any antigenic stimulus except one producing tolerance: bad and confusing usage but probably too late to change it.

We should like to emphasize the operational distinction between the *Allergic state* and the *Allergic reaction.* The allergic reaction is that of, e.g. a patient whose eyes and nose are swollen and dripping in the pollen season: but he is still in an allergic state when he is symptomless at Christmas. (The popular usage is correct here: someone may claim to be allergic to sports cars or girls with red hair even when these objects are not around.) Similarly an animal in 'latent immunity' between a primary and a secondary stimulus is in an allergic state, though producing no detectable antibody. So we can define—*The Allergic State:* a state of specifically altered potential reactivity to a particular chemically definable substance, and—*An allergen* (or antigen): a substance capable of provoking the allergic state '(allergenic').

An immunogen: an allergen when used to elicit protection against something (i.e. a purely operational word).

The use of the word *antigen* for substances used *in vitro* to react with antibodies remains quite clear and precise.

Auto-immune: MEANING: that the subject is satisfactorily protected against his own cells or constituent macromolecules: USAGE: that the subject is not protected from but is indeed (probably) 'attacked' by his own cells or antibody products. BAD USAGE: discard.

Hypersensitive: a loose but useful word meaning that the subject is more sensitive than one would expect: a Greek/Latin hybrid, supersensitive is correct (as employed by the late Carl Prausnitz: Appendix A, B) but seldom used: *Not* equivalent to 'allergic' in our usage, which includes *hypo*sensitivity also.

Auto-allergic: MEANING: the subject's response to 'self' constituents is altered. USAGE: generally that the subject's cells react against, or produce antibodies which react against 'self'-components.

Allergy, allergist, allergic reaction: MEANINGS: defined by von Pirquet who introduced the words, see Chapter 20 and Appendix A. USAGE: so varied and

inconsistent at present that the words actively hinder understanding, but broadly equivalent to 'hypersensitivity', etc. BAD USAGE: we recommend a return to the use of von Pirquet as meaning broadly 'altered in reactivity'. *Allergized:* RECOMMENDED USAGE: specifically changed in some way; includes 'antibody-producing', 'hypersensitive', 'tolerant', 'immune', etc.

We do not wish to be fanatic on this question nor to proselytize, but we feel fairly convinced ourselves that in time the rationality of the terminology we have tried to introduce in this book will speak for itself, for its consistency makes these complicated interactions more comprehensible to physicians and students. We have asked those contributing chapters to this book to use it unless they felt strongly against it, as was the case with the contributors of Chapter 11 and of Chapter 35.

Teaching of Immunology in the Medical Curriculum

An event of considerable significance in the last five years has been the concern of the World Health Organization, not only for research in Immunology and early exploitation of this in disease prevention, but also in the teaching of the subject in the medical curriculum. This concern did not arise primarily in connection with the under-developed countries alone, but because the present position as regards teaching seemed unsatisfactory in many universities and medical schools throughout the world, especially in the light of the rapidly changing relationship of Immunology to medicine.

The recommendation of an expert committee (World Health Organization, 1967) was for an obligatory minimal basic course of about ten lectures with practical classes, towards the end of the pre-clinical studies. This course should be the responsibility of a group of immunologists constituting a distinct unit or sub-department within the framework of the medical school or university. The importance of continuing the teaching of immunology into the clinical years was stressed and ways in which this might be done were suggested. More advanced scientific teaching of the subject in elective undergraduate courses and as postgraduate courses was considered essential as an introduction for postgraduate research.

Such teaching is already available in some universities and medical schools but is still deficient in all too many. The report could be very helpful to Deans of medical schools having to allocate teaching periods.

Allergic Reactions as a Factor in Microbial Pathogenicity

We had hoped to have had another chapter in Section IV of this edition on 'Allergic reactions as a factor in microbial pathogenicity', but a proper exposition was obviously going to take more time than we could find, so we have had to

leave such a chapter to a future edition. Nevertheless, we feel we should draw the attention of those of our readers who bother to read introductions to this important aspect of disease.

When considering microbial pathogenicity, it is only too easy to confine one's attention to toxins, aggressins and other virulence factors of the micro-organism. In the majority of instances the individual allergic responses mounted against these substances and their consequent reactions do indeed act, in a true sense, as reactions of immunity and produce immunity. However, similar allergic responses with their consequent reactions may be mounted against antigenic products of infecting micro-organisms which otherwise are quite non-toxic and only become pathogenic by virtue of initiating allergic reactions resulting in tissue damage by mechanisms Types I–IV (see Chapter 20). Tuberculosis, where no definite toxic principle of the tubercle bacillus is known, has long been adduced as an example of this, but the pathogenesis of many other diseases may be considered in this context.

Thus we have the situation that a micro-organism may be non-pathogenic in an animal which has *not* mounted an allergic reaction to particular microbial antigens, or which is unable to do so (e.g. a tolerant animal, or an animal in which the allergic response has been damped down by X-irradiation, or an animal raised germ-free which has not been previously sensitized by cross-reacting microbial antigens) but is pathogenic—almost 'passively' so—in the allergized host. It is not our intention to treat this thesis in depth here, but some examples may be sufficient to stimulate further thought along these lines. Although intimate specialist knowledge of all forms of these diseases is required for full understanding, it seems to us that the situation is not so complicated if the allergic mechanisms underlying immunity and clinical hypersensitivity in such diseases are viewed as set out in this book.

Matsumura (1963) having assured himself that the symptomatology and pathogenesis of gastro-duodenal ascariasis in man depended on an allergic reaction and not on a so-called ascaris toxin, paid attention to the pathogenic mechanisms of bacilliary dysentery due to *Sh. flexneri* in man and laboratory animals (Matsumura 1962). It had been known that *Sh. flexneri* was not pathogenic for normal rabbits or guinea-pigs. However, if these animals were first sensitized, or allergized, by colonic infusion with *E. coli* 6–13 which has a common antigen with *Sh. flexneri*, then they became susceptible on oral ingestion of *Sh. flexneri* to an infection with the typical symptomatology of human dysentery. Matsumura considers that the allergic reactions play an essential role in the presentation of the human disease.

The similarity between the clinical signs of the acute form of fowl typhoid and anaphylactic shock in birds (e.g. respiratory embarrassment, cyanosis of the wattle and diarrhoea) drew the attention of Buxton and his colleagues (Buxton & Allan 1963; Buxton & Davies, 1963) to the role of allergic reactions

in the pathogenesis and symptomatology of *Salm. gallinarum* infection in birds. Rather than resulting from toxaemia it seemed that the manifestations of the disease could be explained to a large extent by allergic reactions. These workers present evidence that the haemolytic anaemia results from adsorption of bacterial polysaccharide on to the red cells, rendering them reactive with polysaccharide antibody (see Chapter 20); while white cells could be shown to be passively sensitized with antibody rendering them susceptible to an allergic reaction (possibly Type I) with polysaccharide antigen. Similar factors are suggested as being operative in other salmonella infections including *Salm. typhi* infections in man.

Likewise, Thomlinson & Buxton (1963) have discussed and produced suggestive evidence that oedema disease and gastroenteritis in young pigs associated with *E. coli* infection are again not toxaemias. They suggest that the clinical signs and lesions result from a form of anaphylactic reaction set off by rapid absorption of large amounts of polysaccharide antigen produced by rapid growth of *E. coli* in the gut of previously sensitized animals, a mechanism analogous to a 'Herxheimer reaction'.

It should also be remembered that in lobar pneumonia in man due to *Str. pneumoniae* no toxic factor has so far been isolated; many of the observed facts and experimental findings suggest that the pneumonic effusion and lesions can best be explained in terms of allergic reactions involving the microbial products. Heffron (1939) gathers together much of the experimental work on this thesis. In this context it is pertinent to bear in mind the evidence and arguments which have been put forward suggesting that many of the activities of endotoxin are attributable to delayed type cellular allergic reactions rather than to any direct toxicity of the substance itself. For a discussion, see Stetson (1959) and Schaedler & Dubos (1961).

Virus diseases also need to be considered from this aspect. Infection with the virus of lymphocytic choriomeningitis produces minimal lesions in mice rendered tolerant by intra-uterine infection or with their allergic response damped down by other means, whilst infection in allergically (immunologically) competent animals results in brain damage and typical disease (Hotchin, 1962). Webb & Gordon Smith (1966) from their own observations on encephalitis-producing neurotropic viruses also consider that an antibody reaction against viral antigens may be responsible for clinical encephalitis in man. Experimental evidence in mice indicates that injury due to the virus in X-irradiated animals is slight, the virus being within the neurones, while if antibody is also present at a particular time after the virus has disseminated, then the brain lesions are more inflammatory with the oedema and infiltration leading to pressure and neurological manifestations.

A final example is taken from protozoal diseases. The pathogenetic mechanisms in trypanosomiasis have for long been far from clear, yet with the vast

amounts of protozoal antigen detectable in the circulation as exo-antigen (Weitz, 1960) and the high levels of circulating antibody directed against the sequence of variant antigens, the state is set for some serum-sickness-like syndrome. In investigating the pathogenesis of *T. Brucei* infection in the rabbit, Goodwin & Hook (1968) describe the lesions as chronic angeitis and compare these lesions, their physiopharmacology and the clinical signs they produce, with the chronic anaphylactic cachexia described in rabbits by Arthus in 1903.

These are perhaps sufficient examples to illustrate the important role the allergic reactions may play in the pathogenesis of infective diseases and in their clinical presentation. Too often the lesions and disease picture are ascribed *in toto* to toxic properties of the micro-organisms.

Immunosuppression

There are a few situations in which it is desirable to alter allergic reactivity from an active to a tolerant state. These are, firstly, situations where such responses are damaging, and secondly those where normally functioning allergic responses interfere with organ replacement therapy. In such situations before interference is attempted one would like to have answers to two questions—first, is the allergic response we are suppressing really part of the causation of the disease and not a result of it? and secondly, what antigen or antigens are crucially involved?

In damaging allergic responses the answer to the first is often very difficult while that to the second is either known or readily amenable to experiment: in homografting, the reverse holds—we can be reasonably certain that part or all of graft rejection is immunologically determined, but the nature of the antigens involved is highly complex.

There are a number of ways in which such deviations and suppressions of allergic reactivity can be carried out, not all of which have been applied yet to human disease or therapy.

(a) *Feed-back inhibition:* Here we make use of the principle that the presence of small amounts of IgG antibody tends to depress the primary response to the relevant antigen. This is probably the mechanism by which Rhesus sensitization (iso-allergic) can be suppressed by the administration of very small amounts of anti-Rh antibody (cf. Chapter 28). Whether the antibody itself or an antigen-antibody complex is the active agent is still being investigated. Such a mechanism might be exploited in other situations, but has the disadvantage that it applies mainly to the primary response, and therefore can only be used prophylactically.

(b) *'Immune' deviation:* Another mechanism worth considering is that of 'immune' deviation. There are methods being developed by which either antibody production or delayed sensitivity can be 'favoured' (cf. Asherson 1967). If one type of reactivity is crucial in causing damage, one might develop means to favour the other, which might indeed be protective.

(c) *Specific tolerance:* The third mechanism is the production of specific tolerance. In auto-allergy, it appears to be just this protective mechanism of tolerance which has broken down, so on the face of it one would not expect much hope here: but the demonstration by Mitchison (see Dresser & Mitchison, 1968) of two zones of tolerance (in the adult mouse), high-dose and low dose (of which the latter is probably the 'natural' mechanism) might give a loophole. This is even more relevant to the homograft situation, where the production of specific tolerance to the genetic products of the proposed donor is almost certainly the ultimate answer. But for this we shall need much more study of the actual antigens involved in homograft rejection in man, and of the mechanism of rejection.

(d) *Damage to the lympho-reticular system:* The methods which are at present practical and in use for immunosuppression are essentially 'blunderbuss': namely the production of gross damage to the response of the whole lymphoid system by radiation, anti-metabolites or steroids on the one hand, and by anti-lymphocyte antisera (ALS) on the other. The sooner we can avoid the use of such dangerous methods as radiations and anti-mitotic agents the happier everyone will be: but ALS is a rather different matter. Here we may in time have some control of the particular population of lymphocytes or particular allergic response which is affected: at present it is becoming clear that the effects of ALS are multiple, and variable from situation to situation, and indeed from serum to serum. The sorting out of the antigens and effects involved is going on actively, and one may hope that by the next edition of this book, a chapter of practical information and advice to clinicians on the whole subject of immunosuppression may be appropriate.

In the meantime, comprehensive reviews of immunosuppressive drugs are given by Schwartz (1965) and Gabrielsen & Good (1967) and the subject was discussed in a recent Symposium (1967). For current work and ideas on ALS the reader is referred to the *Ciba Foundation Study Group No. 29* (Wolstenholme and O'Connor, Eds., 1967) and for clinical management, Calne (1967), among others.

Conclusion

Of the two main branches of academic immunology, Immunochemistry and Immunobiology, the 'growing-point' of the first is the genetics and molecular biology of antibody synthesis, and of the second, the analysis of all aspects—ontogenetic, phylogenetic and individual—of the allergic response of cells. In considering this more generally as a biological process, however, with special reference to what Burnet has called 'the Integrity of the Body', ideas which have been in the air for some years are becoming increasingly important and apposite. Widening knowledge of auto-allergy has emphasized the two-edged nature of the allergic response, and has made possible for the first time

a 'holistic' approach to disease as a balance between helpful and damaging effects, not all the damaging ones being due to extrinsic agents. It seems indeed that vertebrate evolution has encapsulated a defence mechanism which is still far from ideal; and makes more and more attractive the theory that, in long-lived animals subjected to a constant level of mutagenic irradiation from outside and gene-replication errors from within, the overriding demand on defence is protection against harmful mutants. In addition such defences are possibly needed against invasion of the genome itself by episomal agents derived from micro-organisms. The promiscuity of the nucleus, if one may use the metaphor, once genetic material has succeeded in entering the cell, is vividly illustrated by the cell-hybridization experiments of Harris and his collaborators (Harris *et al.* 1966); and it is understandable that such nymphomaniac cells and their bastard offspring should have to be sternly excised from the body politic, 'never to darken our doors again'.

REFERENCES

ARTHUS M. (1903) Injections repetées de serum de cheval chez le lapin. *C.r. Seane Soc. Biol.* **55,** 817.

ASHERSON G.L. (1967) Antigen-modulated suppression of delayed hypersensitivity. *Brit. med. Bull.* **23,** 24.

BUXTON A. & ALLAN D. (1963) Studies on immunity and pathogenesis of salmonellosis. I. *Immunology* **6,** 520.

BUXTON A. & DAVIES J.M. (1963) Studies on immunity and pathogenesis of salmonellosis. II. *Immunology* **6,** 530.

CALNE R.S. (1967) *Renal Transplantation*, 2nd ed. Edinburgh: Edward Arnold.

CIBA FOUNDATION STUDY GROUP NO. 29 (1967) Anti-lymphocytic serum. London: J. & A. Churchill.

DRESSER D.W. & MITCHISON N.A. (1968) The mechanism of immunological paralysis. In *Advances in Immunology*, vol. 8, pp. 129–81. Eds. DIXON F.J. & KUNKEL H.G. New York: Acad. Press Inc.

GABRIELSEN ANN E. & GOOD R.A. (1967) Chemical suppression of adaptive immunity. *Adv. Immunol.* **6,** 91.

GOODWIN L. G. & HOOK S.V.M. (1968). Blood vessels in rabbits infected with *Trypanosoma (Trypanazoon) brucei. Brit. J. Pharmacol.* In press.

HARRIS H., WATKINS J.F., FORD C.E. & SCHOEFL G.I. (1966) Artificial heterokaryons of animal cells from different species. *J. Cell Sci.* **1,** 1.

HEFFRON R. (1939) Pneumonia with special reference to pneumococcus lobar pneumonia. *Commonwealth Fund N.Y.* pp. 240–5.

HOTCHIN J. (1962) The biology of lymphocytic choriomeningitis infection: virus-induced immune disease. *Cold Spring Harbor Symposia on Quantitative Biology* **27,** 479.

MATSUMURA T. (1962) Studies on bacterial cross allergic reaction on the basis of natural sensitization by intestinal flora. *Gunma J. Med. Sci.* **11,** 319.

MATSUMURA T. (1963) Ascaris allergy. *Gunma J. Med. Sci.* **12,** 186.

SCHAEDLER R.W. & DUBOS R.E. (1961) The susceptibility of mice to bacterial endotoxins. *J. exp. Med.* **113,** 559.

SCHWARTZ R.S. (1965) Immuno-suppressive drugs. *Progr. Allerg.* **9,** 246.

STETSON C.A. (1959) Endotoxins and bacterial allergy. In *Cellular and Humoral Aspects of the Hypersensitive States*, p. 442. Ed. LAWRENCE H.S. New York: Hoeber-Harper.

SYMPOSIUM (1967) Immuno-suppressive agents. *Proc. roy. Soc. Med.* **60,** 125.

THOMLINSON J.R. & BUXTON A. (1963) Anaphylaxis in pigs and its relationship to the pathogenesis of oedema disease and gastro-enteritis associated with *Escherichia coli*. *Immunology* **6,** 126.

WEBB H.E. & GORDON SMITH C.E. (1966) Relation of immune response to development of central nervous system lesions in virus infections in man. *Brit. med. J.* **2,** 1179.

WEITZ B. (1960) A soluble protective antigen of *Trypanosoma brucei*. *Nature, Lond.* **185,** 788.

WORLD HEALTH ORGANIZATION (1967) Teaching of immunology in the medical curriculum. *Wld. Hlth. Org. Techn. Rep. Ser. No.* 358.

SECTION I

DIAGNOSTIC AND ANALYTICAL APPLICATION OF IMMUNOLOGICAL METHODS

CHAPTER 1

DIAGNOSTIC AND ANALYTICAL *IN VITRO* METHODS

R.R.A.Coombs & P.G.H.Gell

INTRODUCTION

It is our aim in this chapter to set out without specialist or technical jargon the basis of the various diagnostic and analytical methods in immunological laboratory practice today. The principles only will be discussed and for all but the minimum of technical details the reader will be referred to other sources.

Most of the present-day serological methods are evolutions and refinements of the classical reactions well known to all students of medicine, namely, precipitation, agglutination, neutralization of toxins and complement fixation. New developments are especially concerned with cellular interactions which are becoming more amenable to examination *in vitro*. Serology is the science of the serum reactions, while immunology deals with all 'immune' or 'allergic' reactions; thus the repertoire of immunological methods is greater.

Demonstration of Antigens

The fact that a substance is antigenic is a casual property of its size and structure (see Maurer 1964) though a convenient one for the investigator; it is present in the cell or body fluids for quite other reasons, as a structural part of a dynamic living and changing system.

It is essential, when looking for antigens in biological materials, to adapt one's method of investigation according to the site of the antigen—as for example the nuclear components within the cell, or the blood group antigens on its surface, or the serum proteins free in the body fluids. Technically, the demonstration of cell-surface antigens is simple only if the cells are suspensible and can be specifically agglutinated: it is difficult to obtain a stable suspension of most tissue cells, so a more elaborate method such as mixed agglutination must be used. Antigens within the cell are even more tricky to deal with: here non-denaturing extraction methods, or, more recently, cell smears or sections treated with fluorescent antibody have to be used. With all these, and especially with antigens in solution, we have the problem of diversity of antigens and of antibodies: and techniques such as immuno-electrophoresis have been devised to demonstrate whether or not we are dealing with a single antigen-antibody system, or with many.

Detection of the Allergic Response of the Host

Before we can measure *fully* the allergic or 'immune' responses and their effects, either protective or damaging, on the host, we still need further insight into the relationship (see Chapters 11 and 12) between antibody production and the cellular responses which are measured in delayed sensitivity (not dependent on humoral antibody). The latter responses enter into diagnostic work in the form of empirically used skin tests such as the tuberculin reaction, but are not, as

yet, amenable to *in vitro* testing although developments in this area are to be expected in the next few years (see later section on 'Reactions *in vitro* of actively allergized cells', p. 39).

Measurement of humoral antibody also requires a full appreciation of the recently recognised distinct immunoglobulin classes of γ globulin and of the further heterogeneity within these classes (see Chapter 13). Antibodies of these different molecular classes and sub-classes have different biological properties (an end to the Unitarian Hypothesis! Zinsser 1921) and so it may be appreciated that different immunological tests may be needed for the detection and titration of antibodies of these different molecular species. For instance certain immunoglobulins do not fix complement, others do not passively sensitize tissues for an anaphylactic reaction, while again some (e.g. IgM) are much better at producing agglutination than others (e.g. IgG). Antibodies, which at appropriate concentrations fail to precipitate with antigen or to produce agglutination, have been called 'incomplete' and require special procedures for detection.

Since the previous edition considerable progress has taken place in the *in vitro* measure of human reagin and these tests are discussed in a later section of this chapter.

In the remainder of this chapter each immunological test is briefly discussed and, as in most tests for antigens good high-titred and specific reagents are indispensable, a small section is devoted to the production of antisera. In what follows the space devoted to any reaction is in no way proportional to the importance of the test as little will be said about the well-known and much worked-over methods.

RAISING OF ANTISERA

In the laboratory antisera are normally made in the rabbit: for large-scale commercial purposes the horse is used. In order to raise antibodies against rabbit proteins, and for other special purposes, the sheep or goat, the fowl and the guinea-pig have also been used. In general, the more remote the species used from that supplying the antigen, the greater the antigenic stimulus: the more closely the species are related the higher the specificity of the antibodies obtained. Within species, antibodies can sometimes be obtained between genetically diverse individuals, e.g. blood group iso-antibodies and iso-antibodies to the 'allotypic γ-globulins' of the rabbit.

Selection of Antigen

For most purposes it is best to use as pure a preparation of antigen as possible, and it is always desirable to know what antigen-antibody systems are present:

for instance 'anti-human globulin' antisera which contain antibodies against a multitude of serum proteins, such as components of complement, may lead to extremely misleading conclusions if used uncritically.

Use of Adjuvants

The use of 'adjuvants' to improve the antibody response has much simplified the process of raising antisera, both by saving time and by 'ironing out' differences in response between individual animals: so that, with a reasonably uniform strain of animals, and with a reasonably powerful antigen, nine out of ten animals in a group will respond with antisera of good and equal potency. Several types of adjuvant are used: that most often used in the laboratory is a variant of the one described by Freund and Macdermott (see Freund 1947, 1951; and below): it is probably the most efficient, at least in rabbits, if antigen is in reasonably good supply. The use of alum-precipitated antigen intravenously (see Kabat 1961) is effective and rather more economical of materials.

If very small amounts of antigen only are available, the technique described by Newbould (1965) of direct injection into the lymph nodes, is extremely effective. This has been adopted for use with precipitation lines cut out of agar and emulsified in complete adjuvant (Smith, Gallop & Tozer 1964; Goudie, Horne & Wilkinson 1966).

In man alum precipitated antigens (APT) have been used for many years: more effective adjuvants, in particular various emulsions, are being worked with but occasionally produce distressing chronic reactions (see Pearson 1965). White (1968) discusses the theoretical aspects of adjuvant action.

Course of Injections

Many different courses may be used according to circumstances and the whim of individuals. Courses which have been found effective by the authors are:

Rabbit

The material required for the Freund's adjuvant preparation is obtainable from Difco (British agents: Baird & Tatlock) in complete and incomplete (without mycobacteria) forms.

It may also be made up from its components:

BAYOL F or 55 (Esso Ltd.)	17 parts
ARLACEL A (Honeywell and Stein Ltd.)	3 parts
Killed dried tubercle bacilli or *Myc. butyricum* (Difco)	0.5 to 1.0 mg per ml (or more)

Equal volumes of adjuvant and of aqueous antigen solution are emulsified together. The final product must be a true water-in-oil emulsion; a drop

should not spread on water. Incomplete emulsification very much reduces the efficiency of the method.

For immunization of rabbits, injections of 1–2 ml of such emulsions containing up to 10 mg of antigen (as little as 10 μg is sometimes effective) are made intramuscularly. A second similar injection may be made 2–24 weeks later and repeated if required. Adjuvant administration may be followed by a short intravenous course of antigen in solution: this should be preceded by a single subcutaneous injection to guard against anaphylaxis. The animals must also be watched carefully after each intravenous injection for any sign of respiratory distress: should this occur the course should be terminated. Up to 80 ml of blood may be taken (from a large rabbit) 5–8 days later. A longer gap (up to 12 weeks) may be left between injection and bleeding after immunization with adjuvant alone.

Sheep and goats

These are very susceptible to anaphylaxis and sometimes develop severe lesions after subcutaneous use of Freund's adjuvant. Three to five intramuscular injections in Freund's complete adjuvant, at monthly intervals, are safe and effective.

Fowl

Intravenous immunization is good but difficult owing to the fragility of the veins. Three to five injections in complete adjuvant into the pectoral muscles have been found to work well.

Guinea-pig

This animal, contrary to the general impression, can produce excellent precipitating antisera, though there is much individual variation and it is of course highly susceptible to anaphylactic death. A course which has been found effective with rabbit proteins consists of two subcutaneous injections in adjuvant 2–4 weeks apart (10–100 μg of antigen per dose) followed by intradermal injections twice weekly of 100 μg in saline. The course is complete when the animal shows a strong local Arthus reaction with haemorrhage and necrosis: it can be bled out 2–5 days later.

Characterization of Antisera

Immuno-electrophoresis (see below) is the most convenient qualitative method for characterizing antisera to soluble antigens. The antiserum should be run both against the purified antigen used for immunization and against two or three widely spaced dilutions (undiluted, ×5 and ×25) of the crude material (e.g. whole serum) from which the purified antigen was derived, in order to show up any 'contaminating' antigen-antibody systems.

PRECIPITATION TESTS AND THEIR MODIFICATIONS

Soluble antigen when mixed with homologous antiserum forms a precipitate which may be seen and measured. It is one of the classical serological reactions (Kraus 1897).

In the flocculation test varying dilutions of antigen are mixed with a constant dilution of antiserum. Sometimes, especially with toxin-antitoxin reactions, it is the antigen which is kept constant. The interaction is shown by a cloudiness becoming a flocculation with time. Precipitation is inhibited by excess antigen and with some antisera by excess antibody. The test is often employed for a qualitative differentiation only, but a semi-quantitative estimation can be obtained by measuring the degree of opacity with a nephelometer (Boyden 1954) or by measuring the volume of the precipitate with arbitrary dosages of antigen (Nuttall 1904). For a truly quantitative measure of the amount of antibody or antigen in a solution see the quantitative precipitin reaction discussed later. Another indirect and semi-quantitative method of estimating the amount of either reactant is the so-called optimal proportions method of Dean & Webb (1926) which depends on noting the first tube of a series to flocculate.

A useful but simply qualitative method is the interfacial or ring technique (Ascoli 1902) where a drop or two of antigen is layered with care above a similar volume of undiluted antiserum. In a positive reaction a ring of precipitate forms at the interface. Trouble is unlikely to arise due to zones of inhibition as the reactants diffuse into each other until they achieve the ratio optimal for precipitation.

The precipitin test is very sensitive for the detection of antigen, but not for antibody. The advantage of the interfacial test is its extreme simplicity and the small quantity of reagents needed. Fairly strong antisera are required and both the antigen preparations and antisera need to be clear. Oblique illumination with a black background is essential for reading the tests.

Although the more recent modifications of the reaction using an agar-gel matrix have largely superseded these ordinary precipitin tests they still have their uses today; for instance, in grouping streptococcal extracts (Lancefield 1933), in urine precipitin tests (see Chapter 6), in forensic work on the identification of blood, flesh and milk (see Chapter 9) following the classical work of Nuttall (1904), also in establishing the feeding hosts of blood-sucking arthropods (Weitz 1960) and in tests for C-reactive protein in blood (see below).

Tests for C-reactive Protein

C-reactive protein is a protein not normally to be found in the circulating plasma of man but appears in the blood fluid early in the course of many infections,

following inflammatory conditions or where there is tissue damage or necrosis (Raffel 1961). Its presence in the serum of a patient may be shown by:

(*a*) an interfacial precipitin test using an antiserum made in a rabbit to human C-reactive protein. Such antisera are available commercially. This is largely superseded by more quantitative methods, for instance:

(*b*) a double diffusion agar-gel precipitation test (see below).
(*c*) the latex fixation test (see below; Singer *et al* 1957).

It is called C-reactive protein because in the presence of Ca^{++} it reacts and precipitates with C-substance, a polysaccharide derived from the pneumococcus and indeed this is how it was first discovered (Tillet & Francis 1930).

The origin and function of this protein are obscure but clinically its appearance in the serum indicates that an inflammatory reaction is going on somewhere in the body. It forms one of the 'acute phase proteins' and its estimation is particularly valuable as an objective and quantitative measure of 'illness' in, for example, clinical trials.

Quantitative Precipitin Reaction

None of the precipitin tests discussed so far can be considered quantitative in the chemical sense and although admirably suited for diagnostic and much other investigative work they fall far short of the needs of the immunochemist.

The amount of antibody in a serum can be determined with an error of about 5% by the quantitative precipitin technique, developed by Heidelberger and Kendall in the 1930s. The method involves determination of the protein, by the micro-Kjeldahl procedure, in the precipitate after complete precipitation of antibody in the zone of slight antigen excess as found in a preliminary titration. Knowing the amount of antigen added, the amount of antibody can be calculated. A comprehensive discussion of the method with full technical details is given by Kabat & Mayer (1961).

This procedure has formed the basis for much of the experimental immunochemical research for which strictly quantitative results have been necessary. It is essentially a research procedure and not often called for in diagnostic serology.

Gel-Diffusion Tests

Gel diffusion is rapidly becoming the method of choice in dealing with soluble antigens and precipitating antibodies when qualitative or semi-quantitative results are needed. It may be used:

(*a*) As a slightly slower substitute for the interfacial 'ring' test, especially when the reagents are cloudy: it is also less apt to give 'false positives'.
(*b*) As a method for demonstrating multiplicity of antigen-antibody systems: for this purpose it is less satisfactory, though quicker and more economical of reagents than immuno-electrophoresis (see below).

(*c*) As a method for comparing crude antigen mixtures one with another, in order to detect antigens which they possess in common: and for following the chemical fractionation of particular antigens.
(*d*) As a qualitative method for demonstrating the cross reactivity of related but not identical antigens.
(*e*) As a quantitative method for the estimation of particular antigens, using 'monospecific' or absorbed antisera.
(*f*) As a method for determining diffusion constants and hence approximate molecular weights, of antigenic proteins (Allison & Humphrey 1959, 1960).

For a general review of such methods see Grant (1964).

Qualitative Use of Simple Gel Diffusion

These may be single diffusion (Oudin 1946) in which antigen and antibody diffuse directly into one another; double-diffusion tube tests (Oakley & Fulthorpe 1953; Preer 1956) in which a blank volume of gel, into which diffusion occurs from both reagents, is interposed between the two; and double-diffusion plate tests (Elek 1948, 1949; Ouchterlony 1948, 1949) in which the reagents diffuse towards one another in a thin layer of gel in a Petri dish, on a lantern slide, etc., usually from holes cut in the gel—in the last case antigens may be allowed to diffuse from growing bacterial colonies or smears, towards a hole or trough containing antibody. Almost every month some new variant of these basic tests is published, specially adapted for particular purposes (see the book by Crowle 1961).

Medium

Agar, gelatin, cellulose acetate, silica gel, acrylamides and other substances have been used as the supporting medium. For most purposes, agar is the simplest to use and the most readily available. A clean, clear agar of good gel strength is essential: a New Zealand agar at 0.8–1%, in saline, or buffered saline, has been found satisfactory.

Method

For almost all purposes some variation of the Elek-Ouchterlony double-diffusion plate test is the simplest to use and the most versatile.

In principle, the nearer the holes are together the quicker and more sensitive the test, but the harder it is to distinguish multiple lines: round holes cut with a cork-borer, 2–15 mm apart, are in general satisfactory. The arrangement of the wells depends upon the system being investigated, though a set of six surrounding a central well is a convenient standard pattern. The nearer to the optimal ratio the mutual concentrations of the reagents are, the sharper the lines: if one or other is in gross excess the blurred lines produced may be misleading or even apparently absent. When the reactions are carried out on lantern plates

the resultant plate may be thoroughly washed, stained with Ponceau red, Azocarmine, Chlorazol black, Wool black, or other suitable dye, and dried down as a permanent preparation.

Applications

The most useful applications of the plate diffusion tests, apart from the mere demonstration of an antigen-antibody reaction, make use of the 'reaction of identity'. If antigen from two contiguous wells diffuses towards antibody, the lines of precipitate which they form will fuse perfectly at the angular junction between them if the antigens in each well are identical (Fig. 1.1a). The converse also holds: if say a rabbit antiserum and a sheep antiserum both directed against ovalbumin diffuse towards an ovalbumin antigen, a 'reaction of identity' is shown by their respective lines (Fig. 1.1b). Even if the shared component is an antigen in one system and an antibody in another (Fig. 1.1c) fusion can occur.

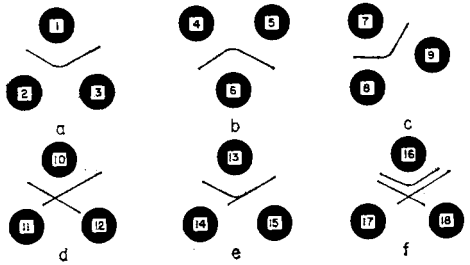


FIG. 1.1. Simple gel-diffusion reactions

a. 1. Anti-A antiserum
2. Antigen A: Prep. I
3. Antigen A: Prep. II

b. 4. Rabbit anti-A antiserum
5. Sheep anti-A antiserum
6. Antigen A

c. 7. Rabbit anti-A antiserum
8. Sheep anti-Rabbit γ-globulin
9. Antigen A

d. 10. Anti-(A+B) antiserum
11. Antigen A
12. Antigen B

e. 13. Anti-A antiserum
14. Antigen A′
15. Antigen A

f. 16. Anti-(A+B+C) antiserum
17. Antigen (A+B)
18. Antigen (B+C)

(*Note:* A, B and C have no reference to blood group factors)

Lines which do not fuse but cross without any apparent decrease in intensity show the 'reaction of non-identity' (Fig. 1.1d): while lines which show a 'spur' (Fig. 1.1e) indicate 'cross reaction', i.e. some of the antibodies in the antiserum will react with antigen A′ as well as with the homologous antigen A, but others, more specifically adapted, will not, and will therefore diffuse through the A′ diffusion-front to react with that of A. Alternatively, there may be some determinant sites which are *identical* on A and A′, but A may possess other

determinants which are entirely lacking in A'. These two quite different types of cross reaction cannot be distinguished by gel diffusion.

Appearances of this sort must always be viewed critically. It cannot be absolutely certain for instance that the picture in Fig. 1.1e is not due to the presence of two distinct antigens in A, only one of which is identically present in A': nor can the picture in Fig. 1.1d exclude the occurrence of a very minor cross reaction between the two antigens.

Immuno-Electrophoresis

This technique was first described by Grabar & Williams (1955) and is reviewed by Grabar & Burtin (1964). It is of value (*a*) for 'screening' antisera to demonstrate multiple antigen-antibody systems, (*b*) for characterizing in a preliminary way unknown antigens, e.g. in plasma, or bacterial extracts, (*c*) for demonstrating subtle abnormalities in proteins such as the myeloma globulins, as compared with 'normal' γ-globulin, with which they cross react. There are many other potential applications.

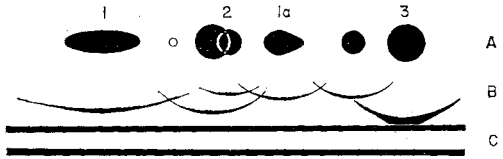


Fig. 1.2. Diagrammatic illustration of the process of immuno-electrophoresis.

A: Distribution of protein spots in gel following electrophoresis.
- 1, 1a: Proteins of broad charge distribution;
- 2: Two different overlapping proteins;
- 3: Protein in high concentration against which there is little antibody.

B: Areas of precipitates which form when diffusing antibody meets antigen.

C: Trough containing 'polyvalent' antiserum.

The method entails a preliminary linear electrophoretic separation in agar of the components of a complex antigenic mixture such as human serum: antiserum is then diffused towards them from a parallel trough cut in the agar. Arcs of precipitate form where the diffusing antibody meets the separated blobs of antigen (Fig. 1.2). Some simplifications and microvariations of the original method have been published (cf. Crowle 1961). The apparatus may be simply adapted in the laboratory from the equipment used for routine paper electrophoresis.

Quantitative Uses of Gel Diffusion

It is not difficult to adapt the gel-diffusion techniques to give quantitative measurements, potentially of very great use in clinical pathology.

Such methods, if they are to be of value for routine clinical use (*a*) should be

simple and quick, (*b*) should be of sufficient accuracy and sensitivity for the purpose required: the sensitivity should be definable as the minimum amount of antigen estimable quantitatively, and the accuracy as the 'coefficient of variation' (or other suitable statistical parameter), using the method on a number of different antigen-antibody systems, since both sensitivity and accuracy vary with individual antisera, (*c*) the method should be economical of antiserum and antigen, and (*d*) should incorporate the necessary control.

Quantitative tests fulfilling some or all of these conditions have been published by Oudin (1953), Augustin & Hayward (1955), Gell (1957), Feinberg (1958), Wright (1959) and Darcy (1960). Soothill (1962) gives a full analysis of one of these techniques, with details of antisera, estimation of coefficients of variation for a number of human plasma protein-antibody systems, and references to the use of the test in clinical investigations. The method of Fahey & McKelvey (1965) in which a small amount of antiserum is incorporated in the plate and the width of the diffusion zone of antigen is measured, is simple and capable of great accuracy and sensitivity. The method of Wright (1959) is useful for comparative estimations of antigens when only multi-specific antisera are available.

Fluorescent Antibody Techniques

Antibodies can be made fluorescent by chemically attaching to them a fluorescent dye, the most generally used being the isocyanate or isothiocyanate of fluorescein itself—a technique introduced by Coons and his co-workers (Coons & Kaplan 1950; Coons 1956). Other fluorochromes have also been used, of which the most useful is the red-fluorescing Lissamine Rhodamine (Chadwick, McEntegart & Nairn 1958). The method and applications have been reviewed by Nairn (1964).

Such fluorescent antibodies can be used to demonstrate specifically both antigen and antibody in tissues, cell suspensions or cell smears, when these are examined under ultraviolet light.

Demonstration of Antigen

Intrinsic tissue antigens such as basement membrane may be made fluorescent in sections by treating them with a specific fluorescent antibody. So may γ-globulins, for example, trapped in tissues owing to a local auto-allergic reaction. Such antibody may in fact be used as a specific stain for any antigen—as long as the original antiserum is specific and non-specific adsorption can be excluded (see Fig. 1.3). Applications of this principle are innumerable, many of which are described in later chapters. Pathogenic bacteria may similarly be identified in direct smears from throat or faeces by the use of the appropriate antisera, and also viruses in and around cells (see Chapters 2, 4 and 5).

A modification of this direct method, which is simpler and more sensitive,

is generally used nowadays, and is known as the indirect technique (see Fig. 1.4). Here, instead of making each specific (rabbit) antiserum fluorescent, the native antiserum is applied to the section, washed off, and then a fluorescent anti-rabbit γ-globulin antiserum, raised in the goat, fowl or guinea-pig, is applied to stain the sites where rabbit antibody has attached itself in the first stage. This has two

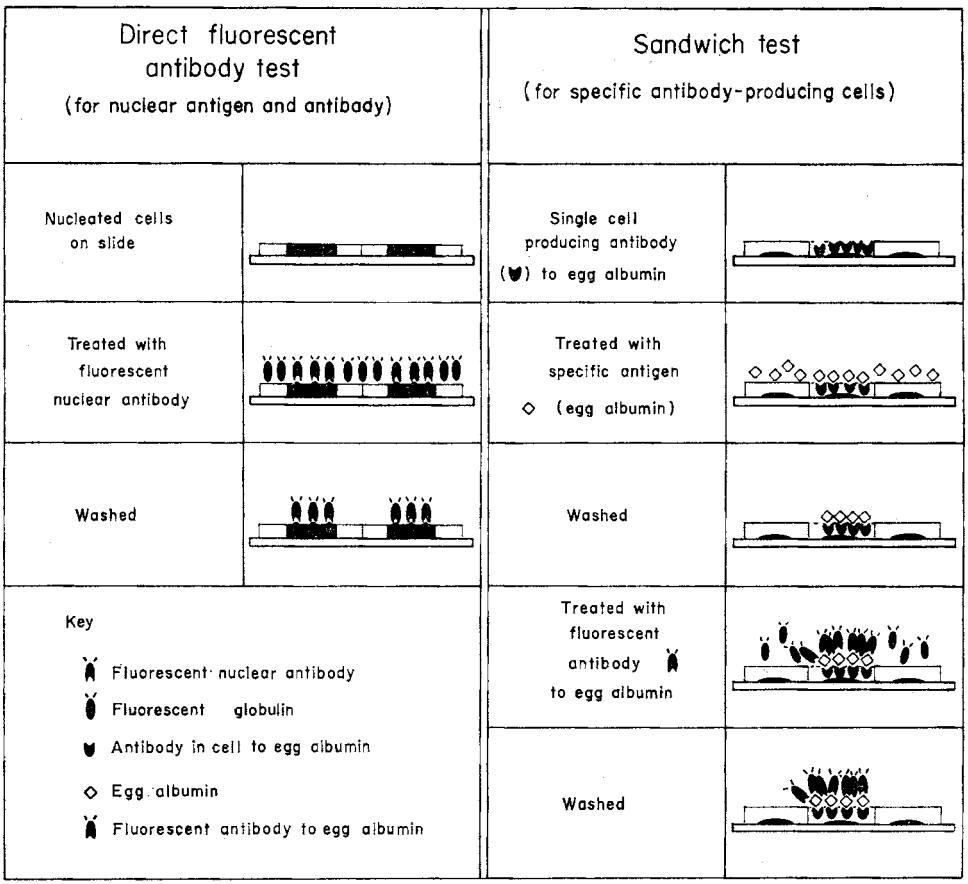


FIG. 1.3. Direct fluorescent antibody test and 'sandwich test' for staining antibody-producing cells.

advantages: firstly, a single preparation of fluorescent serum can be used for a variety of tests; and secondly, since round about ten times as much rabbit γ-globulin antibody by weight becomes attached as there is antigen in the section (in consequence of the usual ratio of antigen to antibody in an immune precipitate), sensitivity is increased by this factor so that much brighter staining results.

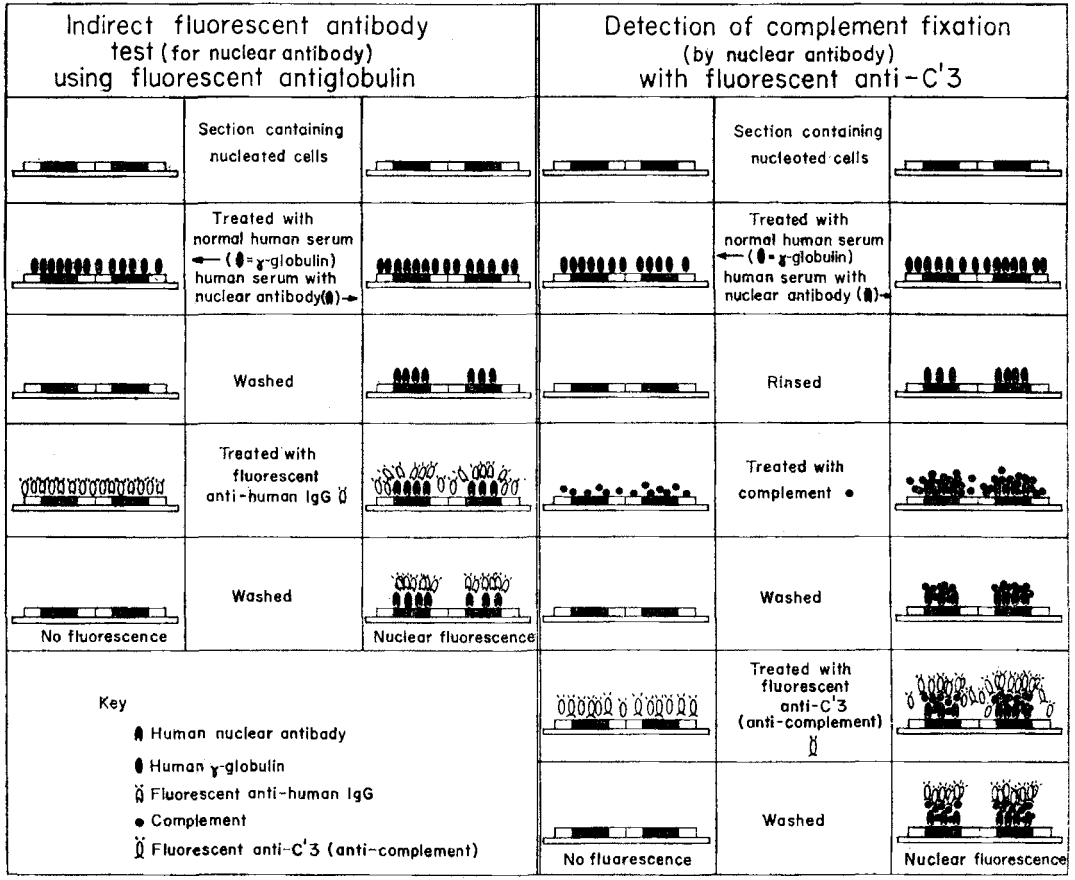


FIG. 1.4. Indirect fluorescent antibody test and detection of complement fixation with fluorescent anti-C'3.

Extreme care, however, must be used to include all possible controls, as non-specific staining is very easy to obtain.

Demonstration of Antibody

The presence of antibodies to tissue components in human auto-allergic sera can be demonstrated with a high degree of sensitivity by the 'indirect' test. A section (usually of normal tissue) is treated first with the suspect serum and then with an anti-*human* IgG antiserum (raised in rabbit, goat, horse or sheep) which has been made fluorescent (cf. Fig. 1.4). Alternatively, if the reaction between the suspect serum and the tissue is carried out in the presence of complement, a fluorescent anti-complement (anti-β1C) antiserum gives a very sensitive test of combination. For the preparation of such an antiserum see Mardiney & Müller-Eberhard (1965).

Demonstration of Antibody-Forming Cells

Antibody forming cells can be elegantly demonstrated (see Chapter 12) by this kind of technique. The production of γ-globulin by cells can be shown by the methods described above: but specific antibody can be shown by what is called the 'sandwich' technique (see Fig. 1.3). Here the cells are first treated with the antigen, and then the free valencies of the antigen are used to combine a fluorescent specific antiserum.

Theoretically the sensitivity of both sorts of method can be multiplied indefinitely by piling on say rabbit anti-goat and goat anti-rabbit antibody alternately, on the principle of one of those excellent Danish sandwiches which constitute a satisfying meal in themselves.

Reactions Using Radio-active Labelling

A technique for measuring very small amounts of antigen and antibody of low avidity, is supplied by the use of the methods described by Farr (1958). He made use of the fact that many antigens are not precipitated by a salt concentration (ammonium or sodium sulphate, etc.) which will bring down all antibody globulins quantitatively. If such an antigen, radioactively labelled, is added to an antiserum, however weak, and the mixture treated with half-saturated ammonium sulphate, the precipitate of globulins will contain no label unless there is some specific antibody present to bind it. The amount of radioactivity bound can be used to quantitate the amount of antibody present. This method can be generalized for all antigens, if the precipitation is made not with salts, but with a specific antiserum against the IgG examined: it can be very sensitive indeed, and, with suitable precautions, highly quantitative (Mitchison 1964). A similar method was used by Ishizaka *et al* (1966) to show the presence of antibody activity in IgE. Some techniques used in immunoassay depend upon the competition by (unlabelled) antigen in a 'test' sample with radio-labelled antigen

for antibody: the unbound antigen is separated by physical methods, e.g. electrophoresis, and its radioactivity, with suitable corrections, can be used as a measure of the amount of antigen in the test sample (see Chapter 10).

AGGLUTINATION TESTS AND THEIR MODIFICATIONS

Simple (Direct) Agglutination Tests

This is one of the classical serological reactions, involving the clumping of a cell suspension by specific antibody and has the advantage of simplicity of execution. According to the Lattice theory the aggregation is brought about by antibody molecules with two or more combining receptors linking across from cell to cell (see Fig. 1.5).

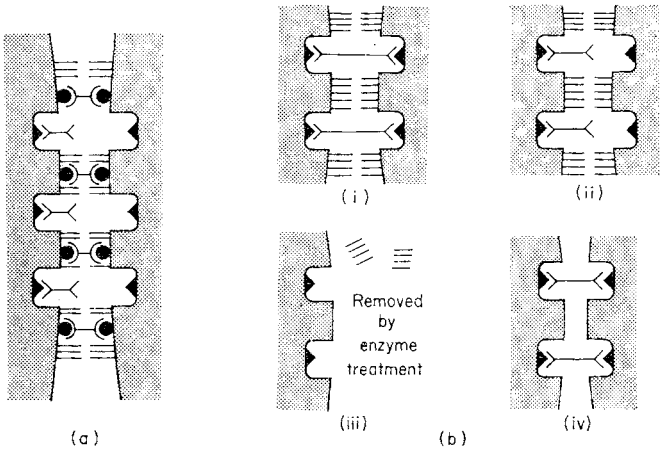


Fig. 1.5. Lattice hypothesis of agglutination and agglutination of enzyme-treated cells by incomplete antibodies.

(*a*) Antibodies with two or more combining receptors linking across from antigens on one cell to those on another. Agglutination only possible with antigens close to the cell surface.

(*b*) Removal of certain structures on the cell surface by enzyme treatment rendering the cells agglutinable by incomplete antibodies.

(i) Agglutination of cells by complete antibodies.

(ii) Inability of incomplete antibodies (possibly smaller molecules) to agglutinate cells.

(iii) Removal of certain structures on cell surface by enzyme treatment.

(iv) These cells now agglutinable by incomplete antibodies.

Certain requirements are necessary in order to carry out an agglutination test. First, one must have a stable cell suspension. It is also obvious that a reaction is only possible with antigens at or close to the cell surface (see Fig. 1.5). Special modifications are required to demonstrate 'incomplete' antibodies which by accepted definition fail to produce agglutination under conditions where

comparable agglutinating antibodies (or agglutinins) do. With Rh blood group antibodies and human brucella antibodies the agglutinins are IgM while the 'incomplete' antibodies are of the smaller IgG and IgA molecular species.

One has always to bear in mind the possibility of prozones of inhibition with the more concentrated serum dilutions. Possible causes of prozones are excess of antibody, admixture of incomplete antibodies or complement interfering with lattice formation.

The test is performed by mixing dilutions of antiserum with the cell suspension. Antibody combines very rapidly with the antigens but no agglutination occurs until the cells come into contact. There are an immense number of diagnostic applications, either using a standard cell suspension to examine sera for antibodies, such as salmonella or brucella antibodies, or using standard antisera, to identify cell suspensions, for instance, typing within the streptococcal groups and blood grouping. The tests may be performed in tubes or on a slide.

Agglutination of Cilia or Flagella of Living Cells Causing Immobilization

The sticking together, by what is essentially agglutination, of the flagella (H antigens) of bacteria curtails the free movement of these organisms. Owing to the small size of these organisms the noticeable effect to the naked eye is simply that of a loose agglutination of the suspension. However, the actual immobilization itself has been used by Nossal (1960) as a delicate means of showing antibody production *in vitro* by single cells derived from an immunized animal.

With much larger unicellular organisms such as the protozoa *Paramecium aurelia* (Beale 1954) or *Trichomonas foetus* (Kerr & Robertson 1941) it is the immobilization of the organism which is obvious to the eye, reflecting as it does the agglutination of the cilia or flagella.

In other so-called immobilization tests, i.e. the *Treponema pallidum* immobilization test (see later), the underlying process is a cytotoxic one involving cell membrane antigens and for which complement is necessary. In such cases the immobilization is not caused by agglutination of appendages.

Agglutination Tests in Media of High Viscosity

For reasons not understood, as yet, (but see Pollack, 1965) some incomplete antibodies which fail to produce agglutination of red cells in saline or dilutions of serum in saline, will do so if this medium is replaced by one of high viscosity such as neat serum, 15–30% bovine serum albumin, a mixture of bovine serum albumin and undiluted human serum, dextran or polyvinyl-pyrrolidone.

For the application of such testing procedures to blood grouping serology the reader is referred to Jandl & Castle (1956) and Stratton & Renton (1967). These testing procedures are important for measuring the H-2 transplantation

antigens on mouse red cells (Stimpfling 1961) and have been used in bacterial agglutination tests (Griffitts 1947) but with little practical importance.

Agglutination of Enzyme-treated Cells

It was discovered by Morton & Pickles (1947, 1951) that treatment of human red cells with certain enzymes rendered the cells directly agglutinable by incomplete rhesus (D) antibody, and that such enzyme-treated cells did not clump in the absence of antibody. Many crude and pure proteolytic enzyme preparations have since been investigated, namely, trypsin, papain, ficin and bromelin and found to be satisfactory for this purpose. In fact the procedure has become a standard one in blood grouping laboratories for the detection of many of the incomplete antibodies.

In performing the test the cell suspension is first treated with the enzyme preparation and is then washed before being added to the serum. The test is read for agglutination in the usual way. Details for performing these tests may be had from any book on practical blood grouping procedures (Stratton & Renton 1967; Dunsford & Bowley 1955; Boorman & Dodd 1966).

The agglutinating Rh antibody is an IgM antibody and the 'incomplete' antibody has been found to be IgG; there is probably no other special characteristic responsible for its 'incompleteness' other than the combination of the fact of it being IgG (smaller molecular weight and structural characteristics) and the particular situation of the corresponding antigen sites on the red cell membrane (see Fig. 1.5). The proteolytic enzymes probably act by hydrolysing mucopeptides on the membrane and releasing into the medium glyco-peptides which, when present as structural components of the cell membrane, prevent the close apposition necessary for the bonding of the cells together by the smaller bivalent IgG antibody molecules.

Very little work has been done with suspensions of cells other than blood cells but there seems to be no reason why its potentialities should not be extended to bacterial agglutination. The procedure cannot be used for all red cell antigen-antibody systems as some antigens are destroyed or digested off the red cell membrane by enzyme treatment (see Morton 1962).

Antiglobulin Test (Also Called Coombs Test)

The principle of this reaction was first described in 1908 by Moreschi but was independently rediscovered and developed as a method for demonstrating incomplete antibodies to red cell antigens by Coombs, Mourant & Race (1945).

Incomplete antibodies, like complete antibodies, are serum γ-globulins. As already mentioned incomplete Rh antibody is an IgG molecule as opposed to the agglutinin which is IgM. It is likely that more red cell incomplete antibodies will be found to be IgG or IgA as was also found to be the case with brucella incomplete antibodies (Kerr *et al* 1967). These incomplete antibodies fail to

produce agglutination of a saline suspension of homologous red cells but nevertheless do combine firmly with the antigens on the red cells. The specifically adsorbed γ-globulin antibodies remain firmly attached to the cell membrane if the cells are washed in saline to remove the unadsorbed serum proteins. Such washed cells with combined antibody γ-globulin, may then be agglutinated by addition of a rabbit anti-human γ-globulin serum, a serum which does not react with red cells devoid of combined antibody globulin (see Fig. 1.6). With recent characterization of the antigenic specificity of the different immunoglobulins, antiglobulin sera may be prepared specific and selective for the different classes of immunoglobulin and antiglobulin sera can be tested for their

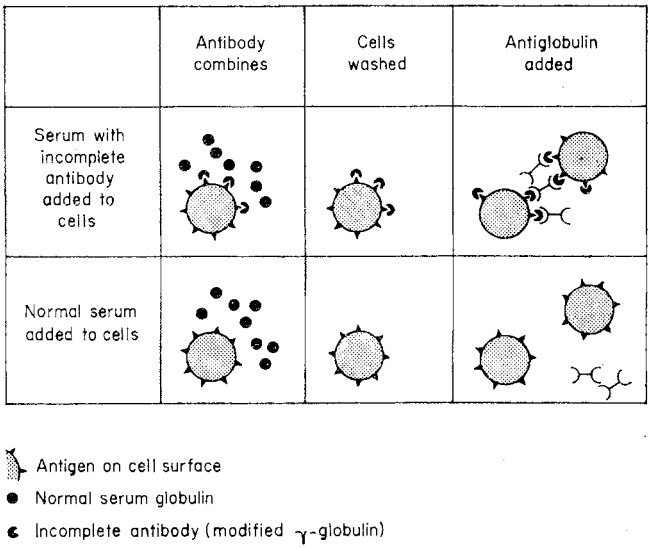


FIG. 1.6. Antiglobulin test for the detection of incomplete antibodies.

polyvalency. Some antibodies remain combined with red cell antigens under *in vitro* conditions only if complement components are present and adsorbed. In these circumstances an antiglobulin reagent reactive with the complement component C'3 (i.e. β1c globulin) has been found to be useful.

The antiglobulin test is simply a serological method of showing globulin firmly attached to cells. The procedure used to examine a serum for antibody is called the *indirect test*, while the shorter procedure, as used in haemolytic disease of the newborn or in acquired haemolytic anaemia, of testing cells taken from the patient with antiglobulin serum, is called the *direct test*.

The test has perhaps found its greatest application in the diagnosis of haemolytic disease of the newborn in man and animals, in blood grouping work and as an aid in investigating acquired haemolytic anaemia. It may, however, also

be used for the detection of non-agglutinating bacterial antibodies and recently the reaction has proved of special value in cases of chronic brucellosis (Kerr *et al* 1966). The antiglobulin reaction is also an essential component of the red cell-linked-antigen-antiglobulin reaction (see later), and the principle is incorporated in the indirect fluorescent antibody procedure of Coons. Experimental studies with the reaction are discussed by Coombs & Roberts (1959) and useful technical experience in the performance of routine tests is gathered together by Dunsford & Grant (1959) in their book *The Antiglobulin (Coombs) Test.* Recently a Sub-Committee of the Blood Transfusion Research Committee of the Medical Research Council (M.R.C., 1966) reported on the production in large animals of specific anti-IgG antisera for use in this test.

Inhibition of Antiglobulin and Antiglobulin Consumption Test

In the early days of the antiglobulin test it was shown that the reaction between antiglobulin serum and red cells sensitized with the Rh (D) incomplete antibody could be inhibited by γ-globulin in solution (Fig. 1.7). This finding confirmed that the mechanism of the antiglobulin reaction was as envisaged (Coombs & Mourant 1947).

The specific nature of this inhibition has been utilized as a means of determining the species-specificity of γ-globulin in fresh serum samples, in blood stains and in ancient cadaver material. The method is considered to have advantages over the direct precipitin technique for forensic investigations (see Wiener, Hyman & Handman 1949; Anderson 1954; and Allison & Morton 1953). Further experience in applying this procedure in the identification of blood stains for forensic purposes is reported by Dodinval (1957). This specific inhibition test may also be applied as a semi-quantitative procedure for estimating the level of γ-globulin in serum (Wiener 1955). Grubb (1956) could show an inhibition with as little as 0.1μg γ-globulin.

Although involving, in the second stage of the test, this same principle—namely specific inhibition of the action of anti-γ-globulin sera on red cells sensitized with incomplete Rh antibody—the test which has been called the 'antiglobulin consumption test' has really had a birth and following of its own. The two workers mainly concerned in the development and elaboration of the method were Moulinier and Steffen. The 'antiglobulin consumption test' offers a way of demonstrating antibody sensitization, or more properly globulin adsorption, on cells such as leucocytes and platelets and even lyophilized tissue homogenates which may be unsuitable for testing by other means. *The testing procedure may be outlined very briefly*: Suspensions of cells are, on the one hand, treated with normal serum and, on the other hand, with the serum under investigation. After allowing time for antibody combination the cells are well washed to remove all unadsorbed serum protein. At this stage there are different

procedures which may be used. In one, aliquots of one or the other washed suspensions are added to each serum dilution of antiglobulin serum with the idea that adsorbed globulin will combine with and neutralize the antiglobulin. After an incubation period the tubes are centrifuged and the clear supernatant tested for unneutralized antiglobulin by adding Rh-positive cells sensitized with incomplete antibody. Consumption of the antiglobulin shows that globulin was present in or on the absorbing tissue suspension and, depending on the circumstances, the consumption may be interpreted as indicating antibody sensitization of the tissue cells (see Fig. 1.7).

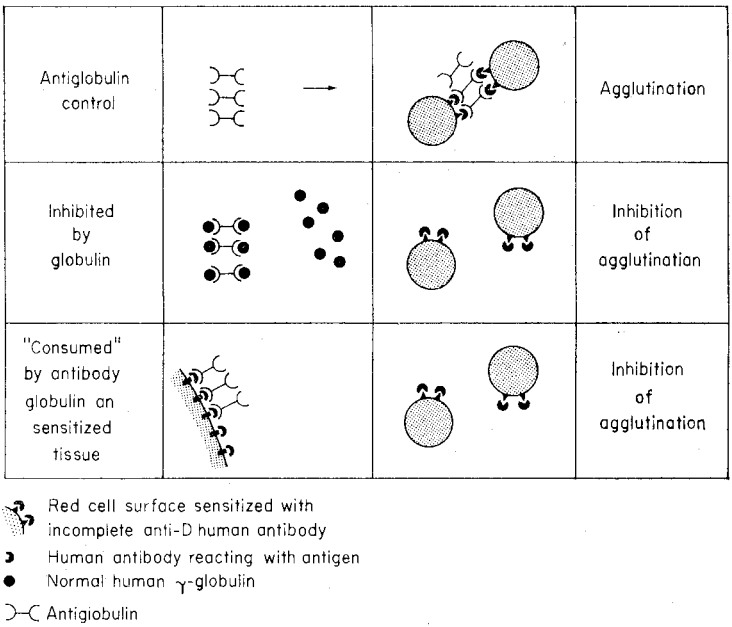


FIG. 1.7. Inhibition of antiglobulin and Antiglobulin consumption test.

It cannot be emphasized too strongly that, as in the antiglobulin test itself, the test is a measure of γ-globulin (if the final indicator cells carry only the γ-globulin Rh antibody), in or on the tissue cells, and even more care and reserve is needed in interpreting the results with the consumption test, as the test is performed on tissue cells often damaged and under any but physiological conditions.

For the procedure of testing recommended by Moulinier (see Moulinier 1956; and Dausset & Brecy 1958) and for a general review of the applications the paper by Steffen (1960) may be consulted. The most promising application of the method has been in testing human sera for platelet and leucocyte iso- or auto-antibodies and perhaps the most outstanding finding has been in idiopathic

thrombocytopenia where the serum is found to give a strong indirect antiglobulin consumption test with normal platelets of any healthy donor.

Dausset has been a keen advocate of the test and together with Colombani (Dausset & Colombani 1964) has written up a full account of the technical procedure.

Passive Agglutination Tests—Agglutination Tests Using Cells or Particles as Passive Carriers of Soluble Antigens

The essential difference between a precipitation and an agglutination reaction is the state of dispersion of the antigen. In the former case the molecules are free in solution and in the latter they are fixed on a surface as on the membrane of a cell. This difference, however, has a profound effect on the sensitivity of the reaction in detecting antibody; agglutination being far more sensitive.

Already in 1927, Jones showed that a precipitin reaction could be transformed into one of agglutination by simply adsorbing the protein antigen on to collodion particles and washing the particles free of excess uncombined antigen before adding the antiserum. In 1940 Cannon and Marshall further developed this 'collodion particle technique' and suggested its application as a routine serological procedure. Unfortunately, however, collodion suspensions were found by many workers to be too unstable for routine use and the method was not further developed.

In more recent years, however, with the great resurgence of interest in immunological problems the need for a reliable 'coated particle technique' was soon realized (Boyden 1951). Methods were needed for detecting antibodies to isolated protein and polysaccharide antigens in amounts too small to be shown by precipitin tests.

In the development of such a technique there are certain important points to be taken into consideration in the selection of the cells, or particles to act as the carrier.

(1) Cells or particles are needed to which antigens of different chemical nature, i.e. protein, polysaccharide or simple chemical may be firmly adsorbed or chemically linked. The antigenic determinants must remain free to react with antibody.

(2) The cells or particles should form stable and agglutinable suspensions. They should be easily deposited by light centrifugation and capable of being shaken up again to an even suspension. This is essential for washing the cells or particles free of excess unabsorbed antigen.

In this connection the red cell itself takes a lot of beating as a 'carrier' particle, and, as will be seen below, it has in fact been used to great satisfaction in passive agglutination tests—in this case the tests being called 'passive haemagglutination tests'.

A great advantage is gained if the antigen-coated particles or cells once prepared can be kept over a period for continual testing. Here the non-cellular particles have an advantage although red cells with adsorbed antigens can be stabilized with formalin.

(3) As a further refinement to which many of the methods do not aspire, failing to get agglutination of the antigen-coated particle, it would be very valuable to be able to proceed the one stage further, as in the antiglobulin test and to look for adsorption of incomplete or non-precipitating antibody to the coating antigen by an antiglobulin serum. This is only possible if the antigen-coated particle does not adsorb γ-globulin non-specifically, a requirement met with polysaccharide-treated red cells and in the case of protein antigens, with the 'red cell linked-antigen-antiglobulin reaction' (see later).

Bentonite Flocculation Test

A carrier which is being used today in passive agglutination tests is bentonite, which is a type of silicaceous earth mined at Wyoming, U.S.A. The particles after being washed and selected for size appear to adsorb most classes of antigen—proteins, carbohydrates and even DNA. If desired the coated particles may be stained with methylene blue and stabilized with a surface-active agent such as Tween 80. The flocculation of such particles by antiserum may be performed on a slide.

The method was first used by Bozicevich *et al* (1951) to detect antibodies in trichinosis and in 1958 Bozicevich *et al* applied the method to the demonstration of rheumatoid factor. Since then, the method has also been used to detect antibodies to DNA-protein and to DNA itself in patients with systemic lupus erythematosus (Bozicevich, Nasou & Kayhoe 1960). DNA may also be absorbed on to formalin-treated red cells (Lawlis 1958) but apparently not on to tanned red cells.

An advantage claimed for the bentonite flocculation test is that once the particles with adsorbed antigen have been prepared, they are stable and may be kept for 3–6 months for routine testing.

It seems likely that this method will receive wide application but it would be the writers' guess that the method has not the sensitivity of the passive haemagglutination tests and also that use of an antiglobulin technique for incomplete antibodies would be precluded.

Latex Fixation Test

In this test, the carrier particle is the perfectly spherical artificial polymer—polystyrene latex. Antigens, which apparently may be protein or polysaccharide (Kabat 1961) adsorb to the surface allowing the particles to be clumped by homologous antibody.

In performing the test the treated particles are often not washed free of excess

antigen before adding the antiserum. In the reaction between adsorbed immunoglobulin G and rheumatoid factor washing is unnecessary as the reacting determinants of the adsorbed IgG are probably only developed or unmasked as a result of the adsorption. In other antigen-antibody systems presence of free antigen probably means that the mechanism by which the particles are clumped is not purely one of agglutination but may involve other principles such as that of co-agglutination. Where the antigen-treated particles are washed free of excess unabsorbed antigen the reaction may be conceived of as one of simple passive agglutination.

Originally the test received most attention as a method for demonstrating rheumatoid factor (Singer & Plotz 1956; Plotz & Singer 1956; Greenbury 1960). Also its application to the demonstration of C-reactive protein has already been mentioned. In Chapters 3 and 6 references are made to recent applications of this latex passive agglutination test in histoplasmosis, cryptococcosis, Farmer's lung, trichinosis and hydatid disease.

Standard antisera are available commercially for agglutination of latex particles coated with purified human chorionic gonadotrophin and human urine may be tested for chorionic gonadotrophin by an inhibition procedure. This is used as a diagnostic test for pregnancy.

Passive Haemagglutination Test using Protein Antigens Adsorbed on Tannic Acid-treated Red Cells (Boyden's Test)

This test was developed by Boyden (1951) and involves the adsorption of protein antigens on to red cells which have been mildly treated with tannic acid. Proteins are not adsorbed on to untreated red cells. The antigen-coated tanned red cells are agglutinated by extremely small amounts of homologous antibody. Boyden's test has had extensive applications in both experimental work with protein antigens and as a diagnostic test in the examination of sera for antitoxins, for antibodies to bacterial and protozoal proteins, or again for antibodies to soluble protein antigens in patients with auto-allergic diseases. Stavitsky & Arquilla (1958) discuss very fully all technical and theoretical considerations and refer to many of these diagnostic applications.

Unfortunately an antiglobulin procedure cannot be used at the end of the test to exclude, in the absence of agglutination, the presence of incomplete antibodies. This procedure is precluded because although the antigen-coated cells are agglutinated only by specific antibody globulin, non-specific globulin is also adsorbed even from a normal serum containing no specific antibody. An attempt to overcome this difficulty has been made by Mathews (1959).

An inhibition reaction permits a semi-quantitative estimation of a protein antigen and has been found very useful in showing, for instance, the specificity

of pollen extracts of different grasses (Gosselin *et al* 1953), the species specificity of blood engorged by blood-sucking arthropods (Weitz 1960) and for this reason it has found application in the forensic identification of blood stains (Ducos 1958).

Fulthorpe *et al* (1961) report a better quantitation with the passive haemagglutination test if the direct test is performed in the presence of a constant amount of free antigen (i.e. 1 μg). The results are expressed in milli-units of antibody; 1 milli-unit being defined as the amount of antibody in 1 ml of serum which would just give clear-cut agglutination of the antigen-coated cells in the presence of 1 μg of free antigen. According to these authors this is a much more precise method of measuring the antibody content of different sera and is less open to day-to-day variation.

For routine diagnostic work the antigen-coated red cells may be treated with formalin which allows them to be kept for long periods, thus considerably shortening the time necessary for the performance of the test. Red cells coated with tetanus toxoid (Fulthorpe 1957) and with thyroglobulin (Fulthorpe *et al* 1961) have been treated and preserved in this way for routine testing. A test system for hydatid disease has also been prepared in this way (see Chapter 6).

Not all red cells have the same agglutinability. The human red cell is the most agglutinable carrier, but cells of other species may also be used although the reaction is a little less sensitive; sheep red cells are perhaps the most commonly used.

Passive Haemagglutination Test With Protein Antigen Linked to Red Cells by Covalent Chemical Bonding

In these procedures protein antigen is coupled chemically by covalent bonds to protein molecules of the red cell membrane. The first and perhaps most successful cross-linking agent used was bis-diazotized-benzidine (BDB) which was used in experimental studies by Pressman, Campbell & Pauling (1942). The method was developed and standardized as a laboratory test by Stavitsky & Arquilla (1955, 1958). Gordon, Rose & Sehon (1958) have published a very satisfactory recipe for performing this test.

The BDB method has similar applications to those of Boyden's test just described. These chemically coupled cells usually undergo lysis within 24 hr. The cell membrane must be damaged to some degree as there is a non-specific adsorption of γ-globulin from normal sera or other globulin containing fluids such as colostrum and this compromises to a degree the employment of a final antiglobulin stage as a test for combination of non-agglutinating antibody. The possibility of coupling the protein antigen to formalin-treated red cells (Cole & Farrell 1955, Csizmas 1960) is worth further investigation although it is likely that this, in turn, would again preclude any subsequent use of an antiglobulin test.

The use of toluene 2.4 diisocyanate (Schick & Singer 1961) as a coupling agent has a certain advantage since a two stage procedure can be used, and Friedmann, Hunter & Coombs (1967) found there was less non-specific absorption of γ-globulin from normal sera on to the coupled cells than in the case of BDB antigen coupled cells. However, the method is much more time consuming and expensive of antigen.

Johnson, Brenner & Hill (1966) report experience with a water soluble carbodiimide as a coupling agent. They considered it produced less damage to the cell membrane, thus leaving the red cell more with its normal stability.

Rose-Waaler Test

This is essentially a passive haemagglutination test using red cells (usually sheep) sensitized with a sub-agglutinating dose of rabbit anti-sheep red cell antibody (or other antibody), as a cell carrying immunoglobulin G to demonstrate the 'rheumatoid factor' (RF, see Chapter 31) in serum. This rheumatoid factor, a 19S immunoglobulin M, which is to be found in the serum of most patients with rheumatoid arthritis and sometimes in other 'collagen diseases', has the property of combining with fixed 7S immunoglobulin G (rabbit or human) and producing agglutination of the cell-substrate complex.

Besides the obvious interest as to the role of this factor in rheumatoid arthritis, its demonstration in serum has been very helpful diagnostically. The test is named from the work of Rose *et al* (1948) and Waaler (1940) who first observed the phenomenon empirically. A comparison of this test and other reactions in rheumatoid arthritis has been made by Greenbury (1960).

Passive Haemagglutination Tests with Polysaccharide Antigens Adsorbed by Natural Bonding on to Red Cells (Passive Haemagglutination Tests with Polysaccharide Antigens)

Many bacterial polysaccharides will combine firmly with a washed suspension of red cells without any special treatment and such cells after washing may be agglutinated by antibodies to the attached polysaccharide. The phenomenon was first described by Keogh, North & Warburton (1947) and has since formed the basis for a very valuable diagnostic test.

Using the very agglutinable red cell as carrier endows the test with very great sensitivity; antibody titres usually being greater than those obtained against the original bacteria from which the polysaccharide has been extracted. The procedure makes it possible to test antibodies against an antigen which is located on the inside of a bacterium by an agglutination test. Again, some bacteria cannot themselves be used in agglutination tests because they form only unstable suspensions. This procedure gets over the difficulty as far as the

polysaccharide antigens are concerned. Also more than one polysaccharide can be adsorbed on to the same cell suspension and so it is possible, in the single test, to screen a serum for several antibodies.

Added to these considerations is the advantage of the extreme simplicity of the method. The red cells are simply mixed with a solution of crude or purified polysaccharide (see Kabat 1961) at 37°C or at room temperature, washed free of uncombined material and added to dilutions of the serum to be tested.

As no damage is done to the red cells in effecting the coupling it is possible to extend the scope of the test with a final antiglobulin stage to show up any non-agglutinating or incomplete antibodies to the polysaccharide (see Hall & Manion 1951; Haberman 1955; Poppe 1959; Sindo 1960).

In most cases, but depending on the red cell used as carrier, haemolysis may be obtained if complement is also added (see test for Chagas's disease, Chapter 5). As in most of these passive haemagglutination tests, an inhibition test is possible. This is a very sensitive method of showing small traces of bacterial polysaccharide and diagnostic use has been made of the procedure to demonstrate antigen in the cerebrospinal fluid of patients with *Haemophilus influenzae* meningitis (Warburton, Keogh & Williams 1949) and rickettsial antigen in urine of scrub typhus patients (O'Connor & MacDonald 1950).

Dysentery, salmonellosis and *Escherichia coli* infections in children are clinical conditions for which the procedure has been found of value in demonstrating serum antibodies. The Middlebrook & Dubos (1948) test for antibodies in tuberculous patients reacting with tuberculin-treated red cells and the test described by Boyden (1950) for detecting antibodies to mallein, are further examples of this procedure. For an extensive review the reader is referred to Neter (1956).

Red Cell Linked-Antigen Antiglobulin Reaction

This reaction was introduced in prototype by Coombs, Howard & Mynors (1953) and Coombs & Fiset (1954). Its purpose was to supply a passive haemagglutination method which was capable of measuring both 'complete' (precipitating) and 'incomplete' (non-precipitating) antibodies to any soluble protein antigen.

The essential idea was to fix antigen to the red cell surface without damage to the cell and so avoid subsequent absorption of globulin non-specifically from the serum to be examined. This was achieved by chemical coupling of the protein antigen to molecules of rabbit non-agglutinating sheep red cell antibody. Exposure of sheep red cells to this reagent results in adsorption of this sheep cell antibody-protein-antigen dimer (complex of two molecules) to the cell surface. This red cell-protein antigen unit, after being washed, is stable in suspension and may be agglutinated by antibodies to the attached protein antigen. Also, as globulin, other than specific antibody to the protein antigen, is not adsorbed

from the serum under examination there is no obstacle to the further employment of a rabbit antiglobulin serum to detect adsorption of incomplete antibody to the attached protein antigen.

In further studies Steele & Coombs (1964) confirmed an earlier observation that photo-oxidation removed the agglutinating capacity of red cell rabbit antibodies and at the same time conjugated them to admixed protein antigens (see Fig. 1.8). Red cells treated with antibody-linked-milk protein reagents have

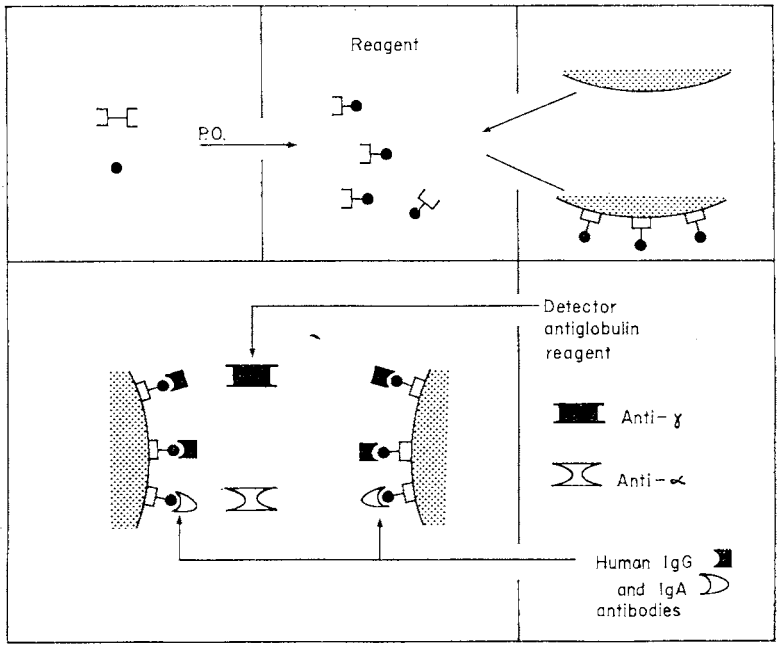


FIG. 1.8. Red cell linked-antigen-antiglobulin reaction for the detection of non-agglutinating antibodies (in human sera) to soluble protein antigens.
The figure illustrates coupling of soluble protein antigen ● to rabbit antibody against human red cells]—[to form the reagent ●–[Human red cells exposed to this solution adsorb the antigen-linked antibody on to their membrane. These stable cells are then used to test sera for antibodies to the coupled protein antigen.

been used to examine human infant sera for agglutinating and non-agglutinating antibodies to milk proteins. Incomplete antibodies could be demonstrated in many sera.

With the feasibility of preparing antiglobulin sera specific for each immunoglobulin class of human γ-globulin it is possible also to identify the immunoglobulin class of the reacting antibodies (Coombs *et al* 1965; see also Fig. 1.8). It will be of great interest to see if human reaginic antibody of the IgE class can be measured by this procedure.

The method would seem to have great potentiality as immunologists are ill-equipped with sensitive methods for detecting incomplete or non-agglutinating antibodies to soluble protein antigens. Use of the red cell as carrier assures the sensitivity of the reaction and the reagents for carrying the antigen on to the cell membrane once prepared are stable and can be stored for long periods at −20°C.

The serum of one particular child with Aldrich's syndrome was tested against casein and the passive haemagglutinating titre was 32 but there was a non-agglutinating IgG titre of 32,000 and a non-agglutinating IgA titre of 128,000.

Investigations are currently in progress (Hunter, Feinstein & Coombs 1967) to improve the manufacture of coupled antigen reagents.

Mixed Agglutination Reaction

Mixed agglutination is the formation of mixed aggregates of two different cell types by antibody reacting with similar antigenic determinants occurring on the

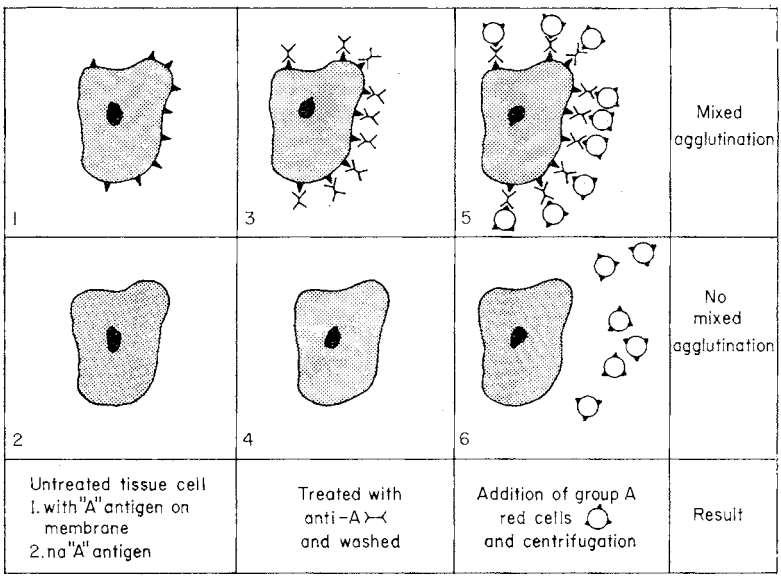


FIG. 1.9. Mixed agglutination reaction: for the detection of blood group A antigen on tissue cells.

two different cells. The antibody molecule makes a specific link bonding the two types of cell together. Using this principle Coombs, Bedford & Rouillard (1956) devised a serological test to show the presence of the A antigen on isolated skin epidermal cells, a demonstration which could be difficult by other methods.

The test which is shown schematically in Fig. 1.9 makes it possible to reveal

the presence of antigens on cells which are unsuited for use in ordinary agglutination tests. For instance, many tissue cells aggregate spontaneously or non-specifically or have shapes and sizes too irregular to give an agglutinable suspension. The second cell type used in the test forms the indicator cell and is chosen as one possessing the antigen under investigation and as one showing no natural affinity towards the cell under study. In most reports up to the present the indicator cell has been a red cell, but this need not be so. Franks and Gurner (1965) have used lymphocytes, Hagiwara (1962) antigen adsorbed on latex particles and we ourselves (Balmforth, Partridge & Coombs, unpublished) have used protein antigens coupled to red cells by bis-diazotized benzidine. The test is read by examining the cells under the microscope so the actual cells may be identified. Clumping of the cells being studied is immaterial, the criterion of a positive reaction is simply the occurrence of mixed agglutination (see Plate 1.1, facing p. 32).

A more detailed account of the reaction is given by Coombs (1964) where mention is made of applications such as the demonstration of blood group antigens on white blood cells and other tissue cells, identification of the species of origin of cells growing in culture (Coombs, Daniel, Gurner & Kelus 1961) and the forensic identification of blood stains (Coombs & Dodd 1961; see also Chapter 9). Using this procedure Kay & Wallace (1961) showed the loss of the A antigen on malignant cells. The reaction has also been used by Edwards, Ferguson & Coombs (1964) in the examination of human spermatozoa for blood group antigens, whilst by its means Franks and Dawson (1966) have been able to show variation in the expression of the A and B antigens within clones of cells growing on glass (see Plate 1.1c).

Mixed Antiglobulin Reaction

The mixed agglutination reaction just described is a method of demonstrating antigens on cell surfaces while the mixed antiglobulin reaction is a means of demonstrating the active sensitization of cell surfaces by antibody. No knowledge of the antigen is required in order to perform the reaction. In essence all the reaction purports to do is to demonstrate antibody globulin adsorbed on a cell membrane as in the ordinary antiglobulin test. Unlike this latter test, however, the mixed antiglobulin reaction can be performed on cells which, by nature clump spontaneously, for a positive result is shown by the formation of mixed aggregates of the cells being studied and the sensitized indicator cells. Clumping of the cells under study amongst themselves is immaterial. The rationale of the method is set out simply in Fig. 1.10. The indicator cells may be red cells sensitized with non-agglutinating antibody or red cells with γ-globulin linked to the cells chemically or after treating the cells mildly with tannic acid (Barron, Milgrom, Karzon & Witebsky 1963). Instead of adding free antiglobulin, some investigators (Fagraeus & Espmark 1961; Barron *et al* 1963) add this

already attached to the indicator cells. Again cells may be tested in suspension or attached to glass.

The mixed antiglobulin reaction was used originally (Coombs, Marks & Bedford 1956) in the examination of human sera for iso-antibodies to leucocytes and platelets (see also Garrett, Giles, Coombs & Gurner 1960). These antibodies were also shown to react with HeLa cells (Chalmers, Coombs, Gurner & Dausset 1959). The potentialities of the method in the study of species-specific antigens on the membrane of tissue cells growing as monolayers has been exploited by Espmark & Fagraeus (1962, 1965) and by Milgrom *et al* (1964). Similarly, Abeyounis, Milgrom & Witebsky (1964) have used the method in experiments on homografted mice to show H-2 antibodies reacting with cell monolayer

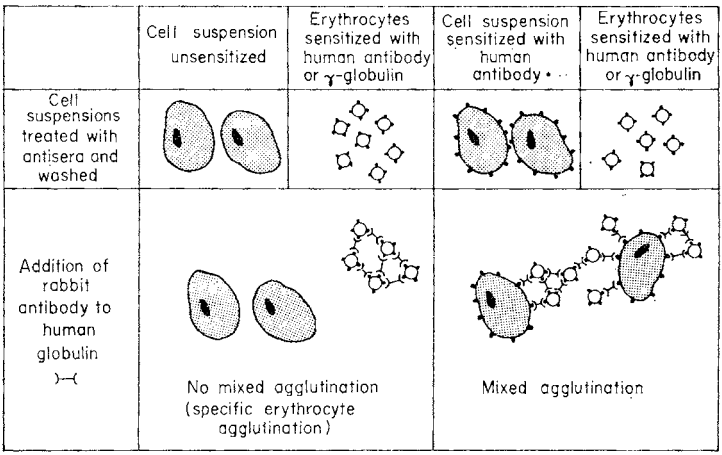
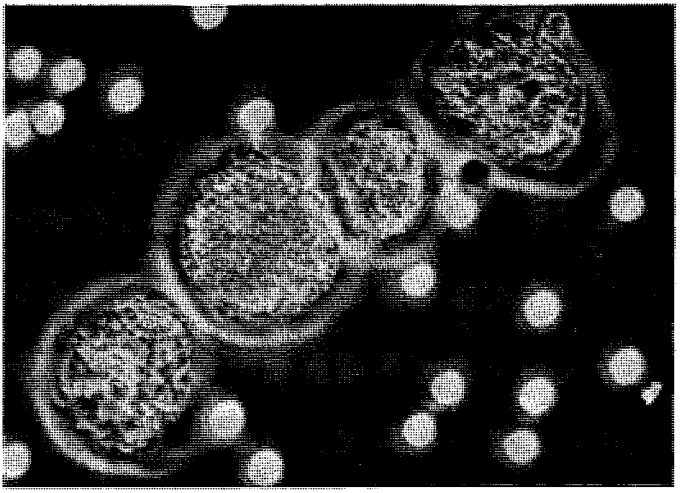


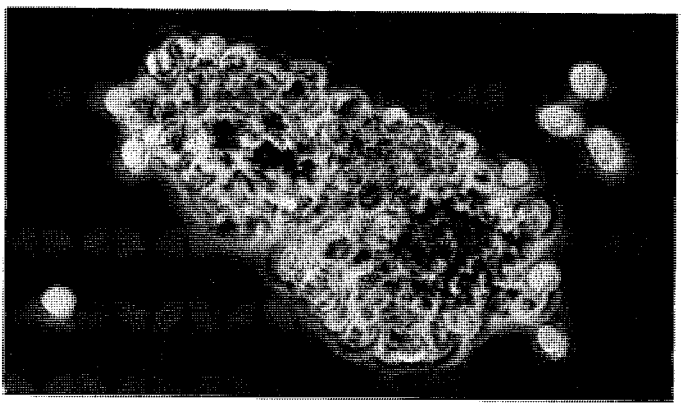
FIG. 1.10. Mixed antiglobulin reaction: for the detection of human antibody on tissue cells.

cultures. Then for organ-specific antigens, Sell *et al* (1968) have successfully used the reaction to show the presence of such antigens on the cell membranes of disaggregated human liver and kidney cells and mucosal cells of stomach and colon.

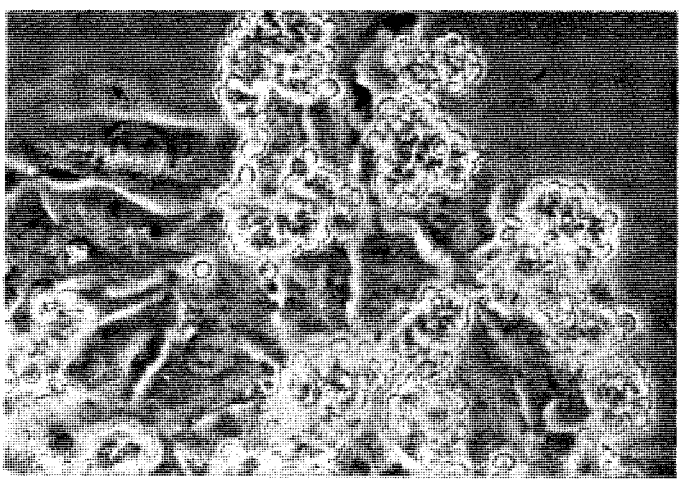
Fagraeus and Espmark (1961) report on what is essentially a mixed antiglobulin reaction (they name it mixed haemadsorption) to show antibody combining with vaccinia and measles virus infected monkey kidney cells in monolayer culture. Barron *et al* (1963) adapted the procedure as a measure for measles antibody in human sera. These studies were extended and further elaborated by Fagraeus, Espmark & Jonsson (1965). In a quite different area again Styles, Dodd & Coombs (1963) found a forensic application for the reaction in the identification of human blood stains (see Chapter 9).



(a)



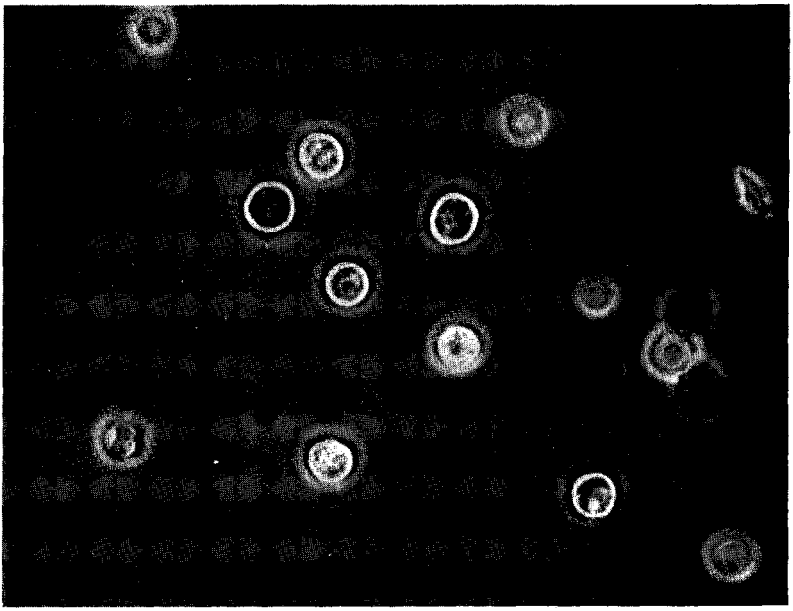
(b)



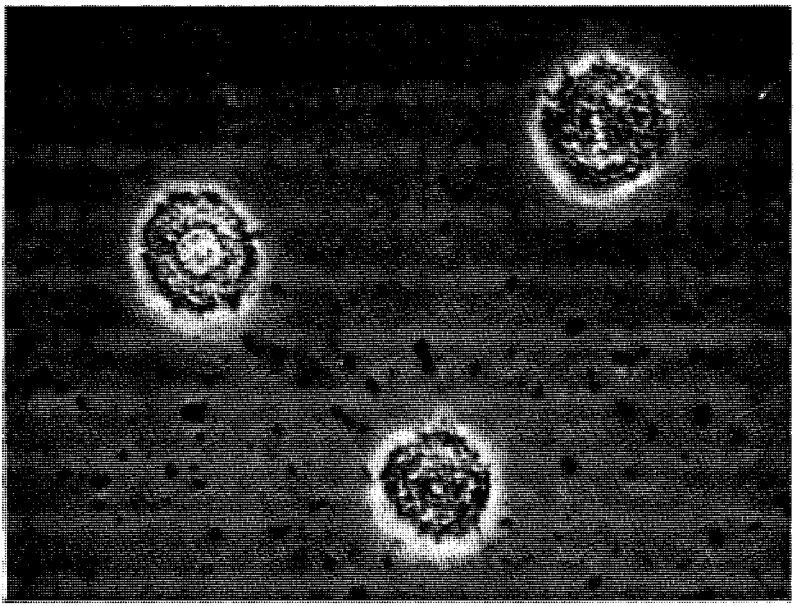
(c)

PLATE 1.1. Mixed Agglutination Reaction.
Mixed agglutination reaction identifying the species of origin of cultured cells (see Coombs, Daniel, Gurner & Kelus, 1961). (*a*) negative reaction, (*b*) positive reaction, (*c*) mixed agglutination reaction on a clone of cells growing as a monolayer on glass. Positively- and negatively-reacting cells within the one clone (taken with permission from Franks & Dawson 1966).

facing p. 32



(a)



(b)

PLATE 1.2. Specific degranulation of isolated mast cells by antigen.
Mast cells pointed with arrow in (*a*) prepared from the peritoneal cavity of rats injected with *Nippostrongylus braziliensis*, degranulated, (*b*) in the presence of an antigenic extract of *N. braziliensis*. Phase-contrast microscopy. Experiments of Hogarth-Scott & Coombs 1967.

facing p. 33

The method is slightly complicated to perform but makes it possible to detect antibody reacting with living or dead tissue cells.

NEUTRALIZATION BY ANTIBODY OF BIOLOGICALLY ACTIVE MOLECULES AND VIRUSES

Bacterial toxins may be considered as soluble bacterial antigens (Oakley 1954) but besides their antigenicity they possess a biological activity or toxicity which an antibody (antitoxin) may be expected, and for clinical reasons is required, to neutralize. In 1954 Pope reviewed the progress since the time of Ehrlich in the work on diphtheria toxin and its antitoxin.

In the analysis of crude toxin preparations gel-diffusion tests are now proving of great value (Ouchterlony 1958). At each stage in the purification the number of reacting systems can be revealed and correlated with the biological activity. Passive haemagglutination procedures are also being harnessed for the measurement of both toxoid and antitoxin (Fulthorpe 1958, 1959, 1962).

Neutralization tests, as already mentioned, still of course provide the final criterion for a protective antiserum, as these tests actually measure the property of the serum with which one is concerned. The test may involve protection against the lethal action of the toxin in mice or guinea-pigs or neutralization of a local action of the toxin in the skin—an example of which is seen in the Schick test. For details on the performance of these tests see Parish (1958). Recommended international standards for different antitoxins are set down by the World Health Organization and most national standards are based on these (see Chapter 49).

'Neutralization tests' are of course frequently used in virological investigations (see Chapter 4) using either protection in the whole animal or inhibition of plaque formation in either chick embryo membranes or in cell culture (Tyrrell 1964; Porterfield 1964).

The neutralizing activity of antitoxins is an important and special illustration of antibody activity against biologically active molecules in general. Further examples are afforded by antibodies directed against enzymes (see Cinader 1963), hormones (see Chapters 10 and 25) and organizers or inducers (Levi-Montalcini 1964). The relatively low association constant for the interaction of antibody and low molecular weight hapten as compared to that existing between the same hapten acting pharmacologically on a cell receptor precludes any successful role for antibody in protection against or neutralization of low molecular weight pharmacologically active substances. It may be remembered that at one time it was thought that an antibody directed against histamine might afford some protection to atopic subjects (Fell, Rodney & Marshall 1943). Similarly an antibody reacting with adrenalin gave no protection against the

pharmacological action of the drug (Went & Kesztyüs 1939; Kesztyüs & Went 1941).

SEROLOGICAL TESTS INVOLVING SERUM COMPLEMENT

Complement, occurring naturally in the serum of man and all animals, must fulfil some physiological role. The present status and recent developments in our knowledge of this substance are discussed in Chapter 14. Besides the significance due to its natural role in the body it is also important as a reagent in diagnostic tests. This has been so since 1901 when the experiments of Bordet and Gengou suggested the complement fixation test which has, over the years, played such a major part in diagnostic serology.

Two very obvious and distinctive *in vitro* manifestations of complement activity are firstly the haemolysis of red cells sensitized with antibody (called haemolysin for this function) and secondly, conglutination (massive aggregation) of red cells sensitized with antibody in the presence of conglutinin. Built around these two properties of complement are four serological tests described below.

Besides these four reactions further tests derive from the participation of complement in opsonization and in the phenomenon of immune adherence. Lastly, with the realization of the possible role of complement in the development of lesions in the body in certain diseased states, increasing attention is being paid to *in vitro* cytotoxic or cytolytic tests on cells other than red blood cells.

Immune Haemolysis

This is the name given to the haemolysis of red cells by the combined action of complement and red cell antibodies. Most of the studies on complement itself have been made on this model (see Chapter 14). It is well suited for kinetic studies for the liberation of haemoglobin, which can be estimated spectroscopically, gives an exact measure of the extent of the reaction at any given time.

The main interest in the reaction for the diagnostic laboratory is in its use in the indicator system of the haemolytic complement fixation test and in the standardization of the reagents for that test (see next section).

Greater interest is now being taken in the level of serum complement in different diseases and the laboratory may be requested to undertake such measurements. In such cases antibody (haemolysin) is held constant and the serum titrated for complement activity. The reverse of this is seen if the haemolysin activity of the Forssman or Paul-Bunnell antibodies are to be measured; in the former case on sheep cells and in the latter case on ox red cells (Mason 1951). Demonstration of the haemolysin activity of the human anti-A isoantibody, as opposed to its agglutinating activity, is another example of this reaction (Mollison 1967).

Red cells carrying adsorbed polysaccharides, as in passive haemagglutination tests, are not only agglutinated by the corresponding antibody to the polysaccharide but are also lysed if complement is present (for a review see Neter 1956).

Haemolytic Complement Fixation Test

In principle this test is still the same as that described in 1901 by Bordet & Gengou. If a standard dose of complement is added to a mixture of antigen and corresponding antibody it is absorbed by the antigen-antibody complexes. This removal of the free complement is made evident in a second stage of the test by adding a mixture of red cells and corresponding haemolysin. Failure of these red cells to lyse indicates that a specific antigen-antibody reaction occurred in the first stage of the test.

The principles underlying the test are very simple but literally thousands of papers and many books have been written about the technical details and it should be stressed that both knowledge and experience are required in the design of tests and in the interpretation of results. In setting up a complement fixation test it is important to consider the 'fixability' of the complement selected (i.e. which species) by the antibodies under test and not simply by the antibody sensitized red cells of the indicator system (Hoet, Blomfield & Coombs 1954).

Despite the encroachment of passive agglutination procedures in investigations which used to be done by the complement fixation test, this latter reaction is still of great value as will be appreciated from Chapters 2–5. It can be used with all types of antigens, soluble or particulate, protein, carbohydrate or lipid. It is specially of value with lipid antigens. Again it is well suited for tests where the antigen is in a far from pure state, for instance virus-containing tissue extracts. For this reason it is frequently used in epidemiological surveys with this sort of antigen.

The Wassermann reaction is a complement fixation test which, besides its diagnostic importance, is interesting in that it uses, as antigen, a lipid extracted from normal tissues against which there is an antibody in the serum of syphilitics. For the use of complement fixation tests in auto-'immune' disease (AICF tests) see Chapter 32.

Technical developments in the complement fixation test involve adaptation to micro-techniques using plastic plates instead of tubes or to automation using equipment such as the Auto-Analyser (Irvine 1966; Gaillon *et al* 1967).

Complement fixation may also be demonstrated on tissue sections using a fluorescein-tagged anti-β1c serum (see section on Fluorescent Antibody Techniques).

Direct Conglutination Reaction

The complement in the serum of some species of animals is not haemolytic but may nevertheless be shown by its property of producing massive aggrega-

tion (conglutination) of sensitized red cells in the presence of conglutinin, the heat-stable factor present normally in bovine serum. This model system gives another forum for the more esoteric studies on complement itself.

Although the direct conglutination reaction has in the past been used to enhance the activity of weakly agglutinating bacterial antibodies it has little application for this purpose at the present time. Its importance in serodiagnosis today, lies in its use in the indicator system of the conglutinating complement absorption test (see next section) and in the measurement of the interesting substances conglutinin and the immuno-conglutinins.

The serology of conglutination and experimental work on the stimulation and activities of immuno-conglutinin formed the subject of a monograph by Coombs, Coombs & Ingram (1961). New work since then on conglutinin, the complement components involved in conglutination and the specificities of immuno-conglutinin is mainly due to Lachmann and has recently been reviewed by him (Lachmann 1967).

Conglutinating Complement Absorption Test (CCAT)

The fact that complement is an essential component for conglutination allows a complement absorption test to be set up in an analogous manner to the more familiar haemolytic complement fixation test. The CCAT differs, however, in that conglutinating complements are used, as well as the appropriate conglutinating indicator system.

The significance of the reaction lies in the fact that it allows a wide range of complements to be used, which is important because Blomfield, Coombs & Hole (1949) showed that antibodies in the sera of certain species of animals could be demonstrated by complement fixation only if certain conglutinating complement were used.

The full details for performing these tests are given by Coombs, Coombs & Ingram (1961).

Cytotoxic or Cytolytic Tests (Mediated by Antibody and Complement)

Haemolysis of red cells and bacteriolysis or bactericidal action (see Muschel 1965) by antibody and complement are special examples of cytolytic tests but because of the distinctive nature of the cells involved they are usually treated and discussed separately.

To get lysis of antibody-sensitized susceptible bacteria it seems that the lesion produced by complement alone is not sufficient to breach the cell wall and plasma membrane, and that the concerted action of lysozyme acting on a mucopeptide substrate is also required to effect this (Wardlaw 1962). There are also other special categories but, in general, cytotoxic or cytolytic tests refer to the reaction of antibody and complement on other somatic cells of the body or

even on unicellular protozoa (Le Page 1968). The words cytolytic and cytotoxic are often used synonymously although this is, strictly speaking, incorrect. A toxic effect may be produced on a cell without 'lysis' or damage of the membrane resulting in leakage of cytoplasmic material. Whatever the damage to the living cells the nature of the injury requires special and careful study and cannot be observed so simply as with leakage of haemoglobin from red cells. As an illustration the reader is referred to the elegant studies of Goldberg & Green (1959); Green, Fleischer, Barrow & Goldberg (1959) and Green, Barrow & Goldberg (1959) on the cytotoxic action of antibody and complement on ascites tumour cells.

Disregarding processes quite divorced from immunology there are in all probability quite a few types of antigen-antibody reactions capable of damaging or disrupting the membrane of tissue cells and so it is important that the designation cytotoxic or cytolytic be qualified to indicate the mechanism in mind.

Although studies on immune cytolysis go back to the very early days of immunology (see Kolmer 1924a) these were not very fruitful because of the difficulty of isolating pure cell suspensions to work on and the crudity of the techniques used. That cells growing in tissue culture might be useful in cytotoxic studies was suggested by Lambert & Hanes (1911) but it is only in recent years that the method has received proper analysis (Ross & Lepow 1960; Lepow & Ross 1960). Boyse, Old and Thomas (1962) described a simple procedure for cytotoxic tests in relation to transplantation research. Cultured cells for use in such tests may be preserved in special media over liquid nitrogen (Spooner 1965) and a micro-titration cytolytic test using such cells is described by Spooner, Bowden & Carpenter (1965). Viability of cells following a reaction may be judged by their appearance under phase-contrast microscopy or by virtue of the property of living cells to exclude certain dyes such as trypan blue or eosin. Terasaki *et al* (1965) have evaluated the use of a cytotoxic test on lymphocytes as a means of sero-typing tissues previous to homo-transplantation in man. For the relevance of model cytotoxic tests to mechanisms of allergic tissue damage in the body, the reader is referred to the section on Type II reactions described in Chapter 20. Chapters 17 and 18 on the allergic responses to transplantation and to tumours are also relevant.

Immobilization Tests (Mediated by Antibody and Complement)

As has already been discussed it is possible with certain organisms to produce immobilization with antibody alone due to simple agglutination of the cilia or flagella. In other cases as in the immobilization of active *Treponema pallidum* the process is a cytotoxic one for which complement is also necessary.

This effect of antibody and complement on treponema has proved most useful and forms the basis of the *Treponema pallidum* immobilization test (TPI)

which, despite the difficulties of setting up the reaction, has achieved a definite place in the routine serological diagnosis of syphilis. It has the great advantage that it measures antibodies to the causative treponema and not just an antibody to a normal tissue component as is measured in the Wassermann reaction. Details for performance of the TPI test are given by Nelson & Mayer (1949) and in a U.S. Public Health Service Publication (1959).

Immune Adherence

Aggregates of antibody and soluble antigen or bacteria sensitized with antibody have the property, in the presence of complement, of adhering to the red cells of primates (Nelson 1953) and this forms the basis of the immune adherence test. The reaction has its roots in the previously described red cell adhesion test for trypanosomiasis (Duke & Wallace 1930; Wormall 1933) and in the even older 'Rieckenberg reaction' (1917).

The method may be used as a sensitive test for antibody. In tests with bacteria these may be seen under the microscope adhering to the red cells. With soluble antigen-antibody complexes the red cells become clumped (Turk 1964). The reaction is proving very valuable in revealing antigenic determinants on the membrane of tissue cells. A positive reaction is shown by adherence of red cells to the tissue cells in question. Sell & Spooner (1966) find it very sensitive and easy to perform.

A full treatment on the reaction itself is given by Nelson (1963) and by Nelson & Uhlenbruck (1967).

Opsono-Phagocytic Tests

It is many years since measurements of the opsonizing property or opsonic index (Wright & Douglas 1903; 1904) of a blood sample or serum were undertaken in routine investigational work (see Kolmer, 1924b) despite the sound rationale of the method. The difficulties arise from there being so many variables in the test—the bacterial suspension, the antibody, complement and the phagocytes and perhaps, above all, from the subjective element in reading the test.

For experimental work, however, it has importance as an *in vitro* model for the functioning of the RE system (Maale 1946). Clearance from the blood of intravenously injected colloidal suspensions or bacteria by phagocytosis may be used as a measure of reticulo-endothelial function (see Chapter 12). Likewise a shortened survival time of transfused red cells indicating heightened removal of the red cells by the reticulo-endothelial system may be an indication of incompatibility even if no antibody is demonstrable by *in vitro* serological methods (Mollison 1967).

The bactericidal test used by Lancefield (1957) to measure type-specific streptococcal antibodies is really an opsono-phagocytic test.

The LE cell reaction, which is a serological test for the auto-antibody to

nuclear desoxyribonucleo-protein in patients with lupus erythematosus (see Chapter 32) is, of course, another example of an opsono-phagocytic test although a further process is also involved and is responsible for the alteration in the texture of the ingested nuclear material. Dacie (1956) describes a technique for the direct test on the whole blood of a patient and an indirect test for antibody in a patient's serum is described by Lachmann (1961).

A much fuller discussion of opsonins and phagocytosis in relation to immunity is given in Chapter 15. For the role of complement see Chapter 14.

REACTIONS *IN VITRO* OF ACTIVELY ALLERGIZED CELLS AND RELATED PHENOMENA

Demonstration of Antibody-Producing Cells by Immuno-Fluorescence

Plasma cells synthesizing specific antibody are actively allergized cells (see Fig. 15.1) and these may be shown in a tissue section by the immuno fluorescence 'sandwich technique' (see section on Fluorescent antibody techniques and Fig. 1.3).

Demonstration of Antibody-Producing Cells by 'Plaquing' Techniques

Jerne, Nordin & Henry (1963) and Ingraham & Bussard (1964) described methods by which single cells producing haemolytic antibodies for erythrocytes could be identified; when mixed with the appropriate erythrocytes in agar or gum, zones of lysis are demonstrable around them, after short incubation, in the presence of complement. Macroglobulin antibodies are most readily demonstrable, though methods have been described (Dresser & Wortis 1965; Sterzl & Riha 1965) by which 7S IgG antibodies can also be shown. Theoretically production of any antibody which can be used in a haemolytic system can be shown by such methods, though they have been most successful with ordinary haemolysins.

Blast Transformation and Stimulation of Lymphocytes to Mitosis

It is well known that immunization of an animal leads to enlargement of its lymph nodes, i.e. the reaction to antigen involves cell proliferation. Similarly if antigen is mixed *in vitro* with lymph node cells from an immunized animal, proliferation occurs, best measured by the uptake of radioactive thymidine into DNA (Dutton & Eady 1964).

'Cell transformation' or 'Lymphocyte-blast-transformation' is simply a special case of this phenomenon applied to a cell population, namely the blood leucocytes, which is constant in its cellular composition, and not initially in a

state of proliferation; this gives the advantages of easy accessibility and negligible numbers of proliferating cells in control populations, so that morphological criteria as well as those of DNA synthesis can be applied. The whole phenomenon seemed initally mysterious and novel simply because the first observations were on the non-specific transforming powers of various vegetable extracts (lectins, poke-weed extract, etc.): there is no good reason to suppose that these are in any way involved with allergic processes, though the final common pathway, blast transformation leading to mitosis, is the same for phytohaemagglutinin (PHA, a lectin) stimulation as for stimulation by antigen. The phenomenon is of clinical interest for several reasons:

(1) in immunological deficiency syndromes the 'transformability' of the cells, by PHA *or* antigen, may be affected (see Chapter 19).

(2) in a few cases the peripheral lymphocytes of man (but not usually of experimental animals) can show a specific response to some antigens, in particular tuberculins, as measured by a significant percentage of 'blasts' and a thymidine uptake markedly above background (Pearman, Lycette & Fitzgerald 1963; Elves, Roath & Israels 1963). The significance of this is still obscure; it is by no means established as originally claimed, that the transformed cells go on to produce antibody.

(3) The mere fact that a very high percentage (up to 80%) of normal blood lymphoid cells, of which the bulk are small lymphocytes, can rapidly proliferate, reinforces the modern concepts of the versatility of this cell.

(4) PHA stimulation is a splendid method for getting chromosome preparations in man—a technique at present irrelevant to immunology but which started up interest in the *in vitro* techniques as such.

Cells of all animal species so far tested can be 'transformed' under suitable cultural conditions: these, especially for cells of small animals are fairly exacting and have been fully described by Knight *et al* (1965). Unless directions for manipulation, cell numbers, dimensions of vessels, etc., are followed fairly exactly results may be negative, since undefined contiguity relations between cells certainly play some part. Much new work on PHA transformation is reviewed in the monograph edited by Elves (1966).

Anti-lymphocyte iso-antisera raised in rabbits have a 'transforming' effect *in vitro* which is often evident at higher dilution than their cytotoxic activity (Ling 1967): it is not known yet whether this phenomenon is general. The striking *in vivo* immunosuppressive activity of anti-lymphocyte sera is considered in Chapter 17.

Mixed Lymphocyte-Culture Reactions

Lymphoid cells both *in vivo* and *in vitro* react directly, i.e. as if 'pre-sensitized', to the contiguity of cells of a different genotype. Strictly since it is a property of

normal cells this is not an allergic reaction at all: but its relevance to allergic reactivity particularly to homograft reactivity is so great that its proponents (Brent, Brown & Medawar 1962) and nearly everyone else, treat it as if it were. *In vivo*, injection of fairly large numbers of lymphoid cells of a foreign genotype intradermally leads to a reaction (called the normal lymphocyte transfer (NLT) reaction) which macroscopically looks like a typical 'delayed' (Type IV) reaction. It is too early to classify it as such, however, since there is no doubt that the reactivity is mediated by the donor cells and not by those of the host (for discussion and references see Brent & Medawar (1967) and Chapter 17). The mechanism has not yet been elucidated.

In vitro, under the same cultural conditions as 'blast transformation' by PHA, etc., mixed blood lymphocyte reactions between humans are often demonstrable by transformation and thymidine uptake (Bain, Vas & Lowenstein 1964) though those between laboratory animals of the same species are sometimes not. Between species technical difficulties arise since heterospecific sera tend to be toxic: where cultural conditions can be made satisfactory, mixed cell reactivity is evident.

Delayed Allergy: Migration-Inhibition Tests

The laboratory still has little to offer as an *in vitro* measure of the cell allergization underlying delayed hypersensitivity. However certain experimental systems, e.g. that involving 'blast' stimulation by antigen (Oppenheim, Wolstencroft & Gell 1966), reaction of immuno-cyto-adhesion (Nota *et al* 1964) and inhibition by antigen of macrophage migration from capillary tubes (David *et al* 1964) are promising if they can be firmly correlated with the delayed hypersensitivity state and are not mediated by irrelevant antibodies, of special type and in low concentration.

Most information is available on the macrophage-inhibition test, which appears to depend upon the presence of allergized lymphocytes in the mixed peritoneal cell exudates used (see discussion by Dumonde 1967) and from which a substance is released on contact with antigen which affects the mobility of macrophages.

REACTIONS *IN VITRO*, AND IN EXPERIMENTAL ANIMALS, OF PASSIVELY ALLERGIZED CELLS

Allergic tissue reactions in the skin involving both actively allergized (delayed hypersensitivity) or passively allergized cells (immediate reactions) have long been used diagnostically (see Chapter 7) in the patient himself or in some cases the serum of the patient may be tested by passive transfer (Prausnitz-Küstner test) in the skin of a test subject.

In the laboratory, antibodies mediating immediate or anaphylactic hypersensitivity are mainly studied by the Schultz-Dale reaction and passive cutaneous anaphylaxis which are briefly discussed below.

Schultz-Dale Reaction in the Guinea-pig

Anaphylactic antibody in the guinea-pig has for long been studied and characterized as regards its activity by the Schultz-Dale reaction (Dale 1913). The uterus or terminal ileum is removed from an actively or passively sensitized guinea-pig and its contraction measured after addition of the corresponding antigen. Antigen acting on the sensitized tissue liberates histamine and other pharmacologically active substances which initiate the muscle contraction. Coulson (1953) discusses and illustrates the usefulness of the technique in general investigational work. Gut or uterus from a normal animal may be passively sensitized *in vitro* (Hartley 1951; Halpern *et al* 1959). This allows a titration of the antibody to be made.

Passive Cutaneous Anaphylaxis in the Guinea-pig

Guinea-pig anaphylactic antibody can also be measured by its ability to sensitize local areas of guinea-pig skin in the reaction of passive cutaneous anaphylaxis (PCA—Ovary 1964). Serum dilutions in 0.1 ml are injected intradermally into an area of skin clipped free of hair and after 4–24 hr antigen, together with a dye such as Geigy blue, Evans blue or Pontamine blue, is injected intravenously. Convenient quantities to use are 1 mg of antigen plus 2.5 mg dye in a total volume of 0.5 to 1.0 ml. Although no whealing is visible, the sites of reaction are shown by escape of dye from the dilated and affected small vessels and these show up as blue areas. These can be seen more distinctly and measured more accurately by first killing the animal, then stripping the skin and measuring the blue area on the underside.

The guinea-pig seems somewhat unusual as regards its antibody giving this reaction and this is discussed by Ovary (1964) and Bloch (1967). In other species, such as the rat and rabbit, only the homologous reagin-like antibody gives a satisfactory PCA reaction in the skin of the corresponding species. Thus although rabbit IgG antibody will give a PCA reaction in guinea-pig skin, in rabbit skin only the reagin-like rabbit antibody will localize and give this reaction.

Active Cutaneous Anaphylaxis

An animal actively sensitized to an antigen will react to the intradermal injection of minute amounts of that antigen by histamine release: the reaction is demonstrable by the previous injection of blue dye as above. Care must be taken however that non-specific reactions due to the trauma of the injection are properly controlled.

Of particular interest clinically, is the fact that human reagin cannot be measured by a PCA reaction in guinea-pig skin. However this antibody can be demonstrated with a PCA reaction performed in the skin of non-human primates. This leads us on to important recent work on laboratory methods for the detection of human reagin.

Laboratory Procedures now Available for the Detection of Human Reagin

Passive Cutaneous Anaphylaxis Reaction in the Skin of non-Human Primates

Layton showed that human reaginic antibody localized in the skin of non-human primates allowing the human reagin to be demonstrated and titrated in passive transfer tests in skin other than human. Details for the performance of the test are given by Layton and his colleagues (1963). It is very valuable to have this reliable method (Rose *et al* 1964) as a measure of reaginic activity in human serum because of the understandable reluctance in many countries to perform the Prausnitz-Küstner reaction with the attendant danger of infecting the test-subject with serum hepatitis. Limiting aspects are that few laboratories have monkeys available for testing, their cost and the dangers associated with working with primates, e.g. infection with B virus—*Herpes virus simiae* (Hartley 1966). Also, in our own experience, occasional baboons are found to be refractory test subjects and the undoubted fact that if many sera are tested at the one time, thus increasing the amount of human serum injected, then the animal cannot be relied on to serve for repeated tests at intervals, since it becomes refractory. This is due in all probability to antibody being produced to some of the human serum proteins injected.

Schultz-Dale Reaction Using *in vitro* Passive Sensitization of Monkey Ileum or Human Appendix

Following the observations by Layton and his colleagues that the tissue cells of non-human primates may be passively sensitized or passively allergized by human reaginic antibody, Arbesman, Girard & Rose (1964) showed that human reagin could be demonstrated and titrated by the Schultz-Dale reaction if monkey ileum was used instead of that of the guinea-pig. This method again is limited to places where monkeys are being regularly killed to supply fresh strips of ileum. Chopra *et al* (1966) have used with success strips of human appendix instead of monkey ileum.

A chain made of bronchial rings from an atopic patient was shown by Schild *et al* (1951) to contract on addition of allergen, and this could be done presumably following passive sensitization *in vitro* although today this would have little of advantage to offer in the way of a routine test.

Mast Cell or Basophil Degranulation

Antigen added to tissues containing mast cells from anaphylactically sensitive guinea-pigs (Humphrey & Mota 1959) or to isolated mast cells from sensitized rats (see Plate 1.2, facing p. 33) causes degranulation of these cells. In man this could form the basis of a test for sensitization by reaginic and possibly other tissue sensitizing antibody if an objective measure of blood basophil degranulation could be established. Shelley & Juhlin (1961) claimed to have achieved this, and again in 1962 Shelley described an '*indirect basophil degranulation test*' which he stated allowed a serum to be examined for antibody capable of passively sensitizing basophils from a normal individual or even from a rabbit. The experience of other investigators has not been so successful although the principle of the method is sound, especially if human basophils are used. A great difficulty is the low percentage of basophils in the blood leucocytes.

Liberation of Histamine and other Muscle-Contracting Mediators from Sensitized Cells or Tissues of Man Himself

Lung

Schild *et al* (1951) showed that on addition of allergen, histamine was released from a chopped lung removed at operation from an atopic subject. This may also be achieved after passive sensitization of lung tissue *in vitro* (O'Sullivan & Augustin 1966). Fresh human lung is frequently available these days following surgical lobectomy.

Blood cells

Katz & Cohen (1941) showed that histamine was released from blood cells (white cells) into the plasma on addition of allergen to whole blood of hay fever patients. Using either a biological assay of histamine (and other active substances) or a microchemical procedure (Lowry *et al* 1954) this phenomenon could form the basis for a reliable investigative procedure (Noah 1964; v. Arsdel *et al* 1958; Lichtenstein & Osler 1964). Hastie (1968) using his own modifications demonstrates the constancy of the method and a definite correlation with basophil involvement.

The difficulties still to be overcome before a satisfactory 'passive sensitization reaction' can be set up to test for reaginic antibodies in a serum sample are illustrated in a paper by Levy & Osler (1966).

Double Layer Leucocyte Agglutination Reaction

In 1964 Ridges & Augustin reported a 'double layer leucocyte agglutination reaction' which according to them measured reagin (see also Fitzpatrick *et al*

1967). In this reaction human leucocytes (polymorphonuclear) passively sensitized with reagin in the body or *in vitro* and reacted on by the specific pollen allergen are agglutinated by a rabbit anti-pollen serum. Appraisal from other laboratories is still awaited. As with tests based on degranulation of mast cells a reaction such as this is sound enough in principle but the technical difficulties are great.

Red Cell Linked-Antigen Antiglobulin Reaction

If reagin is proved finally to be IgE as now seems likely (Ishizaka, Ishizaka & Hornbrook 1966) then its detection in serum may be possible by the red cell linked antigen-antiglobulin reaction (see above and Coombs *et al* 1965) using a specific anti-IgE serum when such a serum becomes available.*

Detection of non-Reaginic Macrophage-Cytophilic Antibody

Besides reagins other cytophilic antibodies are known (see Nelson & Boyden 1967). The macrophage-cytophilic antibody, especially that produced in guinea-pigs, can be visualized as it passively sensitizes the cell membrane of the macrophage by adherence of antigen-carrying red cells (Boyden 1964) in a rosette-forming-reaction (Jonas *et al* 1965). Unlike the case with reagin the significant role of this antibody is still to be determined.

REFERENCES

Abeyounis C.J., Milgrom F. & Witebsky E. (1964) Homotransplantation antibodies detected by mixed agglutination with cell culture monolayers. *Nature (Lond.)* **203,** 313.

Allison A.C. & Humphrey J.H. (1959) Estimation of the size of antigens by gel diffusion methods. *Nature (Lond.)*, **183,** 1590.

Allison A.C. & Humphrey J.H. (1960) A theoretical and experimental analysis of double diffusion precipitin reactions in gels, and its application to characterization of antigens. *Immunology*, **3,** 95.

Allison A.C. & Morton J.A. (1953) Species specificity in the inhibition of antiglobulin sera. *J. clin. Path.* **6,** 314.

Anderson J.R. (1954) Application of antiglobulin reactions to blood stains in determination of animal species. *Amer. J. clin. Path.* **24,** 920.

Arbesman C.E., Girard R. & Rose N.R. (1964) Demonstration of human reagin in the monkey. *J. Allergy* **35,** 535.

Ascoli M. (1902) Ueber den Mechanismus der Albuminurie durch Eiereiweiss. *Münch. med. Wschr.* **49,** 398.

Augustin R. & Hayward B.J. (1955) Standardization of pollen extracts by gel diffusion. *Int. Arch. Allergy* **6,** 154.

Bain, B., Vas M.R. & Lowenstein L. (1964) The development of large immature mononuclear cells in mixed leucocyte cultures. *Blood*, **23,** 108.

Barron A.L., Milgrom F., Karzon D.T. & Witebsky E. (1963) Demonstration of human measles antibody by mixed agglutination. *J. Immunol.* **90,** 908.

* A recent report suggests it may be possible to detect reagin by a radioimmunoabsorbant test. See Wide, Bennich and Johansson (1967).

BEALE G.H. (1954) *The Genetics of Paramecium aurelia.* London: Cambridge University Press.

BLOCH K.J. (1967) The anaphylactic antibodies of mammals including man. In *Progress in Allergy*, vol 10, p. 84. Ed. KALLOS P. & WAKSMAN B.H. Basel: S. Karger.

BLOMFIELD ANNE M., COOMBS R.R.A. & HOLE N.H. (1949) The conglutination phenomenon. V. Further experiments on the importance of the choice of complement when examining antisera for the presence of complement-fixing or complement-absorbing antibodies. *J. Hyg. Camb.* **47,** 132.

BOORMAN KATHLEEN E. & DODD BARBARA E. (1966) *An Introduction to Blood Group Serology*, 3rd ed. London: J. & A. Churchill.

BORDET J. & GENGOU O. (1901) Sur l'existence de substances sensibilisatrices dans la plupart des sérums antimicrobiens. *Ann. Inst. Pasteur* **15,** 289.

BOYDEN A.A. (1954) *Serological Approaches to Studies of Protein Structure and Metabolism*, p. 74, Ed. COLE W.H. New Jersey: Rutgers University Press.

BOYDEN S.V. (1950) Adsorption by erythrocytes of antigens of Pfeifferella mallei and Pfeifferella whitmore. *Proc. Soc. exp. Biol.* (N.Y.) **73,** 289.

BOYDEN S.V. (1951) Adsorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by antiprotein sera. *J. exp. Med.* **93,** 107.

BOYDEN S.V. (1964) Cytophilic antibody in guinea pigs with delayed-type hypersensitivity. *Immunology* **7,** 474.

BOYSE E.A., OLD L.J. & THOMAS GLORIA (1962) A report on some observations with a simplified cytotoxic test. *Transplant. Bull.* **29,** 63.

BOZICEVICH J., BUNIM J.J., FREUND J. & WARD S.B. (1958) Bentonite flocculation test for rheumatoid arthritis. *Proc. Soc. exp. Biol. (N.Y.)* **97,** 180.

BOZICEVICH J., NASOU J.P. & KAYHOE D.E. (1960) Desoxyribonucleic acid (DNA)—bentonite flocculation test for lupus erythematosus. *Proc. Soc. exp. Biol. (N.Y.)* **103,** 636.

BOZICEVICH J., TOBIE J.E., THOMAS E.H., HOYEM H.M. & WARD S.B. (1951) A rapid flocculation test for the diagnosis of trichinosis. *Publ. Hlth. Rep. Wash.* **66,** 806.

BRENT L., BROWN J.B. & MEDAWAR P.B. (1962) Quantitative studies in tissue transplantation immunity. VI. *Proc. roy. Soc. B.* **156,** 187.

BRENT L. & MEDAWAR P.B. (1967) Cellular immunity and the homograft reaction. *Brit. med. Bull.* **23,** 55.

CANNON P.R. & MARSHALL C.E. (1940) An improved serologic method for the determination of the precipitative titers of antisera. *J. Immunol.* **38,** 365.

CHADWICK C.S., MCENTEGART M.G. & NAIRN R.C. (1958) Fluorescent protein tracers: a simple alternative to fluorescein. *Lancet* **i,** 412.

CHALMERS D.G., COOMBS R.R.A., GURNER B.W. & DAUSSET J. (1959) The mixed antiglobulin reaction in the detection of human iso-antibodies against leucocytes, platelets and Hela cells. *Brit. J. Haemat.* **5,** 225.

CHOPRA S.L., KOVACS B.A., ROSE B. & GOODFRIEND L. (1966) Detection of human reagins by Schultz-Dale Technique using human appendix. *Int. Arch. Allergy* **29,** 393.

CINADER B. (1963) Antibody to enzymes—a three component system. *Ann. N.Y. Acad. Sci.* **103,** 493.

COLE L.R. & FARRELL V.R. (1955) A method for coupling protein antigens to erythrocytes. I. Description of method. *J. exp. Med.* **102,** 631.

COOMBS R.R.A. (1961) The mixed agglutination reactions in the study of normal and malignant cells. *Cancer Res.* **21,** 1198.

COOMBS R.R.A. (1964) In *Immunological Methods*, C.I.O.M.S. Symposium, Ed. ACKROYD J.F. & TURK J.L. Oxford: Blackwell Scientific Publications.

COOMBS R.R.A., BEDFORD D. & ROUILLARD L.M. (1956) A and B blood group antigens on human epidermal cells demonstrated by mixed agglutination. *Lancet* **i,** 461.

COOMBS R.R.A., COOMBS ANNE M. & INGRAM D.G. (1961) *The Serology of Conglutination and its Relation to Disease*. Oxford: Blackwell Scientific Publications.

COOMBS R.R.A., DANIEL MARY R., GURNER B.W. & KELUS A.S. (1961) Species-characterizing antigens of 'L' and 'ERK' cells. *Nature (Lond.)* **189,** 503.

COOMBS R.R.A. & DODD BARBARA (1961) Possible application of the principle of mixed agglutination in the identification of blood stains. *Med. Sci. and L.* **1,** 359.

COOMBS R.R.A. & FISET M.L. (1954) Detection of complete and incomplete antibodies to egg albumin by means of a sheep red cell–egg albumin unit. *Brit. J. exp. Path.* **35,** 472.

COOMBS R.R.A., HOWARD A.N. & MYNORS L.S. (1953) A serological procedure theoretically capable of detecting incomplete and non-precipitating antibodies to soluble protein antigens. *Brit. J. exp. Path.* **34,** 525.

COOMBS R.R.A., JONAS W.E., LACHMANN P.J. & FEINSTEIN A. (1965) Detection of IgA antibodies by the red cell linked antigen-antiglobulin reaction: Antibodies in the sera of infants to milk proteins. *Int. Arch. Allergy* **27,** 321.

COOMBS R.R.A., MARKS J. & BEDFORD D. (1956) Specific mixed agglutination; mixed erythrocyte-platelet antiglobulin reaction for the detection of platelet antibodies. *Brit. J. Haemat.* **2,** 82.

COOMBS R.R.A. & MOURANT A.E. (1947) On certain properties of antisera prepared against human serum and its various protein fractions. *J. Path. Bact.* **59,** 105.

COOMBS R.R.A., MOURANT A.E. & RACE R.R. (1945) A new test for the detection of weak and 'incomplete' Rh agglutinins. *Brit. J. exp. Path* **26,** 255.

COOMBS R.R.A. & ROBERTS, FULTON (1959) Antiglobulin reaction. *Brit. med. Bull.* **15,** 113.

COONS A.H. (1956) Histochemistry with labelled antibody. *Int. Rev. Cytol.* **5,** 1.

COONS A.H. & KAPLAN M.H. (1950) Localization of antigen in tissue cells—II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. exp. Med.* **91,** 1.

COULSON E.J. (1953) The Schultz-Dale technique. *J. Allergy* **24,** 458.

CROWLE A.J. (1961) *Immunodiffusion*. Academic Press: N.Y. and London.

CSIZMAS L. (1960) Preparation of formalinized erythrocytes. *Proc. Soc. exp. Biol. (N.Y.)*, **103,** 157.

DACIE J.V. (1956) *Practical Haematology*, p. 146, 2nd ed. London: J. & A. Churchill.

DALE H.H. (1913) The anaphylactic reaction of the plain muscle of the guinea pig. *J. Pharmacol.* **4,** 167.

DARCY D.A. (1960) A quantitative application of the agar-diffusion plate: the estimation of specific proteins in serum. *Immunology* **3,** 325.

DAUSSET J. & BRECY H. (1958) Test direct de consommation de l'antiglobuline sur les leucocytes et les plaquettes de certains malades atteints de pancytopénies idiopathiques. *Vox Sang.* **3,** 197.

DAUSSET J. & COLOMBANI J. (1964) Detection of anti-leucocyte and anti-platelet antibodies by the antiglobulin consumption test. In *Immunological Methods*, p. 575, Ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

DAVID J.R., AL-ASKARI S., LAWRENCE H.S. & THOMAS L. (1964) Delayed hypersensitivity in vitro. *J. Immunol.* **93,** 264.

DEAN H.R. & WEBB R.A. (1926) The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. *J. Path. Bact.* **29,** 473.

DODINVAL P. (1957) Sensibilité de la réaction d'inhibition de l'antiglobuline appliquée à la determination du caractère humain des taches de sang. *Acta clin. belg.* **12,** 169.

DRESSER D.W. & WORTIS H.H. (1965) Use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency. *Nature (Lond.)* **208,** 859.

DUCOS J. (1958) Une réaction d'hémagglutination passive utilisable pour l'identification du sang humain et animal. *Rev. franc. Étud. clin. biol.* **3,** 875.

DUKE H.L. & WALLACE J.M. (1930) 'Red cell adhesion' in trypanosomiasis of man and animals. *Parasitology* **22,** 414.

DUMONDE D.C. (1967) The role of the macrophage in delayed hypersensitivity. *Brit. med. Bull.* **23,** 9.

DUNSFORD I. & BOWLEY C.C. (1955) *Techniques in Blood Grouping.* Oliver & Boyd, Edinburgh.

DUNSFORD I. & GRANT JEAN (1959) *The Antiglobulin (Coombs) Test.* Edinburgh: Oliver & Boyd.

DUTTON R.W. & EADY J.D. (1964) An *in vitro* system for the study of antigenic stimulation in the secondary response. *Immunology* **7,** 40.

EDWARDS R.G., FERGUSON L.C. & COOMBS R.R.A. (1964) Blood group antigens on human spermatozoa. *J. Reprod. Fertil.* **7,** 153.

ELEK S.D. (1948) Thesis. London University.

ELEK S.D. (1949) The serological analysis of mixed flocculating systems by means of diffusion gradients. *Brit. J. exp. Path.* **30,** 484.

ELVES M.W. Ed. (1966) *The Biological Effects of Phyto-Haemagglutination,* Symposium publication the Robert Jones & Agnes Hunt Hospital, Oswestry.

ELVES M.W., ROATH S. & ISRAELS M.C.G. (1963) The response of lymphocytes to antigenic stimulus in vitro. *Lancet* **i,** 806.

ESPMARK A. & FAGRAEUS A. (1962) Detection of antigens in tissue culture with the aid of mixed haemadsorption. *Acta path. microbiol. scand. Suppl.* **154,** 258.

ESPMARK J.A. & FAGRAEUS A. (1965) Identification of the species of origin of cells by mixed haemadsorption: a mixed antiglobulin reaction applied to monolayer cell cultures. *J. Immunol.* **94,** 530.

FAGRAEUS A. & ESPMARK A. (1961) Use of a 'Mixed haemadsorption' method in virus infected tissue cultures. *Nature, (Lond.)* **190,** 370.

FAGRAEUS A., ESPMARK J.A. & JONSSON J. (1965) Mixed haemadsorption: a mixed antiglobulin reaction applied to antigens on a glass surface. *Immunology* **9,** 161.

FAHEY J.L. & MCKELVEY M. (1965) Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* **94,** 84.

FARR R.S. (1958) A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. *J. infect. Dis.* **103,** 239.

FEINBERG J.G. (1958) *Proc. IV Internat. Congress on Biological Standardization.* p. 195. Brussels.

FELL N., RODNEY G. & MARSHALL D.E. (1943) Histamine-protein complexes; synthesis and immunologic investigation. *J. Immunol.* **47,** 237.

FITZPATRICK M.E., CONNOLLY R.C., LEA D.J., O'SULLIVAN S.A., AUGUSTIN R. & MACAULAY M.B. (1967) *In vitro* detection of human reagins by double-layer leucocyte agglutination: Method and controlled blind study. *Immunology* **12,** 1.

FRANKS D. & DAWSON A. (1966) Variation in the expression of blood group antigen A in clonal cultures of rabbit cells. *Exp. Cell Res.* **42,** 543.

FRANKS D. & GURNER B.W. (1965) Leucocyte allo-antigens as markers on cultured human cells. *Exp. Cell Res.* **37,** 693.

FREUND J. (1947) Some aspects of active immunization. *Annual Review of Microbiology,* vol. 1, p. 291. California: Annual Reviews Inc.

FREUND, J. (1951) Effect of paraffin oil and Mycobacteria on antibody formation and sensitization; review. *Am. J. Clin. Path.* **21,** 645.

FRIEDMANN O., HUNTER A. & COOMBS R.R.A. (1967) To be published.

FULTHORPE A.J. (1957) Tetanus antitoxin titration by haemagglutination. *J. Hyg. Camb.* **55,** 382.

FULTHORPE A.J. (1958) Estimation of tetanus toxoid by different methods, including haemagglutination inhibition. *Immunology* **1,** 365.

FULTHORPE A.J. (1959) Factors influencing haemagglutination tests with tetanus antitoxin. *Immunology* **2,** 104.

FULTHORPE A.J. (1962) Multiple diphtheria antigen-antibody systems investigated by passive haemagglutination techniques and other methods. *Immunology* **5,** 30.

FULTHORPE A.J., ROITT I.M., DONIACH D. & COUCHMAN K.G. (1961) A stable sheep cell preparation for detecting thyroglobulin auto-antibodies and its clinical application. *J. clin. Path.* **14,** 654.

GAILLON R., RIPAULT J., STUDIEVIC C. & DAUSSET J. (1967) Use of the Auto-Analyzer to perform serological tests for syphilis. *Int. Arch. Allergy.* **32,** 278.

GARRETT J.V., GILES H. McC., COOMBS R.R.A. & GURNER B.W. (1960) Neonatal purpura with platelet iso-antibody in maternal serum. *Lancet* **i,** 521.

GELL P.G.H. (1957) The estimation of the individual human serum proteins by an immunological method. *J. clin. Path.* **10,** 67.

GOLDBERG B. & GREEN H. (1959) The cytotoxic action of immune gamma-globulin and complement on Krebs ascites tumor cells. I. Ultrastructure Studies. *J. exp. Med.* **109,** 505.

GORDON J., ROSE B. & SEHON A.H. (1958) Detection of 'non-precipitating' antibodies in sera of individuals allergic to ragweed pollen by an *in vitro* method. *J. exp. Med.* **108,** 37.

GOSSELIN M.L., MYNORS L.S., COOMBS R.R.A. & MILNER F.H. (1953) Serological studies on pollen extracts. II *Acta. Allerg. Kbh.* **6,** 96.

GOUDIE R.B., HORNE C.H.W. & WILKINSON C.C. (1966) A simple method for producing antibody specific to a single selected diffusible antigen. *Lancet* **ii,** 1224.

GRABAR P. & BURTIN P. (1964) *Immuno-Electrophoretic Analysis: Applications to Human Biological Fluids.* Elsevier Publ. Co. London.

GRABAR P. & WILLIAMS C.A. (1955) Méthode immuno-électrophorétique d'analyse de mélanges de substances antigéniques. *Biochem. et biophys. acta* **17,** 67.

GRANT G.H. (1964) Gel diffusion techniques. In *Recent Advances in Clinical Pathology.* Series IV, Ed. S.C. DYKE. London: J. & A. Churchill.

GREEN H., BARROW P. & GOLDBERG P. (1959) Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes. *J. exp. Med.* **110,** 699.

GREEN H., FLEISCHER R.A., BARROW P. & GOLDBERG B. (1959) The cytotoxic action of immune gamma-globulin and complement on Krebs ascites tumor cells. II. Chemical studies. *J. exp. Med.* **109,** 511.

GREENBURY C.L. (1960) A comparison of the Rose-Waaler latex fixation 'RA test' and bentonite flocculation tests. *J. Clin. Path.* **13,** 325.

GRIFFITTS J.J. (1947) Agglutination and an agglutination 'blocking' property in serums from known cases of brucellosis. *Publ. Hlth Rep. Wash.* **62,** 865.

GRUBB R. (1956) A method for demonstrating minute amounts of human γ-globulin. *Acta path. microbiol. scand.* **38,** 339.

HABERMAN S. (1955) Detecting non-agglutinating antibodies to the tubercle bacillus by Coombs' test. *Brit. med. J.* **ii,** 620.

HAGIWARA A. (1962) A new method for the detection of blood group antigen and its application to F.L. human amnion cell. *Exp. Cell Res.* **28,** 615.

HALL W.H. & MANION R.E. (1951) Haemagglutinins and haemolysins for erythrocytes sensitized with tuberculin in pulmonary tuberculosis. *J. clin. Invest.* **30,** 1542.

HALPERN B.N., LIACOPOULOS P., LIACOPOULOS-BRIOT MONIQUE, BINAGHI R. & VAN NEER F. (1959) Patterns of *in vitro* sensitization of isolated smooth muscle tissues with precipitating antibody. *Immunology* **2,** 351.

HARTLEY E.G. (1966) 'B' virus: herpes virus simiae. *Lancet* **i**, 87.

HARTLEY P. (1951) The effect of peptic digestion on the properties of diphtheria antitoxin. *Proc. roy. Soc. B.* **138**, 499.

HASTIE A. R. (1968) In preparation.

HOET J.J., BLOMFIELD A.M. & COOMBS R.R.A. (1954) The selective absorption of haemolytic complements by antibodies from different species of animals. *Brit. J. exp. Path.* **35**, 32.

HUMPHREY J.H. & MOTA I. (1959) The mechanism of anaphylaxis: Specificity of antigen-induced mast cell damage in anaphylaxis in the guinea pig. *Immunology* **2**, 31.

HUNTER A., FEINSTEIN A. & COOMBS R.R.A. (1967) In preparation.

INGRAHAM J.S. & BUSSARD A. (1964) Application of a localised haemolysin reaction for specific detection of individual antibody-producing cells. *J. exp. Med.* **119**, 667.

IRVINE W.J. (1966) Automated determinations of thyroid and gastric complement-fixing antibody. *Clin. exp. Immunol* **1**, 341.

ISHIZAKA K., ISHIZAKA T. & HORNBROOK M.M. (1966) Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity with γ E globulin antibody. *J. Immunol.* **97**, 840.

JANDL J.H. & CASTLE W.B. (1956) Agglutination of sensitized red cells by large anisometric molecules. *J. Lab. clin. Med.* **47**, 669.

JERNE N.K., NORDIN A.A. & HENRY C. (1963) The agar plaque technique for recognizing antibody producing cells. In *Cell-bound Antibodies*, Ed. AMOS B. & KOPROWSKI B. Philadelphia: Wistar Institute Press.

JOHNSON H.M., BRENNER K. & HALL H.E. (1966) The use of a water-soluble carbodiimide as a coupling reagent in the passive haemagglutination test. *J. Immunol.* **97**, 791.

JONAS W.E., GURNER B.W., NELSON D.A. & COOMBS R.R.A. (1965) Passive sensitization of cells. I. Passive sensitization of macrophages by guinea pig cytophilic antibody. *Int. Arch. Allergy* **27**, 86.

JONES F.S. (1927) Agglutination by precipitin. *J. exp. Med.* **46**, 303

KABAT E.A., & MAYER, M.(1961) *Experimental Immunochemistry*, 2nd ed, Springfield, Ill., U.S.A.: Charles C Thomas.

KATZ G. & COHEN S. (1941) Experimental evidence for histamine release in allergy. *J. amer. med. Ass.* **117**, 1782.

KAY H.E.M. & WALLACE D.M. (1961) A and B antigens of tumors arising from urinary epithelium. *J. nat. Cancer Inst.* **26**, 1349.

KEOGH E.V., NORTH E.A. & WARBURTON M.F. (1947) Haemagglutinins of the haemophilus group. *Nature (Lond.)* **160**, 63.

KERR W.R., COGHLAN J.D., PAYNE D.J.H. & ROBERTSON L. (1966) Laboratory diagnosis of chronic brucellosis. *Lancet* **ii**, 1181.

KERR W.R., PAYNE D.J.H., ROBERTSON L. & COOMBS R.R.A. (1967) Immunoglobulin class of brucella antibodies in human sera. *Immunology*. **13**, 223.

KERR W.R. & ROBERTSON M. (1941) An investigation into the infection of cows with *Trichomonas foetus* by means of the agglutination reaction. *Vet. J.* **97**, 351.

KESZTYÜS L. & WEST S. (1941) Über die physiologischen Wirkurgen einiger Amino- und Nitrobrenzcatechin-Verbindungen und Adrenalylazoproteine. *Arch. exp. Path. Pharmak.* **198**, 338.

KOLMER J.A. (1924a) *Infection, Immunity and Biologic Therapy*, p. 566, 3rd ed. W. B. Saunders Co.

KOLMER J.A. (1924b) *Infection, Immunity and Biologic Therapy*, p. 176, 3rd ed. W.B. Saunders Co.

KNIGHT S., LING N.R., SELL S. & OXNARD C.E. (1965) The transformation *in vitro* of the peripheral lymphocytes of some laboratory animals. *Immunology* **9**, 565.

KRAUS R. (1897) Ueber specifische Reactionen in keimfreien Filtraten aus Cholera, Typhus und Pestbouillonculturen, erzeugt durch homologes Serum. *Wien. Klin. Wschr.* **10,** 736.

LACHMANN P.J. (1961) A two-stage indirect L.E. cell test. *Immunology* **4,** 142.

LACHMANN P.J. (1967) Conglutinin and immuno-conglutinins. *Adv. Immunol.* **6.** 479.

LAMBERT R.A. & HANES F.M. (1911) The cultivation of tissues *in vitro* as a method for the study of cytotoxins. *J. exp. Med.* **14,** 453.

LANCEFIELD REBECCA C. (1933) A serological differentiation of human and other groups of hemolytic streptococci. *J. exp. Med.* **57,** 571.

LANCEFIELD REBECCA C. (1957) Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. *J. exp. Med.* **106,** 525.

LAWLIS J.F. (1958) Serological detection of desoxyribonucleic acid (DNA) adsorbed to formalinized erythrocytes. *Proc. Soc. exp. Biol (N.Y.)* **98,** 300.

LAYTON J.L., YARNAKA E., GREENE F.C. & PERLMAN F. (1963) Atopic reagins to penicillin, pollens and seeds: thermostability, titer and persistence in the skin of passively sensitized macaque monkeys. *Int. Arch. Allergy* **23,** 87.

LE PAGE R.W.F. (1968) Immune lysis of trypanosomes: description of the method and its use for the detection of antigenic variation in T. congolense. *Ann. trop. Med. Parasit.* In press.

LEPOW I.H. & ROSS A. (1960) Studies on immune cellular injury. 11. Functional role of C'1 esterase in immune cytotoxicy. *J. exp. Med.* **112,** 1107.

LICHTENSTEIN L.M. & OSLER A.G. (1964) Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leucocytes by ragweed pollen antigen. *J. exp. Med.* **120,** 507.

LEVI-MONTALCINI R. (1964) Growth control of nerve cells by a protein factor and its antiserum. *Science* **143,** 105.

LEVY D.A. & OSLER A.G. (1966) Studies on the mechanisms of hypersensitivity phenomena. XIV. Passive sensitization *in vitro* of human leucocytes to ragweed pollen antigens. *J. Immunol.* **97,** 203.

LING N.R. (1961a) The coupling of protein antigens to erythrocytes with difluorodinitrobenzene. *Immunology* **4,** 49.

LING N.R. (1961b) The attachment of proteins to aldehyde-tanned cells. *Brit. J. Haemat.* **7,** 299.

LING N.R. (1967) In *Immunosuppression.* Ciba Foundation study group. In press.

LOWRY O.H., GRAHAM H.T., HARRIS F.B., PRIEBAT M.K., MARKS A.H. & BREGMAN R.U. (1954) The chemical measurement of histamine in blood plasma and cells. *J. Pharmacol.* **112,** 116.

MAALE O. (1946) *On the Relation between Alexin and Opsonin.* Copenhagen: E. Munksgaard.

MARDINEY M.R. & MÜLLER-EBERHARD (1965) Mouse β1c-globulin: Production of antiserum and characterization in the complement reaction. *J. Immunol.* **94,** 877.

MASON J.K. (1951) An ox cell haemolysin test for the diagnosis of infectious mononucleosis. *J. Hyg. Camb.* **49,** 471.

MATHEWS K.P. (1959) Adaptation of antiglobulin test for use with tannic acid treated erythrocytes. *J. Immunol.* **82,** 279.

MAURER P.H. (1964) Use of synthetic polymers of amino acids to study the basis of antigenicity. *Progr. Allergy* (Karger, Basle/N.Y.) **8,** 1.

MEDICAL RESEARCH COUNCIL REPORT (1966) Production of antibody against purified human γG globulin in rabbits, goats, sheep and horses. *Immunology* **10,** 271.

MIDDLEBROOK G. & DUBOS R.J. (1948) Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. *J. exp. Med.* **88,** 521.

MILGROM F., KANO K., BARRON A.L. & WITEBSKY E. (1964) Mixed agglutination in tissue culture. *J. Immuno.* **92,** 8.

MITCHISON N.A. (1964) Induction of immunological paralysis in two zones of dosage. *Proc. roy. Soc. B.* **161,** 275.

MUSCHEL L.H. (1965) *Immune Bactericidal and Bacteriolytic Reactions in 'Complement'*. Ciba Foundation Symposium, p. 155. Eds. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill.

MOLLISON P.L. (1967) *Blood Transfusion in Clinical Medicine*, 4th ed. Oxford: Blackwell Scientific Publications.

MORESCHI C. (1908) Neue Tatschen über die Blutkörperchenagglutination. *Zbl. Bakt.* (1 Abt. Orig.) **46,** 49.

MORTON J.A. (1962) Some observations on the action of blood-group antibodies on red cells treated with proteolytic enzymes. *Brit. J. Haemat.* **8,** 134.

MORTON J.A. & PICKLES M.M. (1947) Use of trypsin in the detection of incomplete anti-Rh antibodies. *Nature (Lond.)* **159,** 779.

MORTON J.A. & PICKLES M.M. (1951) The proteolytic enzyme test for detecting incomplete antibodies. *J. clin. Path.* **4,** 189.

MOULINIER J. (1956) Technique de la réaction de consommation d'antiglobuline. *Rev. franç. Étud. clin. biol.* **1,** 355.

NAIRN R.C. (1964) *Fluorescent Protein Tracing*, 2nd ed. Edinburgh and London: E. & S. Livingstone Ltd.

NELSON D.S. (1963) Immune Adherence. *Adv. Immunology* **3,** 131.

NELSON D.S. & BOYDEN S.V. (1967) Macrophage cytophilic antibodies and delayed hypersensitivity. *Brit. med. Bull.* **23,** 15.

NELSON D.S. & UHLENBRUCK G. (1967) Studies on the nature of the immune adherence receptor. *Vox Sang.* **12,** 43.

NELSON R.A. (1953) The immune-adherence phenomenon. An immunologically specific reaction between micro-organisms and erythrocytes leading to enhanced phagocytosis. *Science* **118,** 733.

NELSON R.A. & MAYER M.M. (1949) Immobilization of *Treponema pallidum in vitro* by antibody produced in syphilitic infection. *J. exp. Med.* **89,** 369.

NETER E. (1956) Bacterial haemagglutination and haemolysis. *Bact. Rev.* **20,** 166.

NEWBOULD B.B. (1965) Production of allergic encephalomyelitis in rats by injection of suspensions and adjuvants into lymph nodes. *Immunology* **9,** 613.

NOAH J.W. (1964) Anaphylactic histamine release and the Schultz-Dale reaction. In *Immunological Methods*, p. 285, Ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

NOSSAL G.J.V. (1960) Antibody production by single cells: IV. Further studies on multiply immunized animals. *Brit. J. exp. Path.* **41,** 89.

NOTA N.A., LIACOPOULOS-BRIOT M., STIEFEL C. & BIOZZI G. (1964) 'L' immuno-cyto-adherence: une methode simple et quantitative pour l'étude *in vitro* des cellules productrices d'anticorps. *C.R. Acad. Sci. Paris* **259,** 1277.

NUTTALL G.H.F. (1904) *Blood Immunity and Blood Relationship*. Cambridge University Press.

OAKLEY C.L. (1954) Bacterial toxins: demonstration of antigenic components in bacterial filtrates. In *Annual Review of Microbiology*, col. 8, p. 411. California: Annual Reviews Inc.

OAKLEY C.L. & FULTHORPE A.J. (1953) Antigenic analysis by diffusion. *J. Path. Bact.* **65,** 49.

O'CONNOR J.L. & MACDONALD J.M. (1950) Excretion of specific antigen in the urine in tsutsugamushi disease (scrub typhus). *Brit. J. exp. Path.* **31,** 51.

OPPENHEIM J.J., WOLSTENCROFT R.A. & GELL P.G.H. (1967) Delayed sensitivity in the guinea pig to a protein hapten conjugate and its relationship to *in vitro* transformation. *Immunology* **12,** 89.

O'SULLIVAN S.A. & AUGUSTIN R. (1966) Demonstration of reagins by histamine release from human lung—in preparation (see *Immunology*, 1967, **12**, 12).

OUCHTERLONY, Ö. (1948) *In vitro* method for testing toxin-producing capacity of diphtheria bacteria. *Acta path. microbiol. scand.* **25**, 186.

OUCHTERLONY, Ö. (1949) *In vitro* method for testing toxin-producing capacity of diphtheria bacteria. *Acta path. microbiol. scand.* **26**, 516.

OUCHTERLONY, Ö. (1958) Diffusion-in-gel methods for immunological analysis. In *Progress in Allergy*, vol. 5, p. 1. Basel and New York: S. Karger.

OUDIN J. (1946) Méthode d'analyse immunochimique par précipitation spécifique en milieu gélifie. *C.R. Acad. Sci. Paris* **222**, 115.

OUDIN J. (1953) In *Methods in Medical Research*, p. 335, vol. 5, Ed. COHN, M. Chicago, Ill.: Yearbook Publishers.

OVARY Z. (1964) Passive Cutaneous Anaphylaxis. In *Immunological Methods*, p. 259. A symposium, Ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

PARISH H.J. (1958) *Antisera, Toxoids, Vaccines and Tuberculins in Prophylaxis and Treatment.* Edinburgh: Livingstone.

PEARMAN G., LYCETTE R.R. & FITZGERALD P.H. (1963) 'Tuberculin-induced mitoses in peripheral blood lymphocytes'. *Lancet* **i**, 637.

PEARSON R.S.H. (1965) Depot injections in the treatment of hypersensitivity and asthma. *Brit. med. J.* **ii**, 1148.

PLOTZ C.M. & SINGER J.M. (1956) The latex fixation test. II. Results in rheumatoid arthritis. *Am. J. Med.* **21**, 893.

POLLACK, W. (1965) Some physicochemical aspects of hemagglutination. *Ann. N.Y. Acad. Sci.* **127**, 892.

POPE C.G. (1954) Diphtheria toxin and antitoxin. A short review of progress since the work of Ehrlich. *Int. Arch. Allergy* **5**, 115.

POPPE L. (1959) Der Tb-Coombstest. *Z. ImmunForsch.* **117**, 419.

PORTERFIELD J.S. (1964) The plaque inhibition test. In *Immunological Methods*, p. 341, Ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

PREER J.R. (1956) A quantitative study of a technique of double diffusion in agar. *J. Immunol.* **77**, 52.

PRESSMAN D., CAMPBELL D.H. & PAULING L. (1942) The agglutination of intact azo-erythrocytes by antisera homologous to the attached groups. *J. Immunol.* **44**, 101.

RAFFEL S. (1961) *Immunity*, p. 71, 2nd ed. U.S.A.: Appleton-Century-Crofts Inc.

RATNER B. (1943) *Allergy, Anaphylaxis and Immunotherapy.* Baltimore: Williams & Wilkins Co.

REDFERN W.W. (1926) Primary toxicity of heterophile immune rabbit serum for guinea pigs, and its apparent relation to phenomenon of anaphylaxis. *Amer. J. Hyg.* **6**, 276.

RIDGES A.P. & AUGUSTIN R. (1964) An *in vitro* test for atopic reagins by double-layer leucocyte agglutination. *Nature* (*Lond.*), **202**, 667.

RIECKENBERG P. (1917) Eine neue Immunitalisreaktion bei experimenteller Trypanosomen-Infektion: due Blutplättchenprobe. *Z. ImmunForsch* **26**, 53.

ROSE H.M., RAGAN C., PEARCE E. & LIPMAN M.O. (1948) Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. *Proc. Soc. exp. Biol.* (*N.Y.*) **68**, 1.

ROSE N.R., KENT J.H., REISMAN R.E. & ARBESMAN C.E. (1964) Demonstration of human reagin in the monkey. *J. Allergy* **35**, 520.

ROSS A. & LEPOW I.H. (1960) Studies on immune cellular injury. I. Cytotoxic effects of antibody and complement. *J. exp. Med.* **112**, 1085.

SCHILD H.O., HAWKINS D.F., MONGAR J.L. & HERXHEIMER H. (1951) Reactions of isolated human asthmatic lung and bronchial tissue to a specific antigen. *Lancet* **ii,** 376.

SCHICK A.F. & SINGER S.J. (1961) On the formation of covalent linkages between two protein molecules. *J. biol. Chem.* **236,** 2477.

SELL K.W., MORI W., RACK J. & COOMBS R.R.A. (1968) In preparation.

SELL K.W. & SPOONER R.L. (1966) Differential effectiveness of complement fixed by IgG and IgM antibodies in the cytolysis of dog kidney tissue culture cells. *Immunology* **11,** 533.

SHELLEY W.B. (1962) A new serological test for allergy in man. *Nature (Lond.)* **195,** 1181.

SHELLEY W.B. & JUHLIN L. (1961) A new test for detecting anaphylactic sensitivity: The basophil reaction. *Nature (Lond.)* **191,** 1056.

SINDO T. (1960) Antigen-antibody reaction in system of M. tuberculosis. *Jap. J. Tuberc.* **8,** 81.

SINGER J.M. & PLOTZ C.M. (1956) The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. *Am. J. Med.* **21,** 888.

SINGER J.M., PLOTZ C.M., PADER E. & ELSTER S.K. (1957) The latex fixation test. III. Agglutination test for C-reactive protein and comparison with the capillary precipitin method. *Am. J. clin. Path.* **28,** 611.

SMITH H., GALLOP R.C. & TOZER B.T. (1964) The production of specific rabbit antibodies by injecting individual antigen-antibody complexes separated from mixed antigens. *Immunology* **7,** 111.

SOOTHILL J. (1962) Estimation of eight serum proteins by a gel diffusion precipitin technique. *J. Lab. clin. Med.* **59,** 859.

SPOONER R.L. (1965) The use of preserved cells in immune cytolytic tests. *Exp. Cell Res.* **38,** 465.

SPOONER R.L., BOWDEN F.W. & CARPENTER R.G. (1965) Description and analysis of a simple micro-titration immune cytolytic test. *J. Hyg., Camb.* **63,** 369.

STAVITSKY A.B. & ARQUILLA E.R. (1955) Micromethods for the study of proteins and antibodies. III. Procedure and applications of haemagglutination and haemagglutination-inhibition reactions with bis-diazotized-benzidine and protein conjugated red blood cells. *J. Immunol.* **74,** 306.

STAVITSKY A.B. & ARQUILLA E.R. (1958) Studies of proteins and antibodies by specific haemagglutination and haemolysis of protein conjugated erythrocytes. *Int. Arch. Allergy* **13,** 1.

STEELE A.S.V. & COOMBS R.R.A. (1964) The red cell linked antigen test for incomplete antibodies to soluble proteins. *Int. Arch. Allergy* **25,** 11.

STEFFEN C. (1960) Les applications de la réaction de consommation de l'antiglobuline aux études cliniques et expérimentales. *Rev. franç. Étud. clin. biol.* **5,** 831.

STERZL J. & RIBA K. (1965) Detection of cells producing 7S antibodies by the plaque technique. *Nature (Lond.)* **208,** 858.

STIMPFLING J.H. (1961) The use of P.V.P. as a developing agent in mouse haemagglutination tests. *Transplant. Bull* **27,** 109.

STRATTON F. & RENTON P.H. (1967) *Practical Blood Grouping*, 2nd edn. Oxford: Blackwell Scientific Publications.

STYLES W.McN., DODD B.E. & COOMBS R.R.A. (1963) Identification of human blood stains by means of the mixed antiglobulin reaction on separated cloth fibres. *Medicine Sci. Law* **3,** 247.

TERASAKI P.I., MICKEY M.R., VREDEVOE D.L. & GOYETTE D.R. (1965) Serotyping for homotransplantation. IV. Grouping and evaluation of lymphotoxic sera. *Vox Sang.* **11,** 350.

TILLET W.S. & FRANCIS T. (1930) Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J. exp. Med.* **52,** 561.

TURK J.L. (1964) The detection of antibodies by immune-adherence. In *Immunological Methods,* p. 405. C.I.O.M.S. Symposium, Ed. ACKROYD J.F. & TURK J.L. Oxford: Blackwell Scientific Publications.

TYRRELL D.A.J. (1964) Demonstration of antibodies to viruses. In *Immunological Methods,* p. 313, ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

U.S. PUBL. HEALTH SERV. PUBL. NO. 411 (1959) *Serologic Tests for Syphilis.* Washington D.C.: U.S. Govt. Printing Office.

V. ARSDEL P.P., MIDDLETON E., SHERMAN W.B. & BUCHWALD H. (1958) A quantitative study on the *in vitro* release of histamine from leucocytes of atopic persons. *J. Allergy* **29,** 429.

WAALER E. (1940) On occurrence of factor in human serum activating specific agglutination of sheep blood corpuscles. *Acta path. microbiol. scand.* **17,** 172.

WARBURTON M.F., KEOGH E.V. & WILLIAMS S.W. (1949) A haemagglutination test for the diagnosis of influenzal meningitis. *Med. J. Aust.* **1,** 135.

WARDLAW A.C. (1962) The complement-dependent bacteriolytic activity of normal human serum. *J. exp. Med.* **115,** 1231.

WEITZ B. (1960) Feeding habits of bloodsucking arthropods. *Exp. Parasit.* **9,** 63.

WENT S. & KESZTYÜS L. (1939) Das Anti-adrenalin. *Arch. exp. Path. Pharmak.* **193,** 669.

WHITE R.G. (1968) Presidential address Royal Society of Medicine 'Antigen and Adjuvant' *Proc. roy. Soc. Med.* **61,** 1.

WIDE, L., BENNICH, H. and JOHANSSON, S.G.O. (1967) Diagnosis of allergy by an *in vitro* test for allergen antibodies. Lancet **ii,** 1105.

WIENER A.S. (1955) Serologic test for human gamma globulin. II. Application in diagnosis and treatment of agammaglobulinemia. *Amer. J. clin. Path.* **25,** 595.

WIENER A.S., HYMAN M.A. & HANDMAN L. (1949) A new serological test (inhibition test) for human serum globulin. *Proc. Soc. exp. Biol. (N.Y.)* **71,** 96.

WITEBSKY E. & NETER E. (1935) Primary serum toxicity as demonstrated by the chicken embryo. *J. exp. Med.* **61,** 489.

WORMALL A. (1933) Some observations on the value and the mechanism of the red cell adhesion test for trypanosomiasis of man and other animals. *Parasitology,* **25,** 64.

WRIGHT A.E. & DOUGLAS S.R. (1903) An experimental investigation of the role of the blood fluids in connection with phagocytosis. *Proc. roy. Soc. B.* **72,** 357.

WRIGHT A.E. & DOUGLAS S.R. (1904) Further observations on the role of the blood fluids in connection with phagocytosis. *Proc. roy. Soc. B.* **73,** 128.

WRIGHT S.T.C. (1959) A quantitative serum-agar technique. *Nature (Lond.)* **183,** 1282.

ZINSSER H. (1921) On the essential identity of the antibodies. *J. Immunol.* **6,** 289.

CHAPTER 2

DIAGNOSIS OF BACTERIAL DISEASES

R. KNOX

INTRODUCTION

Historically microbiology and immunology have grown up side by side and each has owed much to the development of the other. From start to finish the course of an infection in the individual host depends very much on the types of defence that the body can muster against infection and on the speed with which these can become effective, while the course of epidemics is largely determined by the immune state of the community as a whole. Considered as a subject in its own right, immunology of course covers a far wider field than its microbiological aspects; and antimicrobial mechanisms of immunity can be regarded as special examples of immunological principles which run through the whole of biology.

Here we are concerned with the application of immunological principles and methods in the diagnosis of bacterial diseases. Of all the methods used in the identification of pathogenic bacteria, some of the most precise and reliable are those which make use of their antigenic properties. These methods are used for two main purposes—for the identification of infective organisms and for measuring the response of the host in terms of antibodies of different kinds. Where for various reasons it has not been possible to isolate the causal organism directly during the acute phase of the disease, the only method of making a laboratory diagnosis may be by immunological methods, such as the examination of the serum for antibodies. But although this method of diagnosing microbial infections, both viral and bacterial, is often highly reliable and practically very useful, the evidence it provides is always indirect, since it tells us only that the patient or host has developed antibodies of a certain kind. If there has been a history of immunization by vaccines or toxoids then the presence of antibodies is certainly not reliable evidence of actual infection unless

there is a significant change in titre in consecutive blood samples. In the Widal reaction for the enteric fevers the possession of agglutinating antibodies to *Salmonella typhi* or other salmonellae may merely indicate that an individual has received prophylactic inoculations in the past. A positive Mantoux reaction may be the result of BCG vaccination not of experience of tuberculosis. A negative Schick test may be due to active immunization with diphtheria toxoid and not exposure to the diphtheria bacillus. But so long as we are aware of these limitations, the examination of samples of serum for antibodies can be of great value in the diagnosis of bacterial infections both in individuals and in groups.

IDENTIFICATION OF PATHOGENIC BACTERIA

In the identification of pathogenic bacteria the main methods used are direct microscopy, isolation by appropriate methods of culture, investigation of pure cultures so obtained for their growth characteristics, their biochemical reactions, their antigenic structure and their susceptibility or resistance to antibiotics and other agents, and finally, if necessary, demonstration of their virulence or pathogenicity to laboratory animals or to other hosts including man.

Direct Microscopy

Direct microscopy is of limited value but in a few infections may save many valuable hours or days. Examples of infections which can be diagnosed in this way are tuberculosis of the lungs, where a properly controlled examination of sputum can be relied upon to make a diagnosis, Vincent's angina, revealed by the presence of the characteristic fusiform bacilli and spirilla in stained smears of a swab from the mouth or throat, gonorrhoea and meningococcal meningitis, where intracellular Gram-negative diplococci can be recognized in appropriate specimens, and actinomycosis, in which sulphur granules in pus can be seen by the naked eye and the microscopic appearances are characteristic.

In some infections immunological methods can be useful even at this level of direct microscopy, before isolation of micro-organisms by culture. In pneumococcal pneumonia pathogenic pneumococci can be identified by the capsular swelling reaction which occurs when a type-specific antiserum is added to a fresh specimen of sputum. In the days when the identification of the type of pneumococcus was regarded as essential, since the only method of specific treatment known was the use of type-specific anti-pneumococcal serum, this method of direct 'immuno-microscopy' enabled treatment to be started without delay.

Fluorescent Antibody Techniques

The development of fluorescent antibody techniques has now made it possible

to apply immunological methods in the direct identification of many species of pathogenic organisms in pathological specimens (see Chapter 1). Several methods have been used for detecting the union of antigen with antibody by fluorescent techniques. Coons and his colleagues originally used the direct method in which the specific antibody, conjugated with a fluorescent dye, reacts with the antigen. But this method has some disadvantages. For each antigen that may be looked for a specific fluorescent antibody has to be prepared. For many purposes an indirect method is better. In this the antigen or material containing it is first treated with specific unlabelled antibody prepared perhaps in a rabbit. The complex resulting from the union of antigen and antibody is then made to fluoresce by adding to it specific fluorescent anti-rabbit globulin prepared in a goat or other species of animal. In this way a single fluorescent antiglobulin antibody can be used to detect any number of specific reactions occurring between unlabelled antibodies and their corresponding antigens. Another indirect method makes use of fluorescein-labelled anti-complement as an indicator of the formation of a specific antigen-antibody complex. The literature has been well reviewed by Cherry & Moody (1965) who give a balanced account of the most important diagnostic applications of immunofluorescence. Among these are the identification of Group A streptococci, of enteropathogenic *E. coli* serogroups and of *Neisseria gonorrhoeae*, but many other applications have sometimes proved successful. In practice there are many difficulties in the technique and false positive results may be obtained because of non-specific staining and for other technical reasons. But the advocates of the method are convinced that when these technical difficulties have been overcome the method will prove to be of increasing value in diagnostic microbiology, both for the rapid detection of bacteria or viruses in clinical specimens and, in certain cases, for example in the fluorescent treponemal antibody test, in detection of antibody in patients' sera.

Examination of Cultures

With most bacterial infections a diagnosis cannot be made by direct microscopy alone and it is necessary to wait for the results of cultures. The causal organisms can often be identified by the appearance of the colonies on solid media, their other growth characteristics, their biochemical reactions and their sensitivity to antibiotics and other agents. *Staphylococcus pyogenes* for example is regarded as being adequately identified if the colonies and microscopic morphology are typical and if the coagulase reaction is positive. Tubercle bacilli are identified in culture by their characteristic morphology, by the appearance of the colonies, and if necessary by the results of inoculating into experimental animals. But in many infections the most precise and reliable method of identification is by means of antigenic analysis. The methods used vary in different bacterial genera and species.

Precipitation and Toxin-antitoxin Reactions

In some cases precipitation reactions are used. An extract prepared from the culture under investigation is mixed with antiserum containing known antibodies, and homologous organisms are identified by the formation of a specific precipitate with the appropriate antiserum. This method is used for example in the Lancefield method of grouping β-haemolytic streptococci which fall into a number of serological groups (A, B, C, D, G, etc.) and are distinguished by the possession of different carbohydrates. Specific precipitation reactions are also used in the further subdivision of β-haemolytic streptococci into specific types. Antisera are prepared against different serological types and these sera when mixed with extracts prepared from the homologous type containing the type-specific M protein give a specific precipitin reaction. This method can be used in addition to the agglutination method of Griffith.

Precipitation reactions are also used in agar media by taking advantage of the fact that an antigen diffusing from one site on an agar plate and an antibody diffusing from an adjacent site interact at some point between their sites of origin with the production of a thin white line due to the formation of a specific antigen-antibody precipitate (Ouchterlony 1948, 1949; Elek 1949). These techniques have been used for the identification of many different types of antigen and antibody in bacteriology. The toxigenicity of strains of *Corynebacterium diphtheriae* can be well demonstrated by observing the precipitate formed when toxin diffusing from a growing culture meets antitoxin diffusing from a reservoir in the medium. It has been claimed that this method reliably distinguishes toxigenic from non-toxigenic strains of *C. diphtheriae* and comparison of the method with conventional tests of toxigenicity in guinea-pigs is said to give good agreement. Similar methods have also been used for the demonstration of the production of toxins by strains of staphylococci. The size, position, shape and the angle of the diffusion lines are affected by a number of factors. Quantitative interpretation of results is full of difficulties but in principle the method is extremely valuable and it gives useful qualitative information about the antigenic composition of micro-organisms from which diffusible antigens can be obtained.

Mention should be made here of three other ways in which the specificity of toxin-antitoxin reactions can be used in the identification of pathogenic micro-organisms. (1) In the identification of *Clostridium welchii* the reaction described by Nagler (1939) is used. In a modification of Nagler's original test, material suspected of containing *Cl. welchii* is inoculated on to an agar plate containing human serum. The α-toxin of *Cl. welchii*, which is lecithinase, splits lecithin present in human serum into phosphoryl choline and a diglyceride with a resulting opacity in the neighbourhood of the bacterial colonies. That this is specifically due to the α-toxin can be demonstrated by inoculating half of the surface of the plate with antitoxin which specifically neutralizes the

effects of the toxin. The reaction is interesting since the toxin is an enzyme whose substrate is known.

(2) Antitoxins may also be used in the identification of pathogenic micro-organisms in association with tests of pathogenicity to laboratory animals. Corynebacteria resembling *C. diphtheriae* may or may not be toxigenic strains. Generally, if they are *gravis* or *intermedius* in type, they are likely to be toxigenic, but a fairly high proportion of *mitis* strains may be avirulent and for atypical strains also virulence tests are necessary. The organisms are injected into guinea-pigs. Two animals of approximately equal size are used and one of them is injected with diphtheria antitoxin. The unprotected pig will die with haemorrhages in the suprarenals and other characteristic features of diphtheritic toxaema in guinea-pigs but the other animal will be protected by the antitoxin. Sometimes an intradermal test is used in determining the toxigenicity of *C. diphtheriae*. Suspensions of several different strains can thus be inoculated into the skin of two guinea-pigs one of which is protected with antitoxin. Other toxin-producing organisms such as clostridia can also be identified by injection of suspensions into mice or guinea-pigs. In each case one of a pair is protected by specific antitoxin.

(3) Intradermal tests are widely used in man for clinical and epidemiological purposes but in general it cannot be said that they are directly useful for the identification of pathogenic organisms. But the Schultz-Charlton reaction has sometimes been used to prove that an erythematous rash is due to the erythrogenic toxin of *Streptococcus pyogenes*. In this test a small quantity of antitoxin to the toxin of the haemolytic streptococcus injected into the skin gives blanching of the red rash.

Agglutination Reactions

The specific agglutination of bacterial suspensions by homologous antisera is perhaps the most widely used of all immunological methods in the identification of pathogenic bacteria. Slide or tube agglutination tests may be used. Slide agglutination is useful in the identification of members of the salmonella and shigella group, in the typing of β-haemolytic streptococci and in the identification of meningococci and of *Bordetella pertussis*. A suspension of micro-organisms is placed on a glass slide and mixed with the appropriate antisera. Clumping is observed by naked eye or with a low-power lens. If the sera are suitably diluted so that non-specific reactions are eliminated the method can be made highly specific and reliable. However, when slide agglutination tests are carried out on colonies growing on selective media such as deoxycholate citrate, brilliant green or Wilson and Blair agar or even sometimes on MacConkey agar, both positive and negative results must be interpreted with caution and careful controls are necessary. But in dealing with an epidemic in which large numbers

of specimens may have to be examined and rapid results are required the intelligent use of slide agglutination reactions often makes it possible to identify and eliminate dangerous carriers at the earliest possible moment. The pneumococcal capsular swelling reaction already mentioned in connection with direct examination of smears of sputum can also be used with pure cultures, though pneumococci may quickly lose their capsules in sub-culture, and more generally in identifying pneumococcal types slide agglutination reactions can be employed. Now that sulphonamides and antibiotics are available for the treatment of pneumococcal infections typing of pneumococci has lost most of its clinical and epidemiological importance. Slide agglutination is still used in the typing of β-haemolytic streptococci. The antigens concerned are mainly the T antigens, whereas the precipitation reaction detects the M antigen which seems to be more important in determining the virulence of strains. Agglutination reactions can also be used in dividing staphylococci into three main serological groups, though for epidemiological purposes it is usually thought that the method of bacteriophage typing gives more useful information than serological typing of staphylococci. Slide agglutination reactions are useful in the bacteriological diagnosis of whooping cough and *Bord. pertussis* can be distinguished from the rare *Bord. parapertussis*. Meningococci too can be identified by slide agglutination with anti-meningococcal sera and in epidemic times it has been useful to divide them into groups and types.

The results of slide agglutination tests especially in the salmonella and shigella group must be confirmed by tube agglutination tests. In principle of course these are simple. A suspension of the organisms under investigation is mixed with a suitable volume of the appropriate antisera of which a number of dilutions are made ranging from about 1 in 25 to about 1 in 2500 or higher. Each tube in the series now contains a constant volume of bacterial suspension and falling dilutions of antiserum. The sera containing known antibodies are initially adjusted so that a 1 in 250 dilution produces standard agglutination of the homologous organism. Thus *typhi* will be agglutinated to a titre of 1 in 250 by an antiserum containing *typhi* H antibody and also by one containing *typhi* O antibody. By using properly selected antisera containing antibodies against known antigenic components of different groups of salmonellae it is usually possible to arrive fairly rapidly at a correct identification of the different salmonella types. Several hundred of these types are known but they fall into a comparatively small number of main groups characterized by the possession of common somatic or O antigens. They are further characterized by their H or flagellar antigens which can exist in two phases, phase 1 (specific, 'type' phase) or phase 2 (non-specific, 'group' phase). Quite frequently salmonellae when first isolated may be in the non-specific phase and since the flagellar antigens of organisms in the group phase are fairly widely shared among many different members of the salmonella group it is not easy to identify the salmonella

unless it can be prevailed upon to change into the specific phase. This can be done by means of the ingenious technique described by Craigie in which the culture under investigation is inoculated through a small tube embedded in a test tube containing sloppy agar and also antiserum to the non-specific H antigens. Organisms in the non-specific phase are agglutinated and trapped by the antiserum and the small number of organisms in the specific phase are able to swim through the sloppy agar and appear outside the inner tube on the surface of the medium in the test-tube. Once salmonellae have been obtained in the specific phase it is comparatively easy then to identify their specific H antigens. In some salmonellae the Vi antigen is also important, especially in *typhi*. There has been a good deal of variation in the methods by which these different tests are carried out and in the temperatures and in the type of tubes used. H agglutination tests are usually carried out in the conventional narrow Widal tube with rounded (not conical) bottoms, but the Vi and O tests are usually carried out in wider tubes and the characteristic agglutination is observed by holding the tube over a mirror so that the pattern of the deposit at the bottom of the tube can be clearly seen. The temperature used for the tests should be the lowest consistent with obtaining a rapid result; if the temperature is too high, the antigens or antibodies involved may be damaged or destroyed. Vi and O agglutinations are usually carried out at 37°, H agglutinations usually at 50°.

The same principles as those used in the identification of salmonellae are used for identifying entero-pathogenic *Estherichia coli* now known to be responsible for many cases of acute gastro-enteritis in infants. The serological classification of *E. coli* involves identification of somatic O, surface K and flagellar H antigens. The techniques and some of the difficulties are well discussed by Taylor (1960). It has already been mentioned that fluorescent antibody techniques have been useful in this group of organisms and in some series good correlation has been obtained between the positive results given by fluorescent antibody techniques and other serological methods of identifying pathogenic *E. coli* of types O 111, O 55, O 26 and others.

It is thus possible by serological methods to identify many bacterial species with considerable precision. But often, especially in the investigation of epidemics, even more refined methods have to be used—such as typing by means of bacteriophages or colicins (bacteriocins). Bacteriophages have been extensively used particularly in typing *S. typhi* and staphylococci. In *S. typhi* use is made of the fact that certain specially adapted bacteriophages specifically attack different types of *S. typhi* containing Vi antigen. Thus the method, though not itself a serological technique, has an immunological basis. Strains of *S. typhi* without Vi antigen cannot be typed by Vi phages but, if they do possess Vi antigen, then bacteriophages can be used to differentiate strains indistinguishable by serological methods. Phage typing is thus a highly sensitive tool for the epidemiologist.

ANTIBODY RESPONSE IN INDIVIDUALS

In patients who have suffered from an infectious disease it may be impossible to isolate the causal organisms. In such cases serological methods are often essential for making a diagnosis.

Agglutination Reactions

The most commonly used serological reaction is the agglutination or Widal reaction in which the patient's serum is mixed in falling dilutions with a suspension of various organisms suspected of causing the infection. The diseases in which this technique is especially useful are suspected salmonella infections, brucellosis, tularaemia, leptospirosis and typhus fever. In all these conditions the development of high titre antibodies in a patient's serum may under properly controlled conditions be highly suspicious or diagnostic. In some infections such as bacillary dysentery it is unusual for such high titres to be reached and in general the development of agglutinating antibodies in the patient's serum is not of much value in the diagnosis of bacillary dysentery. In a suspected case of enteric fever the patient's serum is tested against suitably prepared suspensions of *S. typhi* and *paratyphi A, B, C* and possibly other members of the salmonella group. H and O suspensions of these organisms are prepared and the titre to which the agglutination occurs gives a rough measure of the antibody content of the serum. In clear-cut cases a retrospective diagnosis can easily be made; for example a patient who has a febrile illness suggestive of typhoid fever and who 3 or 4 weeks later is found to have a titre of 1 in 500 to *S. typhi* H and to *S. typhi* O has almost certainly suffered from an attack of typhoid fever. The situation is greatly complicated if there have been any previous inoculations of typhoid or paratyphoid vaccine and the interpretation of agglutination reactions in such inoculated individuals is difficult. In any case unless very high titres are obtained it is usually unwise to rely on a single Widal examination and much more valuable information is given by examining two or three samples of serum taken at intervals and observing whether there is a rise or fall in the titre of antibodies to the suspected organisms. In the search for temporary or chronic carriers of *S. typhi*, repeated tests for Vi antibodies may be of great value.

In some agglutination reactions difficulties arise owing to the phenomenon known as prozone. Here agglutination may not occur in the highest concentrations of serum but only starts when the serum is quite highly diluted. This is thought to be due to the blocking effect of the presence of excess antibody in the serum or to the presence of incomplete antibodies or to complement in high concentration. It is particularly apt to occur in brucellosis and therefore the serum dilutions must be carried down to at least 1 in 2500 in any suspected case of brucella infection. Interpretation of these agglutination reactions is not always easy and due attention must be paid to the patient's occupation, since those who

habitually handle brucella-infected milk or herds consistently show rather higher antibody titres than the general population. Agglutination reactions are sometimes especially useful in brucellosis because of the difficulty of isolating the causal organisms. But, of course, the most direct method of diagnosis is to isolate the causal organism from the blood, inject it into guinea-pigs and if necessary investigate the agglutinins in the guinea-pig sera against known brucella suspensions. Brucella agglutinins may also be looked for in samples of milk and this is sometimes a useful method of detecting the presence of brucellosis in a herd.

In some conditions in which for one reason or another there is not a high titre of antibodies in the patient's serum the passive haemagglutination method greatly increases the chances of detecting antibody (see Chapter 1). In this a suspension of organisms of the serological type required is boiled to extract sufficient antigen; the supernatant fluid obtained after centrifugation is then treated with red blood cells from man or sheep and after allowing these to react in a water bath for 30 minutes the serum under investigation is added. If antibodies are present then the red cells will be agglutinated because of the antigen adsorbed on to them. It is possible that a similar technique used in the investigation of serum from patients with dysentery might greatly increase the sensitivity with which antibodies can be detected.

The antiglobulin reaction which has been used so effectively in the identification of red blood cell antigens and their antibodies is sometimes useful for detecting antibacterial antibodies. Bacteria or red cells treated with extracts of salmonellae, shigellae or brucellae are mixed with the patient's serum, washed and subsequently exposed to an anti-human globulin serum prepared in a rabbit. Agglutination may sometimes be demonstrated by this technique when direct agglutination tests are doubtful or negative (Kerr *et al* 1966).

In the diagnosis of typhus fever two types of agglutination test can be carried out. It is now possible to use rickettsial suspensions which make it possible to distinguish between the different types of rickettsia but before these were available it was found that the serum of patients suffering from typhus fever gave a positive agglutination with suspensions of different strains of *Proteus* known as *Proteus* X19, XK, and X2—presumably due to the sharing of some common antigen between these *Proteus* strains and rickettsiae. The reaction, although empirical, is reasonably specific and reliable.

In tuberculosis measurement of antibodies in the patient's serum is on the whole of little diagnostic value. At one time it was thought that the haemagglutination test of Middlebrook and Dubos would be useful, since antisera from patients with tuberculosis were found to agglutinate red cells treated with tuberculin or with polysaccharide from tubercle bacilli. But in general humoral antibodies do not seem to play an important part in the development of immunity to tuberculosis and the most useful immunological test is the Mantoux

test or variants of it. But this test is not of immediate value in diagnosing tuberculosis infection but merely as evidence that the individual has been exposed to tubercle bacilli at some time either in the form of the disease or in the form of active immunization with BCG.

A number of other agglutination reactions of a more ill-defined kind are used in several infections of doubtful or unknown aetiology. These are mainly non-bacterial, for example the Paul-Bunnell reaction for glandular fever, the test for cold agglutinins in primary atypical pneumonia possibly of viral origin and the agglutination of streptococcus MG suspensions also in primary atypical pneumonia. In practice these reactions are of great value and interest although the determining cause of the specificity is still unknown.

Precipitation Tests

Specific precipitation reactions are not commonly used in the detection of antibodies in human sera, since generally other more sensitive serological methods are available. An important exception to this is the Kahn test used in the diagnosis of syphilis. In theory, treponemal antigen should give a specific precipitate when mixed under suitable conditions with antibody-containing serum of patients. In practice, as in the Wassermann reaction, the antigen used is not treponemal antigen at all but material prepared from normal heart muscle. In the usual test a constant volume of undiluted patient's serum is mixed with different proportions of Kahn antigen and the strength of the positive reaction is judged by the size of the floccules formed and the number of tubes in which flocculation occurs. The test may be carried out on serum or on cerebrospinal fluid, though, according to Stokes (1960), in cerebrospinal fluid the Wassermann reaction is more reliable.

Complement Fixation Tests

The most important example of the complement fixation reaction in bacteriology is the Wassermann test used in the diagnosis of syphilis. Here as in the Kahn test the antigen used is not treponemal antigen but extract of normal heart muscle and what is measured appears to be an auto-antibody to a normal tissue component and not an antibody to the treponemata. The fact remains that the tests work and provided that sufficient precautions are taken are extremely reliable in practice. In principle the complement fixation reaction is simple. The serum to be tested (previously heated to destroy complement) is mixed with antigen in the presence of added guinea-pig complement. If the serum contains a syphilitic antibody and an antigen-antibody reaction occurs then the complement is fixed in the process. The presence or absence of complement can then be detected by adding the second system containing an antigen and antibody which will react only in the presence of free complement. The system commonly used is one consisting of sheep's red cells sensitized with specific haemolytic serum

prepared in a rabbit. Thus, if complement is fixed as the result of a combination between the Wassermann antigen and the antibody present in the patient's serum no complement is left over for the haemolytic system, and so no haemolysis occurs. If on the other hand the patient's serum contains no syphilitic antibody, then the complement is not fixed in the first reaction and is free to take part in the second reaction and so haemolysis occurs. In practice elaborate controls are necessary since the amount of complement must clearly be suitably adjusted, while all the reagents must be carefully standardized.

Complement fixation reactions are used in a number of other diseases but not very frequently. Gonococcal complement fixations have been widely used but there seems to be considerable doubt as to their actual value and reliability. Apart from the Wassermann reaction it is probably true to say that complement fixation reactions are of much less value in the diagnosis of bacterial diseases than they have proved to be in many virus infections. For details of both these techniques Stokes (1960) should be consulted.

Other Serological Tests for Syphilis

The Wassermann and Kahn reactions, despite their great value, are not quite as specific as is desirable in a disease so important as syphilis. Recently several other tests have been introduced in which the antigens used are derived from treponemata—either from pathogenic strains of *Treponema pallidum* maintained in animals or from cultivated non-pathogenic strains of uncertain pedigree. It is generally believed that in patients with syphilis several different antibodies can be demonstrated. One of these presumably corresponds to the antigen present in normal heart muscle and is the one detected in the Wassermann and Kahn reactions. But it is probable that others are detected in the various tests with treponemal antigens such as the treponemal immobilization test of Nelson & Mayer (1949) and the fluorescent antibody test of Deacon, Falcone & Harris (1957). Other tests for detecting anti-treponemal antibodies in human sera have been described such as the adherence and agglutination tests—all of which use preparations of treponemata as antigens, but the general view is that the immobilization test and the fluorescent antibody test are the most sensitive and specific (Sequeira 1960; Wilkinson 1961).

Serum Reactions in 'Rheumatic' Diseases

In the diagnosis of conditions such as the connective tissue diseases including acute rheumatism and rheumatoid arthritis there are a number of tests involving examination of the serum for antibodies or antibody-like substances of an antibacterial nature. The anti-streptolysin O test makes use of the fact that the serum of patients who have had infections with β-haemolytic streptococci may contain antibodies which inhibit the haemolysis of suitable red blood cells by β-haemolytic streptococci. To give reliable results the test must be carefully

standardized, and Johnson (1955) recommends that all tests on patient's sera should be carried out by reference to a globulin of known anti-streptolysin content. Using this technique Johnson regards titres of below 200 units per ml as being within normal limits and those of 200 units or above as being raised. It is generally accepted that rheumatic fever is a manifestation of or at least a sequel to infection with group A haemolytic streptococci, and the anti-streptolysin O titre is raised in a high proportion of patients with active rheumatic disease. But, as Bywaters & Scott (1960) point out, a raised anti-streptolysin titre occurs in uncomplicated infections of streptococcal origin and is therefore not specifically diagnostic of rheumatic fever. A similar test for anti-staphylococcal haemolysin (Lack 1957) has also been used in cases of osteomyelitis.

The test for C-reactive protein is another test used as an aid in the diagnosis of a number of inflammatory diseases. Originally it was found by Tillet & Francis (1930) that the serum of patients with pneumonia gave a precipitin reaction with the C polysaccharide of the pneumococcus but it was later found that the protein responsible for this reaction, though specific in the sense that it reacts with C polysaccharide of the pneumococcus, is not a true antibody. It is now known to be a β-globulin and requires the presence of calcium ions for its reaction with C polysaccharide (Bywaters & Scott 1960; Hedlund 1961). The C-reactive protein test gives a good indication of the activity of a number of processes mostly of an inflammatory nature such as rheumatic fever, rheumatoid diseases and others; but it is also positive where there has been extensive tissue damage as in recent myocardial infarction and even in some cases of malignant disease. It is regarded as perhaps being a more sensitive index of rheumatic activity than the erythrocyte sedimentation rate, which, though it often indicates activity of an infective process cannot, of course, be described as a serological reaction.

CONCLUSIONS

An attempt has been made here to give a broad outline of the value of immunological methods in the identification of pathogenic bacteria and in the diagnosis of bacterial diseases. Both in the direct examination of bacterial cultures and in the more indirect method of examining the serum of patients or others for antibodies it is clear that the value of serological methods depends on their high degree of specificity and it is precisely because serological methods are so specific that they are of value in diagnosis, not only for clinical purposes but also in the control and investigation of epidemics. Striking examples of such specificity are found in the salmonella group of organisms where by elaborate methods of serological analysis it is possible to show that even strains whose major antigenic components are identical may differ in minor antigenic components, which, however, may be of great importance from the epidemiological point of

view. The antigenic pattern of any bacterial species may be regarded as the finger-prints by which they can be accurately traced and their spread through the community followed in the greatest detail. The rarer the type and the more finely types can be subdivided, the greater is the value of this method in epidemiology. This aspect of bacteriology has perhaps become even more important since the general introduction of chemotherapeutic drugs and antibiotics, since any given bacterial species exposed to such drugs may be capable of retaliating by the selection of resistant mutants. When this occurs it is not always easy to say whether the resistant survivors are in fact mutants of the originally drug-sensitive population or whether they represent organisms of another race or of another strain which have found their way as it were into the vacuum created by the destruction of the originally drug-sensitive population. The study of the antigenic structure of these strains, therefore, is of the greatest importance. With some organisms, notably *S. typhi* and staphylococci, phage typing gives even greater accuracy than antigenic structure—though in many species of micro-organism phage type and antigenic structure are closely related (Oeding 1960). In general, these are more stable characters than the antibiotic sensitivity pattern, since this, by its very nature, is bound to be much more sensitive to the factors of adaptation and selection.

One of the difficulties in being certain whether a given type of infecting organism is or is not being replaced by another is the obvious difficulty of sampling. In general we assume that if a patient is infected with a pathogenic micro-organism the causal organism is present in pure culture. For example, typhoid fever is usually caused by a single strain of *Salmonella typhi*, staphylococcal septicaemia by a single strain of *Staphylococcus pyogenes* and tuberculosis by a single strain of *Mycobacterium tuberculosis*. When such cultures are isolated from clinical specimens it is of course essential to obtain pure cultures both for identification and for epidemiological purposes. This is usually done by picking single colonies from culture plates and if necessary repeating the process and finally carrying out the appropriate biochemical, serological and other tests on the progeny of a single colony. But in cases where there is reason to believe that there may be mixed infections or there may even be a number of organisms to be investigated as for example in the examination of dust and air samples and water supplies or sewage, or even in nasal carriers of staphylococci or streptococci or diphtheria bacilli, it then becomes obvious that our accepted methods for identifying pathogenic organisms can be seriously deficient. If single colonies are picked from a primary culture plate, there is no guarantee that the colony picked is representative of the rest of the culture. If a general sweep is taken there is no guarantee that the culture is pure. Even if several colonies are picked there is no guarantee that other species which may be present in quite high proportions may not be missed. It is known that the faeces of normal individuals contain many different races of coliform organisms and that the serological

types are far from constant from one day to another. Similarly if the faeces of salmonella carriers are examined carefully it may sometimes be found that not one but several different types are present, and, as has been mentioned already, in samples of food such as dried egg, in air, in water and in sewage, many different varieties of pathogenic micro-organisms may be present. Some of these may be closely similar to each other in colonial form and therefore cannot be recognized easily. The most hopeful way of dealing with this kind of problem is to develop the replica plating method of Lederberg & Lederberg (1952) as suggested by Runnels & Wilson (1960). In certain cases this method would be particularly useful if the master plate used were in fact the primary plate obtained by direct plating of pathological material or other material under investigation. If properly spaced colonies are obtained then these can be replicated on to a variety of media, so that it is possible to tell at a glance what proportion of the colonies resulting are similar to each other in biochemical characteristics and, if suitable techniques can be devised, in antigenic structure.

REFERENCES

Bywaters E.G.L. & Scott F.E.T. (1960) Rheumatism and the connective tissue diseases. In *Recent Advances in Clinical Pathology*, p. 278. London: J. & A. Churchill.

Cherry W.B. & Moody M.D. (1965) Fluorescent-antibody techniques in diagnostic bacteriology. *Bact. Rev.* **29,** 222.

Deacon W.E., Falcone V.H. & Harris A. (1957) A fluorescent test for treponemal antibodies. *Proc. Soc. exp. Biol. (N.Y.)* **96,** 477.

Elek S.D. (1949) The serological analysis of mixed flocculating systems by means of diffusion gradients. *Brit. J. exp. Path.* **30,** 484.

Hedlund P.E.R. (1961) Clinical and experimental studies on C-reactive protein (acute phase protein). *Acta med. scand.* **169,** suppl. 361.

Johnson G.D. (1955). The determination of anti-streptolysin. *J. clin. Path.* **8,** 296.

Kerr W.R., Coghlan T.D., Payne D.T.H. & Robertson L. (1966) Laboratory diagnosis of chronic brucellosis. *Lancet* **ii,** 1181.

Lederberg J. & Lederberg E.M. (1952) Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63,** 399.

Lack C.H. (1957) Staphylococcal antibody in osteomyelitis and suppurative arthritis. *Proc. roy. Soc. Med.* **50,** 625.

Nagler F.P.O. (1939) Observations on a reaction between the lethal toxin of *Cl. welchii* (type A) and human serum. *Brit. J. exp. Path.* **20,** 473.

Nelson R.A. & Mayer M.M. (1949) Immobilization of *Treponema pallidum in vitro* by antibody produced in syphilitic infection. *J. exp. Med.* **89,** 369.

Oeding P. (1960) Antigenic properties of *Staphylococcus aureus. Bact. Rev.* **24,** 374.

Ouchterlony Ö. (1948) In vitro method for testing the toxin-producing capacity of diphtheria bacteria. *Acta path. microbiol. scand.* **25,** 186.

Ouchterlony Ö. (1949) Antigen-antibody reactions in gels. *Acta path. micribiol. scand.* **26,** 507.

Runnels J.L. & Wilson J.B. (1960) Isolation of staphylococci by replica plating. *J. Bact.* **80,** 727.

SEQUEIRA P.J.L. (1960) Developments in the serology of syphilis. In *Recent Advances in Clinical Pathology*, p. 58. London: J. & A. Churchill.

STOKES E.J. (1960) *Clinical Bacteriology*. London: Arnold.

TAYLOR J. (1960) Enteropathogenic *Escherichia coli*. In *Recent Advances in Clinical Pathology*, p. 78. London: J. & A. Churchill.

TILLETT W.S. & FRANCIS T.J.R. (1930) Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J. exp. Med.* **52**, 561.

WILKINSON A.E. (1961) Fluorescent treponemal antibody test. A preliminary report. *Brit. J. vener. Dis.* **37**, 59.

CHAPTER 3

DIAGNOSIS OF FUNGAL DISEASES

JOAN L. LONGBOTTOM, I. G. MURRAY
& J. PEPYS

INTRODUCTION

Fungi may stimulate different immunological responses in man. They can act solely as sources of allergen, causing rhinitis and asthma, in which reaginic antibody is responsible; for example, the spores of *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp. and species of other genera. They may also be responsible for the appearance of precipitins as well as reagins where there is growth of the fungus without tissue invasion, and in these patients the asthma becomes complicated by allergic pulmonary infiltrations as, for example, with *Aspergillus fumigatus* and perhaps *Candida albicans*. The truly pathogenic fungi, which invade the tissues, may lead to the appearance of a variety of antibodies, (reagins, precipitins, agglutinins, complement fixing) or specifically reacting cells and appropriate hypersensitivity reactions may therefore be elicited. The relationship of these reactions to any increased resistance is, however, not clearly understood. It is convenient to deal with the fungi individually where possible, since they may play different roles in varying circumstances.

Variation in the morphological and biochemical characteristics of fungi is governed by many different inherent, acquired and environmental factors, all of which may influence antigenicity. The parasitic phase of growth is often of a specialized form commonly showing morphological simplification, whereas the

saprophytic and hence cultural phase may include a variety of vegetative and fruiting structures. These, together with differences in stages of development and maturity in both phases, may result in antigenic differences, as for example, between the yeast and mycelial phases of *Histoplasma capsulatum* and *Blastomyces dermatitidis* (Kaufman & Kaplan 1963) and in the capacity of antigens of *Coccidioides immitis* at one stage in development to induce immunity, whereas the antigens at another stage do not protect against inhalation of vegetative spores (Campbell 1965). Genetic factors are also responsible for variation in fungi, either through alternation of sexual and asexual stages or by exchange of nuclear material following the fusion of hyphae of different strains, and variations may also occur by spontaneous or induced mutation.

The polymorphism of fungi, which has been most extensively studied from the point of view of their structure, complicates the morphological basis on which they are traditionally identified. Such variations in morphology and in biochemical behaviour which often complicate the task of accurate identification, may also produce variation in antigenicity. This is clearly important when fungal diseases are studied serologically. Taxonomists do not as a rule use serological methods in classifying fungi, and therefore named strains from culture collections do not claim to resemble one another in this respect. That there are strains of fungi, identical in most respects, which do not give rise to identical antigens has been well established for a number of species in several genera, for example *H. capsulatum* (Schubert & Ajello 1957), *B. dermatitidis* (Abernathy & Heiner 1961) and *Candida albicans* (Hasenclever 1965).

Cultural conditions and the nature of the tissues invaded may profoundly influence the morphology and physiology of pathogenic fungi. The age of the culture and the conditions for growth have been shown, for example, to affect the antigens produced by *Aspergillus fumigatus* (Longbottom & Pepys 1964), and by *Coccidioides immitis* (Rowe, Landau & Newcomer 1965), and antigenic differences have also been noted even under apparently identical cultural conditions. Furthermore, even minor differences in extraction procedure can affect the composition of the extract. All these factors make it difficult to produce successive identical batches of fungal antigen, and, with few absolute or relative standards for comparison, each worker is left to his own devices.

The major problems of antigen standardization are due, on one hand, to the multiplicity of antigens of varying specificity and potency and the presence of differing amounts of non-antigenic material in the commonly used crude extracts, and on the other hand to the different individual patterns of antibody response in exposed subjects, for example, in atopic and non-atopic subjects to *A. fumigatus* (see Chapters 7 and 36). The assessment of the relative importance of the antigens and their better chemical characterization are needed for effective resolution of these problems.

In spite of these difficulties, mycoses are comparable to other microbial

diseases in terms of the serological diagnostic procedures available and their accuracy. Serological tests are particularly valuable where the agent isolated may be either a chance contaminant or a pathogen.

DIAGNOSTIC METHODS

A laboratory diagnosis of fungal disease may be made by three complementary methods:

Direct Microscopic Examination

The causal organism may be demonstrated in fresh preparations of exudates or secretions or in histological sections of fixed material. Special staining, such as by periodic-Schiff stain or silver impregnation, may be of help and, increasingly in recent years, specific staining with fluorescent antibody techniques. The presence of an habitual pathogen such as *Coccidioides immitis* in pus, sputum or tissue, or of *Cryptococcus neoformans* in cerebrospinal fluid is reasonably conclusive evidence of disease. On the other hand the demonstration of *Candida* or *Aspergillus* species in sputum may not in itself be of consequence, though their presence in normally sterile tissue such as brain or kidney is significant. Whilst in many instances the microscopic appearance of the fungus may be sufficiently characteristic for firm identification, a final decision is generally made by isolation of the fungus in pure culture.

Isolation of Causal Organism

The diagnosis may be made if an habitual pathogen is isolated but, as often happens in medical mycology, the organism may lead a dual life varying between saprophytism and parasitism. In such cases confirmation must be sought elsewhere, commonly either by demonstrating the organism *in situ* in a normally sterile tissue or by immunological methods.

Immunological Methods Consisting of Serological and Skin Tests

These provide information on diagnosis, epidemiology and prognosis. The immunological procedures adopted with the appropriate fungal antigens are like those in other diseases. In general the presence of antibodies is evidence of past or present disease caused by the fungus, or of considerable contact with it. The chief example is the demonstration of antibodies to *Candida* species, principally *C. albicans*. Most adults possess agglutinins and the great majority give delayed, Type IV, skin test reactions, whereas neonates usually do not have such agglutinins (Winner 1955), and their appearance later in life is attributed to oral, intestinal and vaginal exposure to *C. albicans*. High titres of agglutinating and precipitating antibody to *C. albicans* may indeed be present in rabbits fed

on live cultures and remaining in excellent health (Murray, unpublished). Fungal disease may, however, be present in the absence of a consistently demonstrable antibody response.

Diagnosis

Serological tests

Tests on the serum are diagnostic in certain diseases and will be discussed in relation to them. In general, however, precipitin tests are usually indicative of active disease, often of relatively recent onset; complement-fixation tests also usually indicate active disease but the responsible antibodies tend to be slower to appear and to disappear than the precipitins, the titre being frequently approximately proportional to the degree of infection.

A variety of fluorescent antibody tests are among the other serological methods which are now being applied for diagnosis.

Skin tests

The analysis of the different types of hypersensitivity reaction to fungal antigens, as indeed to other antigens as well, is becoming increasingly important. For example, Type I reactions are given in atopic patients with rhinitis and asthma; Types I and III reactions are given in patients with asthma and pulmonary eosinophilia caused by *A. fumigatus*, and Type IV reactions to histoplasmin and coccidioidin in infected subjects. Much more information is needed on correlations of the same type concerning skin test reactions to fungi in general with the serological findings and in particular with the clinical manifestations (see Chapter 7).

Epidemiology

Immunological tests have been applied to the study of the distribution of histoplasmosis and coccidioidomycosis, and the same procedures, where appropriate, should be used for other diseases. Serological tests consisting mainly of complement-fixation and precipitin tests have been very useful. Skin tests giving Type IV, delayed, reactions have shown, contrary to what was previously thought, that these two diseases are common and rarely fatal.

Prognosis

Adequate, accurate information is available mainly for histoplasmosis and coccidioidomycosis both of which have a long history of immunological investigation. In histoplasmosis, for example, a positive precipitin test suggests that the infection has been recently acquired, although the positive reaction may persist for 8 months or longer. A high and rising titre in the complement-fixation test indicates that the disease is widespread or that the patient's resistance

is poor, whilst a low or falling titre is frequently a favourable sign. A positive skin test reaction indicates past infection and immunity or present infection which the patient is resisting well. A change of skin test reaction from positive to negative during the course of the disease is an unfavourable sign, though this may be due to the physiological effects of severe illness on the skin test reaction (see Chapter 7 on the tuberculin skin test), rather than, or as well as, altered immunological reactivity of the subject.

The intensity of the precipitin reaction may be important as, for example, in pulmonary aspergillosis. Thus in patients with aspergilloma, surgical removal or expectoration of the aspergilloma is followed by a rapid decrease, often to negativity, of the previously intense reaction. In allergic subjects, on the other hand, weak precipitin reactions may be associated with the more intense Type III skin reactions, although it is not known if the pulmonary infiltrations thought to be mediated by Type III reactions as well have the same correlation. In farmer's lung, in which the higher the precipitin titre the greater the clinical sensitivity and severity of disease, avoidance of *Micropolyspora faeni* leads to a decrease in the precipitin reaction.

The possibility of enhancing resistance and perhaps preventing disease by active immunization has been shown to exist in experimental animals treated by injection of the killed spherules and endospores of *Coccidioides immitis* (Levine, Miller & Smith 1962; Levine, Kong & Smith 1965; Levine & Kong 1965). Better understanding of the immunopathology of fungal disease will improve their diagnostic and prophylactic management.

Fungal Antigens

The many factors responsible for the variable antigenic content of fungal extracts have been discussed. Two main types of antigen have been in general use.

1. *Fungal particles.* These consist of whole cells or spores, e.g. yeast phase of dimorphic fungi, or of homogenized mycelium, which must be obtained in homogeneous suspensions for use in direct agglutination and complement fixation tests.

2. *Soluble antigens* extracted from mycelium and culture media. The mycelial antigens such as cell sap, lysates and extracts of cell walls and cytoplasm consist of mainly protein or polysaccharide components. Simple extraction of defatted mycelium with carbol-saline is a standard and effective method for obtaining useful extracts. Culture filtrates are usually good sources of antigen, e.g. coccidioidin, histoplasmin and blastomycin. Antigenic fractions have been extracted by a wide variety of chemical procedures (Pepys & Longbottom 1967).

Much attention has been given to polysaccharide type antigens, often present in large amounts in fungi, although most of them have contained small quantities of firmly bound nitrogen. It seems that much of the cross-reactivity, sometimes

found between taxonomically unrelated species, may be due to the chemical similarities which exist between these polysaccharides (see Chapter 37). Protein antigens may be very important, for example in *A. fumigatus*, being responsible for most of the precipitin and skin test reactions and showing greater specificity (Longbottom 1964).

Standardization of Antigens

The fungal antigens most frequently used are complex mixtures and it is difficult to obtain comparable preparations even under carefully controlled conditions (Campbell 1965). For standardization, comparative skin and serological tests are needed of each new preparation and since different antigen-antibody systems are often involved the responsible antigens are likely to be different.

There is an urgent need for standardization of fungal antigens. It should, however, be noted that differences, however slight, in the methods of testing in different laboratories may give varying results even where the same antigens are used, such as those available commercially, for tests in histoplasmosis and coccidioidomycosis (Smith, *et al* 1957) and in aspergillosis. For comparative purposes test conditions need to be controlled, for example the ratio of antigen and antibody, temperature and duration of the test, and other factors which may influence the sensitivity of the test (Kaufman 1966).

Cross-Reactions

These may be marked and extensive, for example, with antigens from species of *Histoplasma*, *Coccidioides* and *Blastomyces*. As with mycobacterial antigens, cross-reactivity, for example, to skin tests with fungal antigens, has made it necessary to base assessments on patterns of cross-reactivity rather than on a specific reactivity to any one antigen (Campbell 1965).

Non-Specific Factors

There are a number of reasons why many of the crude, complex fungal antigens may be unsuitable for serological tests. They may contain components which are haemolytic, haemagglutinating, or anticomplementary, whilst others are rich in enzymes, especially proteolytic, which may cause interference. Problems may also arise from the presence of media constituents.

An important possible source of diagnostic error is the presence in the genus *Aspergillus* and the dermatophytes, as well as in other unrelated sources such as nematode parasites, of substances similar to pneumococcal C-substance (see Chapter 36) in that they give precipitation reactions with C-reactive protein. Although such reactions in agar gel may look like true antigen-antibody reactions, the C-substance reaction with C-reactive protein is dependent upon calcium and can be prevented by incorporating a citrate buffer in the agar, when only the genuine precipitin reaction will develop (Longbottom & Pepys 1964).

These findings are of importance in the critical assessment of reports of precipitin reactions to fungal antigens in some investigations in which the presence in the serum at the same time of C-reactive protein and specific precipitins was known (Fava Netto, Ferri & Lacaz 1959; Longbottom & Pepys 1964). The transitory presence of precipitation reactions, often found to have appeared and disappeared early, should also be re-examined for the same reason since it is likely that C-substance-like materials may be present in many other fungal extracts.

Nature of Fungal Antibodies

Attempts have been made to identify the immunoglobulins produced in response to fungal antigens and all three classes IgG, IgM and IgA have been reported. Precipitins and reagins were separated in the serum of a patient with pulmonary allergic aspergillosis (Hayward, Augustin & Longbottom 1960) and further work showed that the precipitins in aspergilloma patients appeared to be IgG, mostly with multiple arcs occurring in the slow γ_2 mobility range but in some sera one arc extending into the faster γ_1 range (Longbottom 1964). Similar results were reported using both immuno-electrophoretic and radio-active tracer techniques (Tran van Ky, Havez, Biguet & Leys 1966; Biguet & Tran van Ky 1966) although some 4/9 sera from aspergilloma patients were also shown to contain IgM and 1/9 sera IgA antibodies. In the sera of patients with coccidioidomycosis the complement fixing antibodies appeared to be IgG whereas the precipitins were IgM globulins (Pappagianis, Lindsey, Smith & Saito 1965; Sawaki *et al* 1966) and similarly the precipitins in patients with candidiasis were also associated with IgM globulins (Taschdjian *et al* 1964).

FUNGAL DISEASES

Aspergillosis

Fungi of the genus *Aspergillus* are filamentous, and produce spores which readily become airborne. Aspergillus species are ubiquitous and are commonly found in soil and decaying vegetable matter. Their ability to grow on many substrates under a wide range of environmental conditions and, in particular, those of the living body may account, especially in the case of *Aspergillus fumigatus*, for their capacity to produce disease in man. Whilst there are reports of infection in almost every organ in the body (Austwick 1965), the respiratory tract is the most important (see Chapter 36). Briefly, *A. fumigatus* may act as an allergen, mediating Type I reactions in the bronchi and causing asthma; it may mediate Types I and III reactions in atopic subjects causing asthma and pulmonary eosinophilia; it may grow saprophytically in damaged areas of lung producing the aspergillus mycetoma or aspergilloma; and it may, in patients with reticulo-endothelial disease, invade the tissues of the body.

Precipitating antibodies against aspergillus antigens were first reported in three patients with respiratory disease by Vallery-Radot and Giroud (1928) using the ring precipitin test. The clinical significance in asthma, pulmonary eosinophilia and aspergilloma of the immunological changes induced by *A. fumigatus* and notably the importance of the precipitins were determined using agar gel double diffusion tests (Pepys, Riddell, Citron, Clayton & Short 1959).

Detailed study of factors influencing growth and production of antigens by *A. fumigatus* showed that mycelial extracts (cell saps) are a richer source of antigens than culture filtrates, although the latter are a very good source of antigens (Longbottom 1964; Longbottom & Pepys 1964). Surface culture at 37°C on Sabouraud liquid medium gave slightly better yields of antigen than in Czapek-Dox medium, and culture for 3 to 5 weeks was optimal. As with other fungi, antigen production varied from batch to batch even under constant cultural conditions, and similar antigenic variations occurred between different strains. Ideally a series of different extracts are needed to obtain the maximum number of positive reactions. The most effective method for obtaining purer antigens was found to be salt precipitation followed by filtration through Sephadex G-25 to remove inorganic ions. In this way a highly antigenic protein precipitate was separated from the other, mainly polysaccharide, components (Longbottom 1964).

Two sorts of specific antigen-antibody precipitation reactions were observed in agar gel, consisting of sharply defined arcs regarded as 'R' or rabbit type, due to protein or glycoprotein antigens, and a contrasting diffuse broad precipitation arc of the 'H' or horse type due to polysaccharide-type antigens. Both reactions were due to IgG immunoglobulin, the 'R' reactions usually being multiple and more important for diagnosis, the 'H' reaction occurring infrequently and usually in association with 'R' reactions. The 'H' reaction is obtained with those sera which show extensive cross-reactions with extracts of species of other fungal genera (Pepys, Riddell & Clayton 1959; Longbottom, Augustin & Hayward 1960; Feinberg & Temple 1963). This cross-reactivity was attributed to relative structural similarities of the polysaccharide antigens. By comparison the 'R' reactions were more species-specific, showing only limited cross-reactivity within the genus *Aspergillus* (Longbottom 1964).

A third, strictly speaking non-immunological precipitation reaction, an important possible source of diagnostic error, occurs between substances in the fungal extracts which behave like pneumococcal C-substance and precipitate with C-reactive protein (Plate 3.1). This reaction, which has a characteristic appearance in the agar-gel tests, can be inhibited by incorporation of citrate buffer in the agar. The reaction with C-reactive protein was found to depend upon the peptide moiety of a somatic glyco (glucogalactomannan) peptide in extracts of *A. fumigatus* (Longbottom 1964), and was produced also by a galacto-

mannan peptide of *Trichophyton mentagrophytes* prepared by Barker, Cruickshank, Morris & Wood (1962), as well as with crude extracts of other *Aspergillus* species and of *T. rubrum* and *Epidermophyton floccosum* (see also Dermatomycoses). The capacity of all these C-substance-like extracts to give Type I skin test reactions in patients with aspergillus sensitivity was attributed to their peptide moiety and has been discussed (see Chapter 36), whereas the polysaccharide moiety of these substances was responsible for 'H' type antigen-antibody precipitation reactions.

The demonstration of the precipitating antibodies is of diagnostic value in patients with pulmonary disease caused by *A. fumigatus* and in the infrequent infection with other members of the genus (see Chapter 36). Immuno-electrophoretic tests are helpful in grading the intensity of the reactions according to the number of arcs (Longbottom & Pepys 1964). Passive haemagglutination tests have not proved useful because of the presence in the extracts of spontaneous haemolysins, haemagglutinins and/or anti-complementary factors (Longbottom 1964), though latex agglutination tests may be more satisfactory (Murray, unpublished). Determination of antibody titres would be helpful, but is complicated by the multiplicity of the antigen-antibody systems involved.

In agar gel precipitin tests with extracts of *A. fumigatus* negative reactions were obtained in normal subjects and positive reactions of weak intensity (one to four arcs) in 9% of patients with uncomplicated asthma and in 63% of patients with asthma and pulmonary eosinophilia (Longbottom & Pepys 1964). In the latter group, concentration of the serum or of the immunoglobulins raised the total of positive reactions to 93% (see Chapter 36), whilst negative reactions were given by normal sera concentrated in the same way for control purposes. Skin tests in these patients gave dual reactions, consisting of Type I followed by Type III responses (see Chapters 7 and 37). Therefore a very close correlation exists between asthma and pulmonary eosinophilia, dual skin reactions and the presence of precipitins against *A. fumigatus*. Only a small proportion of patients in the United Kingdom with asthma and pulmonary eosinophilia fail to give these reactions, and in the patients with aspergillus allergy it is unusual for dual reactions to be obtained in the absence of precipitins.

In patients with aspergilloma, precipitins against extracts of *A. fumigatus* are of high diagnostic value (Longbottom & Pepys 1964; Drouhet, *et al* 1963; Gernez-Rieux *et al* 1963). Almost all patients with proven aspergilloma have given strong precipitation reactions with multiple arcs, up to fifteen on occasion (Plate 3.2). Specific reactions have also been obtained to other *Aspergillus* species, such as *A. flavus*, *A. niger* and *A. nidulans*, where these have been responsible (Longbottom, Pepys & Temple-Clive 1964) though cross-reactions have occurred and in one case a positive reaction to *A. fumigatus* was obtained with the serum of a patient with an *A. nidulans* aspergilloma (Helluy *et al* 1963). The precipitin test rapidly becomes weaker and even negative in these patients if

the aspergilloma is removed or if it dies and is expectorated. Also in a patient with aspergillosis involving bone, specific precipitins to *A. nidulans* have been found (Redmond, *et al* 1965).

In a report on aspergillus precipitins (Feinberg & Temple 1963) the reactions obtained with the sera of asthmatic patients, who were not further classified, appeared to be those of the widely cross-reacting H-type, the clinical significance of which has not been determined. In another survey (Campbell & Clayton 1964) precipitins to *A. fumigatus* were reported in 69%, and positive immediate (Type I) skin reactions in 99%, of patients with allergic aspergillosis, of whom 88% had pulmonary infiltrations. The conclusion was drawn that the precipitins were immunologically unimportant, being merely evidence of active or recent infection. However, a better understanding of the possible role of the precipitins in allergic subjects can be obtained by comparison of their low incidence in patients with uncomplicated asthma (a comparison not made by the above authors) and their high incidence in patients with asthma and pulmonary eosinophilia.

In infection with *A. fumigatus*, usually associated with colonization of damaged lung, precipitins are indeed present, but the very strong and highly statistically significant correlation between the appearance of pulmonary infiltrations in asthmatic subjects, and the presence together of reagins as well as precipitins, and the production of dual skin test reactions indicates that these antibodies are of pathogenetic importance.

Candidiasis

Candidiasis is the term commonly applied to all aspects of infections caused by species of *Candida*. These occur most frequently in skin, nail and mucous surfaces, but no tissue is immune. On occasion a septicemia may be produced and with the introduction of cardiac catheterization and open heart surgery, candida endocarditis is now a well recognized entity. The nature of the antibody response may depend on the depth and site of the infection.

A number of tests have been investigated in the past, generally with disappointing or inconclusive results. However, as a means of classifying and identifying *Candida* species, immunological methods have been widely and successfully applied.

The great frequency of positive skin tests in healthy individuals makes it difficult to assess their diagnostic significance, although all types of reaction, whether immediate or delayed, have been regarded as specific and due probably to the early and repeated contacts of man with this organism (Sclafer & Hewitt 1960). In a recent report (Holti 1966) the incidence of delayed Type IV hypersensitivity reactions in humans without any evidence of clinical infection was shown to increase markedly with age, i.e. 14% in an 11–20 year age group, to 83% in the 50 year and over age group. Immediate, Type I, hypersensitivity

reactions were much less common, preliminary studies suggesting an incidence of 10 to 15% in the adult population.

Some workers have attempted complement fixation tests (Peck *et al* 1955; Rimbaud *et al* 1960) but the procedure has not been developed as a test of practical value. The same is true of haemagglutination (Goldin 1957).

Agglutinins to *C. albicans* in human sera were first reported in 1937 (Todd) and were reviewed by Winner in 1955, who found that agglutination titres of 1/16 or higher were obtained with the sera of 20% of healthy males, of 26% of healthy females but only 3.6% of neonates; in a parallel series of patients with superficial candidiasis the comparable figures were males 39% and females 40%. In all groups, individual titres ranged from less than 1/16 to 1/128 or higher, but it has also been shown that the mean titre is higher in infected than in healthy individuals although a low titre did not exclude infection (Comaish, Gibson & Green 1963). High agglutination titres have been noted in three cases of idiopathic hypoparathyroidism associated with moniliasis (Sjöberg 1966).

There is some evidence that children are born free of antibodies to *C. albicans* (Winner 1955; Brody & Finch 1960), that these develop quite rapidly, presumably because of commensal contact, and that their level, as determined by an immune-adherence test, is not affected by the presence of lymphomas, leukemias and similar disorders with which an increased incidence of disseminated monilial infections is associated (Brody & Finch 1960).

Precipitin tests in man have been made with oidiomycin or culture filtrate (metabolic) extracts, cytoplasmic extracts from sonically disrupted cells (Taschdjian *et al* 1964; Taschdjian, Kozinn & Caroline 1964), and crude cell wall polysaccharide extracts prepared by extraction with phenol (Akiba, Iwata & Inouya 1957), β-naphthol (Elinov & Zaikina 1959), and formamide (Stallybrass 1964a) and a purified mannan (Chew & Theus 1967; Pepys *et al* 1967). Precipitin reactions, chiefly to the polysaccharide antigens, in ring and agar gel tests have earned the reputation of being positive only in heavily infected patients with extensive superficial or deep-seated systemic candidiasis (Akiba *et al* 1957; Elinov & Zaikina 1959; Taschdjian *et al* 1964; and Stallybrass 1964). These findings must however be re-examined.

In double-diffusion tests, with mannan A (Chew & Theus 1967) on unconcentrated serum, reactions were given by 3% of the normal sera and by 10% of heavily infected subjects, whereas tests with concentrated immunoglobulins gave positive reactions in 30% of the normals. In very sensitive double-diffusion tests of concentrated immunoglobulins (fifteenfold) in capillary (Preer) tubes containing a mixture of agar-gel and the mannan A, they obtained positive reactions in all the normal sera tested.

By contrast, Pepys *et al* (1967) have found in double-diffusion tests, as used by Longbottom & Pepys (1964), that reactions to the mannan A were obtained in about equal numbers of unconcentrated sera and of the remaining sera after

threefold concentration, in a total of 18% of healthy subjects, 22% of non-asthmatic patients with respiratory disease, 55% of asthmatics and 72% of asthmatics with pulmonary infiltrations, that is with pulmonary eosinophilia (see Chapter 36).

The varying results are probably due to technical differences. Pepys *et al* (1967) found that the mannan A at 10 mg/ml gave reactions in double-diffusion tests with 3 out of 8 sera which had given positive reactions to a concentration of 1 mg/ml. The precipitation arc of the mannan A reaction was of the fuzzy 'H' type which is readily soluble in excess antigen. The sera of heavily infected subjects would be expected to contain more antibodies and therefore be capable of giving a visible reaction with high concentrations of antigen. Only little, if any, information is given in the other reports on the antigen concentrations used for the double-diffusion tests.

Cross-reactions in one-quarter of the positive reactions to the mannans of *C. albicans* group A were given by the mannans of *C. albicans* group B (Pepys *et al* 1967), as reported in experimental animals by Hasenclever (1965). *C. albicans* group A has been cultured more frequently in man than group B. Precipitins against protein antigens contained in the culture filtrate of *C. albicans* group A were found in about one-third of the sera which had mannan A precipitins as well. It was rare for a serum to react to the protein antigen only.

The double-diffusion test employed by Pepys *et al* (1967) would appear to measure significant levels of precipitating antibody as shown by skin and inhalation tests. Of twenty-two subjects giving positive precipitin reactions, seventeen gave Type III, Arthus, reactions to intracutaneous tests with mannan A, compared with four out of twenty-eight giving negative reactions. A single intracutaneous test of 0.01–0.02 ml of mannan A 10 mg/ml resulted in all subjects tested, whether normal, asthmatic or non-asthmatic, in the appearance after several days to 3 weeks of precipitin reactions or of stronger reactions (see Chapter 7). In view of the report by Chew & Theus (1967) of precipitins in all subjects, this appearance of precipitins must be regarded as a secondary response.

On repetition of the skin test with the mannan A after 1 week, both stronger Type III reactions were produced and the number of reactions increased, for example from two up to five out of ten subjects tested. The mannan A is clearly an antigen of remarkable potency. Care must be taken in assessing the serological and skin test findings in previously tested subjects. Inhalation tests with the culture filtrate extract provoked, in six out of ten subjects, febrile reactions appearing after about 6 hr and accompanied by a leucocytosis and blood eosinophilia within 24 hours. In two out of three subjects, an immediate was followed by a late asthmatic reaction, and one other subject gave a late reaction only. The mannan A gave, in one of three subjects, immediate asthma only to the first, and only late asthma to a repeat test. Of the six patients giving reactions, five

had precipitins against protein antigens present in the culture filtrate extract. There were no precipitins against the protein antigens in the four non-reactors.

A possible relationship of the precipitins to disease is suggested by the findings in patients with asthma and pulmonary eosinophilia which in the U.K. is commonly due to *A. fumigatus* (see Chapter 36). In eleven selected patients who had no precipitins against *A. fumigatus*, 9 had *C. albicans* mannan A precipitins. In seventeen with precipitins against both polysaccharide and protein antigens of *A. fumigatus*, all had mannan A precipitins. No cross-reactions were found between the *C. albicans* mannan A and *A. fumigatus* polysaccharide antigens. In the *A. fumigatus* polysaccharide the reacting determinant has been found to be galactose (Longbottom 1964), which is not present in the mannan A. The presence of precipitins against the polysaccharides of both fungi may be evidence of infection by both of them, or it may be that some subjects are particularly sensitive to polysaccharide antigens. In nine who had precipitins only against the protein antigens of *A. fumigatus*, none had precipitins against the mannan A.

Thus there is evidence that precipitins against *C. albicans* are common in man, that they may participate in tissue-damaging reactions of the Arthus, Type III, and that they may be related to allergic pulmonary infiltrations in man. It would be surprising if the potency of the mannans and the frequent presence of *C. albicans* in other organs as well as the respiratory tract were not related to disease processes in these organs.

Immunological methods have been extensively used for taxonomic studies of the genus *Candida* (see Hasenclever 1965). Agglutination inhibition tests have shown that there are two groups, A and B, of *C. albicans*, group A being closely related antigenically to *C. tropicalis* and group B to *C. stellatoidea*. *C. albicans* group A and *C. tropicalis* contain one antigen not present in similar extracts of the group B yeast-cells (Summers, Grollman & Hasenclever 1964). Close, though not identical, antigenic relationships of the two groups have recently been confirmed (Murray & Buckley 1966). The slight discrepancies of these results with those obtained in the past may be due to the use of disintegrated yeast cells for absorption of sera rather than intact cells which led to comparison of surface antigens only.

Quantitative precipitin and agar gel diffusion tests with cell-wall polysaccharides have confirmed the presence of groups A and B of *C. albicans* (Hasenclever & Mitchell 1964a and b; Summers, Grollman & Hasenclever 1964; and Stallybrass 1964a). Antigenic and chemical similarities have been shown between the *Candida* cell-wall polysaccharides, in particular of the isolated mannans, and extracts of species of *Saccharomyces*, *Hansenula*, *Torulopsis* and *Cryptococcus*. Specific thermolabile antigens in the cytoplasm of certain species of *Candida* have also been reported (Kemp & Solotorovsky 1964; Stallybrass 1964a, 1965) and the antigenicity of *C. albicans* has been extensively studied by

immuno-electrophoretic methods (Tran van Ky, Biguet & Andrieu 1963; Biguet *et al* 1965).

Fluorescent antibody tests have been applied to the differentiation of the *Candida* species (Gordon 1958a and b) and also to demonstrate that cell-wall material becomes incorporated into the walls of daughter cells and hyphae (Goos & Summers 1964). Using an indirect fluorescent antibody technique, the sandwich method of Vogel & Padula (1958), patients suffering from candidiasis were shown to have titres greater than 1/16 whereas carriers had titres up to 1/16 and controls up to 1/8 (Lehner 1966). Investigation of saliva by the same method produced parallel results, a titre of 1:1 or above being indicative of clinical disease (Lehner 1965).

There do not appear to be marked differences in the pathogenicity of the groups A and B of *C. albicans*, although there are differences between individual strains of the organisms concerned (Hasenclever 1961, Isenberg *et al* 1963). The majority (68–75%) of human isolates belong to group A (Hasenclever & Mitchell 1963a; Stallybrass 1964b).

Immunization of experimental animals against *C. albicans* has not met with much success (Winner 1956), though some degree of protection has been observed in mice (Mourad & Friedman 1961; Hasenclever & Mitchell 1963b). Whilst there is general agreement that the human antibodies are no measure of resistance, it has been found that normal human serum inhibits the growth of *C. albicans in vitro* (Roth & Goldstein 1961).

Cryptococcosis

The causal organism of cryptococcosis, *Cryptococcus neoformans*, is quite widely distributed in nature but overt disease itself is relatively infrequent. It may attack skin, subcutaneous tissues, lung or brain, though it is best known for its effect on the latter. *C. neoformans* is an imperfect yeast with no tendency to form hyphae or pseudohyphae and most strains, at least *in vivo*, possess a thick polysaccharide capsule (Einbinder, Benham & Nelson 1954) which, according to some, has the power of inhibiting phagocytosis (Drouhet & Segretain 1950). The organism has been divided into a number of serotypes, generally three or four, by means of agglutination and precipitation reactions (Evans 1949, 1950). The capsular polysaccharides have been studied as antigens (Evans & Kessel 1951; Evans & Theriault 1953) and cross-reactivity demonstrated between them and the polysaccharides of *Candida albicans*, *Saccharomyces cerevisiae*, trichophytin and gum tragacanth (Evans, Sorenson & Walls 1953) and also between *C. neoformans* Type A capsular polysaccharide and polysaccharides of Type II and XIV pneumococci (Rebers *et al* 1958). Most workers have experienced difficulties in producing good antisera to *Cryptococcus* species and it has been reported that the weakly encapsulated strains do best in this respect (Neill, Abrams & Kapros 1950) but opinions differ (Seeliger 1960). Slide agglutination

methods have been recommended for rapid identification of species of this genus (Tsuchiya, Kawakita & Udagawa 1963).

Serological diagnosis of cryptococcosis has not, as yet, been developed to the level of routine reliability. Whilst the classical methods of agglutination, precipitation and complement fixation have failed, several other serological tests, with some degree of success, have now been reported.

The presence of cryptococcal antigens in the spinal fluid, blood and urine of a patient with cryptococcal meningitis has been demonstrated by both precipitin and complement fixation tests using *C. neoformans* antisera. Absorption of the antiserum with purified capsular polysaccharide inhibited the reaction (Neill, Sugg & McCauley 1951). Similar results in two further patients with meningitis have been reported (Anderson & Beech 1958). More recently a slide test with antibody-coated latex particles was employed to detect antigens in the serum or spinal fluid (Bloomfield, Gordon & Elmendorf 1963; Gordon & Vedder 1966). Antigens were found, sometimes in high titre in initial specimens of cerebrospinal fluid from proven cases and the method appeared to have adequate specificity and prognostic as well as diagnostic value. Latex particle agglutination with antigen sensitized particles has also been used successfully to detect *C. neoformans* antibodies (Gordon & Vedder 1966). Similarly, significant titres of serum antibodies have been reported in a passive haemagglutination method with human type O red blood cells coated with crude *C. neoformans* A capsular polysaccharide (Pollock & Ward 1962).

Both direct and indirect fluorescent antibody techniques have been applied to the study of cryptococcal infections. The direct test (Marshall *et al* 1961) is useful to identify *C. neoformans* cells irrespective of their degree of encapsulation, and also to detect the presence of cryptococcal polysaccharide lining the bronchial epithelium, in alveolar exudate and within macrophages participating in the granulomatous reaction (Kase & Marshall 1960). However, cross-reactions with several *Candida* species have recently been reported (Kaufman & Blumer 1965).

In the indirect method of fluorescent-antibody staining, heat-killed *C. neoformans* cells are fixed on a slide and covered with heat-inactivated patient's serum. After incubation the slide is washed and the preparation is covered with a fluorescein labelled anti-human globulin (Coombs reagent) serum to detect the fixed serum antibodies. By this method cryptococcal antibodies were demonstrated in seven out of eight proven cases, but some false positive reactions were obtained with sera containing agglutinins to *C. albicans* (Vogel & Padula 1958; Vogel, Sellers & Woodward 1961). Positive reactions to this test have been reported in 18/23 patients with proven disease, and negative results with six control sera (Kaufman 1966).

Delayed skin reactions have been elicited in guinea-pigs sensitized with minute amounts of purified protein from *C. neoformans* (Salvin & Smith 1961). Most

patients who have recovered from the disease give delayed reactions to extracts of *C. neoformans*, but reactions are obtained in other subjects as well, and the value of the test has not yet been determined (Bennett, Hasenclever & Baum 1965).

Most mammals seem to have a fairly high degree of innate resistance to cryptococcosis and the relatively high incidence of this disease in people with disorders of the reticulo-endothelial system suggests a humoral explanation. It has been observed that titres of properdin drop very rapidly in experimentally infected mice (Gadebusch 1961), and that an increased resistance has been conferred on mice by injection with formalin killed cells (Abrahams & Gilleran 1960).

Coccidioidomycosis

The causal organism of coccidioidomycosis, *Coccidioides immitis*, is a dimophic fungus of somewhat uncertain affinities but probably belonging to the Phycomycetes. It adopts a filamentous form in soil where it divides into numerous small arthrospores which, when inhaled, produce the disease. In mammalian tissue the fungus grows into a thick-walled spherule, rather like a sporangium, containing numerous endospores. The clinical manifestations of coccidioidomycosis are variable, and laboratory confirmation can often be given by cultural and microscopic means, though immunological tests play a vital part in diagnosis, prognosis and epidemiology.

The three most valuable tests are the skin test, the complement fixation test and the precipitin test. Coccidioidin, which is obtained from a pool of culture filtrates of 10 strains of *C. immitis* grown under carefully standardized conditions, is the antigen most frequently used in all these tests (Smith *et al* 1948), though other extracts have been successfully tested (Huppert & Bailey 1965a and b). After the appearance of symptoms due to *C. immitis* the first test to become positive is the skin test (Type IV), generally within 2 to 20 days (80% within a week), followed by the precipitin test (50% in the first week and up to 90% by the third week). The skin test normally remains positive indefinitely, but the precipitin test becomes negative some 12 to 16 weeks after infection. A positive complement fixation test may appear during the first 3 months, but as it is a measure of the severity of infection it may remain negative throughout the disease.

Apart from its value in epidemiology, the coccidioidin skin test, a typical delayed hypersensitivity response, is of great diagnostic value, and it becomes positive sooner than the other tests. Positive serological tests were not obtained in patients with primary coccidioidomycosis or with impending dissemination in the absence of a positive skin test (Smith *et al* 1950; Smith, Saito & Simons 1956). The limitations of the test are that it remains positive indefinitely after an attack of the disease, overt or subclinical, and may become negative in the

disseminated disease. Coccidioidin skin testing does not seem to elicit humoral antibodies, though positive skin test sensitivity may be induced thereby (Walraff, Snow & Wilson 1965) and a correlation has been found between the N content of the coccidioidin and its skin reactivity (Walraff & Wilson 1965).

Results with complement fixation tests have been rather variable, varying from 56% positive (Smith *et al* 1950; Smith *et al* 1956) to 100% (Walraff *et al* 1965) in proven cases. On the whole the best results ensue from micro- or semi-micro-techniques with careful attention to end-points and complement dose. High complement fixing titres seem to indicate disseminated disease, but the significance of low titres is not clear. Most workers have emphasized that titres as low as 1/2 or 1/4 can be significant (Smith *et al* 1950, 1956), but such titres have occasionally been obtained in patients known not to have coccidioidomycosis (Schubert & Hampson 1962; Huppert & Bailey 1963). A low titre of this order can only be regarded as presumptive evidence of coccidioidomycosis, and confirmation is needed from clinical and mycological studies. Negative serological tests do not exclude the disease.

Since precipitin tests are considered best for detecting primary coccidioidomycosis at a relatively early stage, and complement fixation tests for detecting disseminated disease, a combination of both tests is better than either alone. In a study of 3219 patients with uncomplicated primary coccidioidomycosis, 56% gave positive complement fixation tests and 78% positive precipitin tests, but no fewer than 90% were positive by one test or the other (Smith *et al* 1950). Cross-reactions occur with histoplasmosis and blastomycosis, but seldom present any problem (Campbell & Binkley 1953).

Immunodiffusion tests have now been described which correlate well both with complement fixation tests when a concentrated (tenfold) culture filtrate antigen is used, and with tube precipitin tests when a toluene lysate antigen is used (Huppert & Bailey, 1963, 1965a and b). Since these diffusion tests give results quickly and are easily performed in any laboratory, they are potentially very valuable and may help to overcome technical laboratory discrepancies which were reported with the other tests (Smith *et al* 1957).

Recent findings indicate that a fluorescent antibody inhibition test can also be used for the rapid detection of antibodies to *C. immitis*. In these tests use was made of a conjugate specific for the tissue form of *C. immitis* prepared from the serum of rabbits either infected by the fungus or immunized with killed arthrospores (Kaplan & Clifford 1964). The procedure involved the comparison of intensity of staining of endospores of *C. immitis* formed *in vivo* in mice, by covering heat-fixed smears of infected mouse lung tissue with a mixture of conjugate and patient's serum, and assessing the intensity of resulting fluorescence. Results very closely paralleled those of complement fixation tests and, although in a much more limited number of sera, tube precipitin tests (Kaplan *et al* 1966).

Coccidioidomycosis is the only fungal disease in which real progress towards a practical vaccine has been made. Earlier experiments with killed arthrospore suspensions as vaccines were not particularly successful (Converse *et al* 1962), but later developments using suspensions of killed spherule walls and endospores have conferred a high level of immunity in mice and monkeys (Levine, Miller & Smith 1962; Levine, Kong & Smith 1965; Levine & Kong 1965). The use of a viable vaccine is also being investigated (Converse 1965).

The antigens and antigenic production of *C. immitis* have also been extensively studied (Rowe, Newcomer & Wright 1963; Rowe, Newcomer & Landau 1963; Rowe *et al* 1965; Pappagianis *et al* 1961).

Histoplasmosis

Two forms of histoplasmosis exist—one caused by the small celled *Histoplasma capsulatum* and the other by the large celled *Histoplasma duboisii*. It is very doubtful whether *Histoplasma farciminosum* of Equidae belongs to this genus at all. Small cell histoplasmosis is widely distributed in the Americas, Africa and Asia, whereas the large cell variety has not so far been recorded outside Africa.

Both *H. capsulatum* and *H. duboisii* are dimorphic fungi, filamentous in the vegetative state and yeast-like in the parasitic phase in mammalian tissues. The two fungi are virtually identical in both phases *in vitro* and frequently also in experimental animal infections. In man, however, the yeast phase of *H. capsulatum* is of the order of 1–4μ whereas that of *H. duboisii* is 10–15μ. The principal reasons for regarding them as different species are that the diseases produced are different, that the parasites in man differ greatly in size and that there are certain subtle serological differences between them. Very little is known about the serology of *H. duboisii* infections in man but *H. capsulatum* has been extensively studied for many years.

Very many tests have been recommended for the diagnosis of histoplasmosis, e.g. skin tests, complement fixation tests, precipitin tests, latex and other agglutination tests, agglutination tests of particles of the causal organism and fluorescent antibody tests. Reviews of some of these tests have recently been published (Schubert & Wiggins 1963; Mays, Hawkins & Kuhn 1964). The only tests that are in regular diagnostic use at present are skin tests, precipitin tests and complement fixation tests, though a latex agglutination test probably deserves a place in routine laboratory practice.

In small cell histoplasmosis, antibodies make their appearance in the order and at about the times already quoted for coccidioidomycosis. Also, the skin test again remains positive indefinitely, the precipitin test is relatively short lived and the titre of complement fixing antibodies is approximately proportional to the degree of infection. The latter may never become positive or may become transiently positive or may persist for many months.

Histoplasmin is used for skin testing, giving typical delayed hypersensitivity

reactions of the tuberculin type. A positive response indicates that the patient has or has had histoplasmosis and a negative response can mean that there is no infection, that the infection is very recent, or that the patient is in a state of anergy. The histoplasmin skin test suffers from the same limitations as coccidioidin skin testing, and in addition the test itself is capable of provoking the appearance of circulating antibodies (McDearman & Young 1960; Nicholas *et al* 1961; Campbell & Hill 1964). The foregoing remarks on skin tests apply only to small cell histoplasmosis as the information available on *H. duboisii* infections is very scant. One of the authors' (I.G.M.) impression with regard to the latter is that histoplasmin skin tests are generally negative and that this disease behaves more like *B. dermatitidis* infections than *H. capsulatum* infections.

Complement fixation tests may be carried out with histoplasmin or suspensions of the yeast phase cells as antigen, the two tests being complementary to one another, since sera from culturally confirmed cases of histoplasmosis may react with only one of these antigens (Hill & Campbell 1956; Campbell 1960). Complement fixation tests are a highly reliable guide and titres of 1/8 or greater are commonly regarded as presumptive evidence of histoplasmosis, with the proviso that histoplasmin skin tests themselves may provoke titres as high as, or greater than, this and these may persist for many months. Positive tests with titres ranging from 1/8 to 1/32 have also been reported in cases of tuberculosis (Mays, Hawkins & Kuhn 1964) but these were usually transient. In one of the authors' (I.G.M.) experience the histoplasmin complement fixation test is positive in at least some cases of large cell histoplasmosis.

The latex agglutination test is, relatively speaking, a newcomer (Saslaw & Carlisle 1958) and successor to the collodion agglutination test, but its simplicity is attractive and a commercial antigen suspension, reasonably specific and stable, is available (Hill & Campbell 1962). It appears to have the same order of sensitivity as the precipitin test, a titre of 1/32 or greater being considered very significant with very few false positives, and it sometimes succeeds where other tests fail, being particularly useful with anticomplementary sera. The antibodies mediating this test do not seem to persist for as long as those responsible for complement fixation, but frequently appear earlier (Bennett 1966).

Heiner (1958) described the use of precipitin tests in agar gel with concentrated histoplasmin and pointed out that both specific and non-specific precipitates occur, the so-called H and M bands. Both were found in the sera of patients with active histoplasmosis, although the latter was also observed to develop in sensitive patients, without active or current disease, as a result of histoplasmin skin testing. Nevertheless the M band is regarded as the most significant, since the sera of patients with proven histoplasmosis may contain only this band (Wiggins & Schubert 1965). In the absence of a recent skin test, the M band may, therefore, be an early indication of disease, appearing before the H band and disappearing more slowly (Kaufman 1966). Heiner also described a C band

which was not specific for *H. capsulatum* but cross-reacted with other fungi. The pattern of precipitating antibodies is, however, even more complex as up to eight bands were found to occur with human sera using an acetone-precipitated fraction of mycelial broth (Klite 1965), and histoplasmosis was present in subjects whose sera gave minimally positive or even negative tests, but here too the M band occurred more frequently. The H and M components which have been separated on DEAE-cellulose (Green, De Lalla & Tompkins 1960) are also present in the broth from yeast-phase cultures, but these were shown to contain an additional antigen for some patients (Tompkins 1965). Nothing is known of the pattern of precipitating antibodies in large cell histoplasmosis.

Immunofluorescence tests have proved useful in several ways. Antigenic relationships have been revealed between both phases of *H. capsulatum* and *B. dermatitidis* (Kaufman & Kaplan 1963) and also between the mycelial phase of *H. capsulatum* and several morphologically similar species of *Sepedonium* and *Chrysosporium* (Kaufman & Brandt 1964). By various absorptions of fluorescein-labelled rabbit anti-*H. capsulatum* globulins, e.g. twice with *B. dermatitidis* or twice with *C. immitis* antigens, a conjugate specific for yeast phase *H. capsulatum* could be prepared (Kaufman & Kaplan 1961). These reagents have been used to identify *H. capsulatum* in the sputum, with an 88% correlation with cultural methods, as well as in some specimens culturally negative although from patients with proven histoplasmosis (Lynch & Plexico 1962). Other similar reports (Carski, Cozad & Larsh 1962) also indicate the existence of some cross-reactions, e.g. with *C. albicans* which may be eliminated by absorption techniques.

Fluorescent antibody inhibition tests in which the reaction of specific labelled fungal antisera with the fungal antigens is blocked by the reaction of specific antibodies in the test serum, have proved to be simple and effective for the rapid detection of yeast-phase antibody and in the differentiation from other serologically related mycotic infections. The method consists of adding the conjugated antiserum together with the patients serum to heat-fixed smears of formalin-killed, 7-day-old yeast-phase cultures of *H. capsulatum*. Positive fluorescence inhibition reactions were given with 26/30 sera containing CF-antibodies to the yeast phase and with 2/11 sera containing CF-antibodies against histoplasmin only, whereas 16/30 of the former and 9/11 of the latter sera gave positive reactions with histoplasmin in agar gel tests. Although the complement fixation test was best for showing antibodies against both histoplasmin and yeast-phase antigens, of the sera giving complement fixation titres of 1/8 or more, 87% gave positive fluorescence tests and 53% positive agar gel tests (Kaufman, Schubert & Kaplan 1962). Similar comparative results have been reported (Shipe, Williams & Vann 1963). These tests were especially valuable for detecting antibodies in anticomplementary sera from patients with histoplasmosis, giving a 97% agreement in patients whose subsequent sera gave complement fixation reactions (Kaufman, Brand & McLaughlin 1965).

Fluorescent antibody methods have been employed to differentiate between *H. capsulatum* and *H. duboisii*. An absorbed conjugate of antiserum to the yeast phase of *H. capsulatum* gave specific homologous reactions and did not react with the yeast phase of *H. duboisii* (Pine, Kaufman & Boone 1964).

Attempts to produce immunity to histoplasmosis have been limited and not very successful. An attack of the disease is known to confer a very high degree of resistance but to date no stable avirulent strains have been produced and the killed products do not form satisfactory vaccines. A small measure of success has been reported in experiments with mice (Knight, Hill & Marcus 1959; Hill & Marcus 1959).

North American Blastomycosis

The causal organism of North American blastomycosis or Gilchrist's disease is a dimorphic fungus, first recorded in the Americas but now known also to occur in tropical Africa, known as *Blastomyces dermatitidis*. The mycelial phase is seen on laboratory media at lower temperatures (*ca.* 26°C) as a filamentous fungus reproducing by conidia; no perfect state is known. The yeast phase occurs on certain laboratory media at higher temperatures, e.g. blood agar at 35–37°C, and in mammalian tissues. From these two phases four basic antigens have been used, viz. culture filtrates of a broth supporting the mycelial phase (blastomycin), extracts of mycelium, suspensions of yeast-phase cells and extracts of yeast-phase cells.

As well as antigenic differences occurring, as might be expected, between mycelial and yeast-phase antigens of *B. dermatitidis*, there are many reports of antigenic cross-reactivity between antigens of both phases and corresponding antigens of other somewhat similar fungi, in particular *Histoplasma capsulatum* and *Coccidioides immitis*. Fluorescent antibody techniques have demonstrated antigenic overlap with *H. capsulatum* (Kaufman & Kaplan 1963), *C. immitis* (Kaplan & Clifford 1964), and *Paracoccidioides brasiliensis* (Silva & Kaplan 1965). However, by various absorption procedures of fluorescein-labelled rabbit globulins against *B. dermatitidis* which initially stained both yeast and mycelial phases of the homologous and other heterologous fungi, a conjugate was obtained specific for the yeast phase of *B. dermatitidis* in cultures and clinical material (Kaplan & Kaufman 1963).

Although such experimental studies of *B. dermatitidis* have been useful, from a diagnostic point of view serological results have proved rather disappointing, due both to the relatively large percentage of negative reactions in the tests attempted and to the incidence of cross-reactions with the sera of patients with histoplasmosis and coccidioidomycosis.

Skin tests with blastomycin as antigen have frequently been associated with histoplasmin reactivity. However, in patients known to have blastomycosis these have been regarded as useless (Balows 1963) although positive reactions

have been reported in some 41% of cases (Veterans Administration co-operative study, Busey 1964). On the other hand skin tests in similar patients using a heat-killed yeast-phase vaccine were regarded as more satisfactory, giving positive reactions in 84% of active cases (Balows 1963) and in 35–38% of those who had recovered (Kaufman 1966).

The complement fixation test is in a similarly unsatisfactory state, typical results consistent with confirmed diagnosis being obtained in 46% of tests with blastomycin (Busey 1964) and 38% with yeast-phase antigens (Kaufman 1966). In eighty-six patients, only some 20% had both skin and complement fixation tests positive and in 36% both tests failed to elicit an immunological reaction. Apparently better results have been obtained (Martin 1953) using an antigen from sonically disrupted, washed yeast-phase cells of *B. dermatitidis* in that 71% of the sera from affected patients gave positive complement fixation tests but 73% of the sera also fixed complement in the presence of sonically treated yeast-phase cells of *H. capsulatum*. Furthermore, false positive complement fixation tests occurred with sera from patients with histoplasmosis, coccidioidomycosis, phycomycosis, tuberculosis, sarcoidosis and malignant diseases (Kaufman 1966).

In agar gel diffusion tests, using concentrated soluble antigens derived from mycelial growth, precipitins were present in the sera of fourteen out of twenty-two patients with blastomycosis. Although cross-reactions occurred with sera of patients with coccidioidomycosis and histoplasmosis these heterologous reactions could easily be distinguished from the specific reaction (Abernathy & Heiner 1961). The precipitin test is regarded as more specific though probably less sensitive than the complement fixation test and therefore both tests are recommended when blastomycosis is suspected.

Paracoccidioidomycosis or South American Blastomycosis

The causal organism *Paracoccidioides brasiliensis* (*Blastomyces brasiliensis*) is a dimorphic fungus displaying a filamentous morphology under most conditions but a yeast-like morphology in mammalian tissue and under certain cultural conditions. The disease is chronic and commonly manifests itself as a granulomatous condition of skin, mucous membranes, lymph nodes and internal organs. Clinical relapses are relatively frequent.

Immunological investigations in this disease, mainly reported by a group of workers in Brazil, indicate that skin tests in general have proved unsatisfactory but that precipitin and complement fixation tests are more valuable for diagnosis (Fava Netto 1965).

Skin tests with a wide variety of culture filtrate and cellular antigens have produced discordant results. However, a polysaccharide antigen (Fava Netto 1955), gave positive results in 87% of patients with confirmed disease, but there was also a high positivity rate among relatives of the patients (Fava Netto &

Raphael 1961). These tests, in the patients with disease, resulted in a subacute inflammation lasting for 7 days, with granuloma formation after 10–15 days. In biopsy specimens small granulomas of foreign body type were seen beneath the epithelium and considered to be formed in response to a primary collagen alteration. Fibrinoid damage of collagen was also seen at 4 days. In the control group of uninfected subjects the initial inflammation was less marked, and decreased after 48 hours without granuloma formation (De Brito *et al* 1961). Although positive tests may be expected in clinically well people living in endemic areas, the test is valuable for a rapid differentiation between this disease, cutaneous leishmaniasis and sporotrichosis.

The complement fixation test has been practised with varying results for many years (Moses 1916; Fava Netto 1955; Lacaz *et al* 1959). Most workers report that the highest titres occur in disseminated disease and low or negative results in localized disease (Fava Netto 1965). Using a combination of precipitin and complement fixation tests, antibodies were demonstrated in 98.4% of patients, the precipitins being the first to appear and to disappear, whereas the complement fixing antibodies persisted for a longer time although with decreased titres in cured patients (Fava Netto 1961). The polysaccharide antigen was used satisfactorily for both these tests (Fava Netto 1961), but it did not diffuse well in agar (Lacaz *et al* 1959). In a study of five different antigenic preparations of *P. brasiliensis*, specific complement-fixing antibodies were demonstrated in the sera of patients with confirmed disease with all five antigens and in a comparative assay with a soluble antigen from yeast phase of *H. capsulatum*, using the sera of fifteen patients suffering from South American blastomycosis and two patients with histoplasmosis, cross-reactions were observed but higher titres were always recorded with homogolous antigens (Maekelt 1960).

Only recently described, and likely to prove the most useful test, is an agar gel precipitin test using a (tenfold) concentrated culture filtrate of the yeast phase of growth as antigen (Restrepo 1966). This test was positive in 89%, i.e. sixteen out of eighteen patients with active disease, and negative in three patients clinically recovered and also negative in cases of North American blastomycosis, histoplasmosis, coccidioidomycosis, sporotrichosis, tuberculosis and healthy individuals.

In fluorescent antibody studies it has been shown that *P. brasiliensis* cross-reacts with *B. dermatitidis*, *H. capsulatum*, *C. immitis*, *S. schenckii* and *H. duboisii*. By multiple absorptions a reagent specific for yeast phase of *P. brasiliensis* was produced and used to demonstrate the fungus in the sputum of three cases of pulmonary South American blastomycosis (Silva & Kaplan 1965).

Sporotrichosis

Sporotrichosis is a comparatively rare chronic infection caused by *Sporotrichum* (*Sporothrix*) *schenckii* which is a dimorphic fungus, filamentous in the vegetative

state and yeast-like in mammalian tissues and under certain conditions of culture. Most commonly the disease presents as a lymphangitis and lymphadenitis with a tendency to cutaneous ulceration. This disease, though chronic, is not usually progressive. More rarely dissemination may occur, sometimes to lung, sometimes to other tissues.

Most of the usual tests have been employed by one worker or another (Norden 1951), but the skin test is the one regarded as being of diagnostic value, although it does not necessarily indicate actual disease or the severity of the disease. Generally speaking, the serological tests are only positive in disseminated cases and in these rare cases complement fixation and agglutination tests may be of the greatest value (Post *et al* 1958; Scott, Peasley & Crymes 1961). In one patient with pulmonary sporotrichosis positive complement fixation tests were obtained with histoplasmin and blastomycin but with much lower titres than to the homologous antigen (Post *et al* 1958).

In the much commoner lymphatic variety the skin test is the most useful and this has been extensively reported on from Brazil (Pereira *et al* 1962; Silva *et al* 1963; Wernsdorfer *et al* 1963). These papers indicate that a positive sporotrichin skin test is almost invariably a sign of infection with *S. schenckii*, either past or present, possibly unrecognized since positive reactions were obtained in 6–24% of subjects in endemic areas compared with 0% (0/55) in a non-endemic area. Similar reports have appeared from America suggesting that positives may be expected in 11–30% of healthy individuals (Schneidau, Lamar & Hairston 1964) the higher percentage being obtained in groups of nursery gardeners more likely to be exposed to the organism. Skin tests with polysaccharides, rather than a concentrated culture filtrate extract, may be more specific since there was a low incidence of positive reactions (Gonzales-Ochoa & Soto-Figueroa 1947). Nevertheless, the test is useful in the differential diagnosis of cutaneous leichmaniasis.

In an attempt to find a reliable diagnostic serological test a variety of antigen preparations was compared in precipitin complement fixation and agglutination tests with sera from eleven patients with sporotrichosis (Norden 1951). The most specific antigens were prepared from yeast-phase cells either by autoclaving or by grinding acetone treated cells, and of the tests the precipitin test appeared most useful.

Direct fluorescent antibody methods have proved to be of value in detecting *S. schenckii* cells in lesion exudates of patients suspected of sporotrichosis, e.g. in twenty-four out of twenty-seven patients (89%) for whom positive cultures were obtained, and in one patient culturally negative but skin test positive (Kaplan & Gonzales-Ochoa 1963). Furthermore, homologous *S. schenckii* fluorescein labelled antisera stained both yeast and mycelial phase cultures of eight different strains and showed no cross-reactivity with heterologous species (Kunz 1959; Kaplan & Ivens 1960) and even the detritus of disintegrated or

phagocytized elements of the fungus gave specific staining. This technique is therefore recommended as a rapid and useful procedure.

The homogenicity of the *S. schenckii* species has also been studied by agglutination (Lurie 1948) and agar gel methods (Kaden 1957) confirming the antigenic similarity between pigmented and unpigmented strains. However, serological cross-reactions have been demonstrated between the specific capsular polysaccharide of *S. schenckii* and similar antigens from a number of strains of pneumococci (Neill, Castillo & Pinkes 1955).

Chromomycosis and Cladosporiosis

A range of fungi relegated to genera such as *Phialophora*, *Fonsecaea* and *Cladosporium* have been identified as causes of chromomycosis. The nomenclature of these organisms is at present in a state of confusion; they all belong to the Dematiaceae and are differentiated by variations in spore formation, but whatever the true nature of the causal organism the tissue appearances are identical. The classical histological appearance is of a small cluster of ovoid, brown bodies in a zone of tissue reaction. The clinical picture is usually fairly striking, but confusion can arise with cutaneous leishmaniasis and sporotrichosis. The diagnosis is normally confirmed by culturing the organism and microscopically demonstrating it in tissue.

The earliest reports on the serological investigations of chromomycosis were those of failures. Montpellier & Catanei (1927) were unsuccessful in attempts to agglutinate the spores of *Phialophora* (*Hormodendron*) *pedrosoi* with the sera from their patients, and complement fixation reactions using suspensions of ground mycelium (Meriin 1930, 1932) were regarded as non-specific owing to a variety of cross-reactions. Precipitin tests (Costello, De Feo & Littman 1959) with saline extracts as antigens have also failed. However, successful specific complement fixation tests were demonstrated (Baliña *et al* 1932; Martin, Baker & Conant 1936) and the titres were found to diminish with clinical improvement.

More recently, in a group of thirteen patients known to be infected with *Phialophora pedrosoi*, twelve were found to have precipitins to extracts prepared by ultrasonic destruction of the cells; eight of the twelve also reacted with antigens of *P. compacta* and two with those of *P. verrucosa* (Buckley & Murray 1966). The cross-reactions are in keeping with the presumed relationships between these three fungi. The sera of the same thirteen patients contained no detectable antibodies to a wide range of other fungi including eight *Cladosporium* species and two other *Phialophora* species.

Attempts to classify the organisms that cause chromomycosis and related fungi have been made with precipitation and agglutination tests (Seeliger, Lacaz & Ulson 1959) and with fluorescent antibody tests (Al-Doory & Gordon 1963; Gordon & Al-Doory 1965), but there is as yet no general agreement on nomen-

clature, though the work to date has shown that the serological tests are useful tools.

Cerebral cladosporiosis is a rather rare disease usually attributed to *Cladosporium trichoides*, a fungus unquestionably related to, but distinct from, some of those that cause chromomycosis. Nothing is known of the patients' serological responses.

Mycetoma

A mycetoma is a granulomatous lesion of fungal or aerobic actinomycete origin, on the skin overlying which may be scattered healed and discharging sinuses. It is customary to subdivide mycetomata into two groups based on the nature of the causal organism:

(*a*) Maduromycetoma caused by several true fungi, e.g. species of *Madurella* and *Allescheria*.

(*b*) Actinomycetoma caused by several aerobic actinomycetes, e.g. *Nocardia* and *Streptomyces* species.

Thus, in this section, a disease caused by a variety of fungi and actinomycetes will be discussed, rather than diseases produced by specific fungi, as in the other sections.

A fully developed mycetoma can be diagnosed by its appearances, but early closed lesions present difficulties. Since for most of them the only effective treatment is surgery, the sooner the diagnosis is established the better the result and the less the chance of recurrence. This can be achieved early by cultural and histopathological means but such procedures require a biopsy. The serology of mycetoma is in an experimental stage, but a number of promising results have been reported.

Specific agglutinins, precipitins and complement-fixing antibodies, together with skin sensitivity to *Allescheria boydii* (*Monosporium apiospermum*) have been reported in a single patient (Seeliger 1956). No overlapping was found with other causes of maduromycosis although in complement fixation tests, cross-reactions occurred with antigens from the genus *Trichophyton*. More recently specific precipitins have been demonstrated in a similar case (Baxter, Murray & Taylor 1966). Using agar gel diffusion methods specific precipitating antibodies were demonstrated in fourteen sera from patients with actinomycetoma caused by *Streptomyces somaliensis*, *S. madurae* and *S. pelletierii* and nine with maduromycetoma due to *Madurella mycetomi*. Although there was a tendency for the sera of the former to react with one or more of the *Streptomyces* antigens, there was no cross-reactivity between the two groups (Mahgoub 1964) and as yet it is not known at what stage in the disease the precipitins develop. Skin tests with appropriate antigens proved of some value in patients with actinomycetoma but those with maduromycetoma failed to react (Murray & Moghraby 1964).

It has been claimed (Zamora, Bojalil & Bastarrachea 1963) that two types of

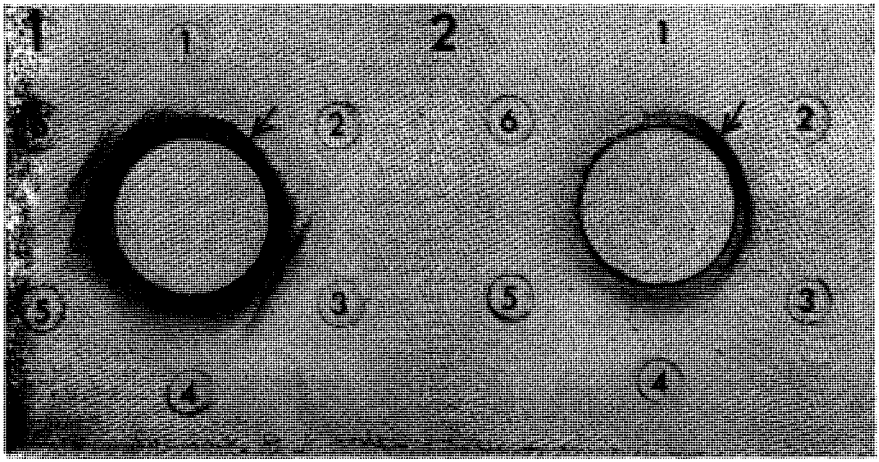


PLATE 3.1. Double diffusion tests with serum from patient with allergic aspergillosis (No. 1) and serum from patient with lung carcinoma (No. 2).

Antigens: (1) *T. mentagrophytes* galactomannan peptide (2) *E. floccosum*, (3) *A. fumigatus*, (4) *A. fumigatus* glycopeptide, (5) *T. rubrum*, (6) *A. fumigatus*.

Note: Serum 1, specific precipitation reactions to *A. fumigatus* and, curving round the central serum well, a C-substance/C-reactive protein reaction (marked by arrow).

Serum 2, the C-substance/C-reactive protein reaction only. (The arrows point to the C-protein line.)

Taken from LONGBOTTOM, JOAN L. & PEPYS J. (1964). *J. Path. Bact.* **88**, 141.

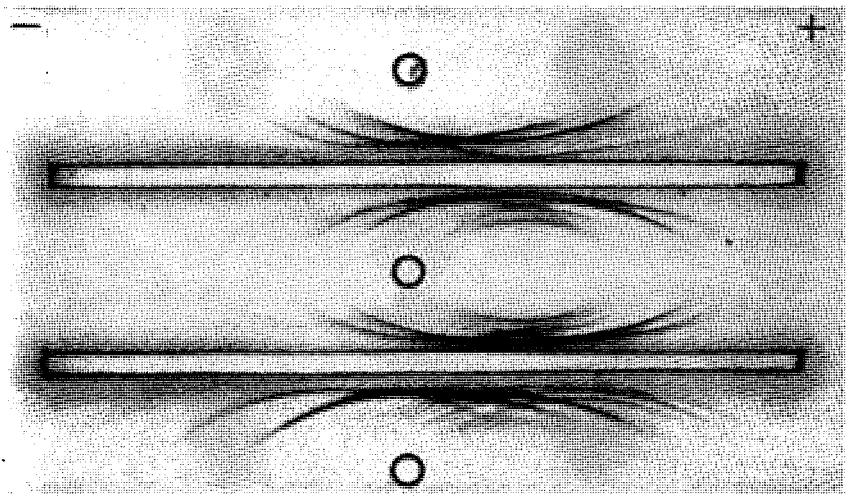


PLATE 3.2. Immunoelectrophoresis test of serum from patient with aspergilloma against extracts of 3 strains of *A. fumigatis*.

facing p. 96

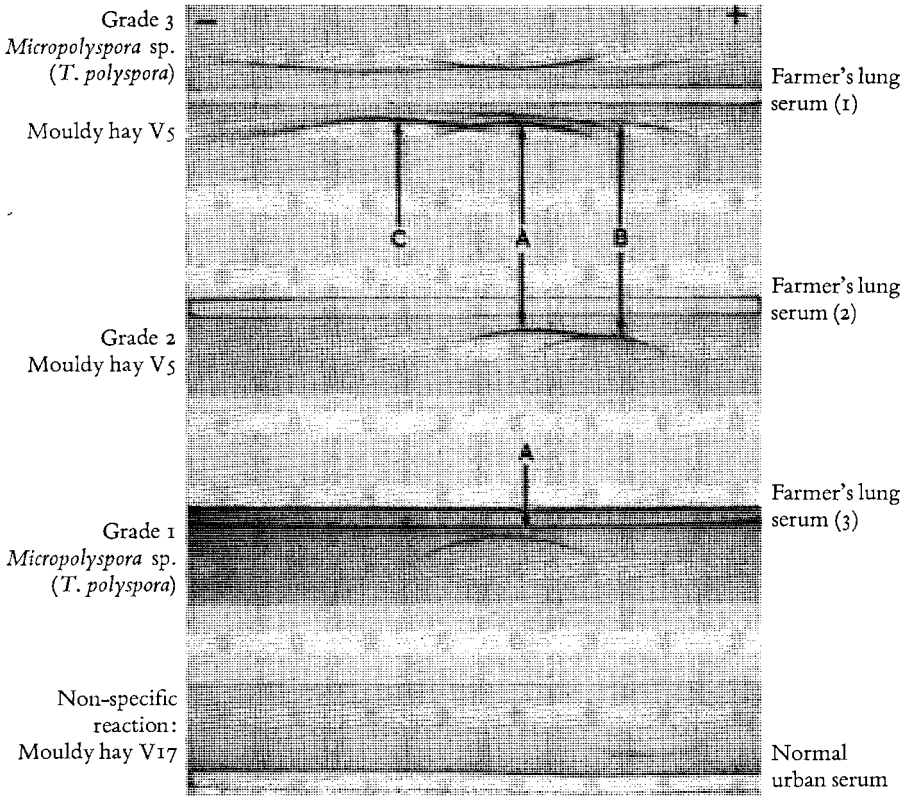


PLATE 3.3. Reactions to FLH antigens on immuno-electrophoresis
Taken from PEPYS J. & JENKINS P.A. (1965) *Thorax* **20**, 21.

facing p. 97

immunologically active polysaccharides have been isolated from the cell-extracts of both *Nocardia brasiliensis* and *N. asteroides*, although Mahgoub (1965) failed to confirm this. One of the polysaccharides from each of these two species contained arabinose, galactose and mannose, in different molar ratios and these polysaccharides were found to be species-specific, whereas the other polysaccharides were identical, containing similar molar ratios of arabinose and galactose, and because of their cross-reactions with heterologous sera these were regarded as group-specific. In agar gel tests the purified species-specific polysaccharide from *N. brasiliensis* gave specific precipitin reactions with the sera of patients with nocardial mycetoma whereas the group-specific polysaccharide was shown to react as well, with sera from patients with acute tuberculosis and leprosy (Bojalil & Zamora 1963; Estrada-Parra, Zamora & Bojalil 1965). In skin tests on patients with mycetoma caused by *N. brasiliensis*, specific positive (Type IV) reactions were obtained using purified protein antigens (Bojalil & Zamora 1963) although earlier workers claimed similarly specific reactions with an antigenic carbohydrate fraction (Gonzalez-Ochoa & Baranda 1953).

Actinomycosis is caused by anaerobic or micro-aerophiic spores of *Actinomyces* and is generally excluded from the classical mycetoma because the organisms are anaerobic and because a wider range of tissues may be affected. *Actinomyces bovis* and *israelii* have been found to be closer to the anaerobic corynebacteria than to *Nocardia* spp. (Slack, Winger & Moore 1961) and to differ from *Nocardia* in cell wall constitution (Cummins 1962). Diagnosis of actinomycosis is by demonstrating the organism; serological tests having little or no value, though they are of use for taxonomic reasons.

Dermatomycoses

Fungi of the dermatophyte group, which includes the genera *Trichophyton*, *Epidermophyton* and *Microsporum*, invade only the superficial skin, hair or nails and not the deeper tissues or internal organs, tending therefore to thrive in keratinized areas and not in active cell layers. Perhaps because of this superficial nature of the disease, circulating antibodies to appropriate fungal antigens have not been demonstrated in the sera of patients with dermatomycoses.

Skin tests, however, may prove helpful, the trichophytin test being regarded as similar to the tuberculin test although there is less evidence of a correlation between the test and present or past infection than in the case of the tuberculin test. Immediate reactions may also occur in infected patients, although this type of reaction has frequently been observed in atopic patients as well (Jillson & Huppert 1949; Wood & Cruickshank 1962) and therefore cannot be regarded as an index of infection. Intense oedematous reactions (probably Type III) have also been noted in some patients (Jillson & Huppert 1949). In a survey of skin reactivity to trichophytin, 40% of patients with infection gave immediate Type I, and 71% gave delayed Type IV, reactions as compared with 19% and 38%

respectively in a control group (Wood & Cruickshank 1962). Strongly positive delayed reactions are generally associated with the presence of deep inflammatory lesions in the patients (Neves 1962).

Methods for preparation of trichophytin for skin testing have varied with regard to the organism used, the culture medium, the mode of extraction and the standardization, but it usually consists of a crude water soluble extract of mycelium. Active fractions isolated have proved to be nitrogen containing polysaccharides, e.g. from *T. purpureum* (Thompson 1942) and from *T. mentagrophytes* (Barker & Trotter 1960; Codner *et al* 1961; and Ito 1965); a galactomannan peptide being isolated when the latter fungus was grown in submerged culture and a glucomannan peptide when grown in surface culture (Barker *et al* 1962). The galactomannan peptide has been shown to share many of the immunological characteristics of a glycopeptide isolated from *A. fumigatus*, in particular the production of immediate skin reactions in patients sensitive to *A. fumigatus*; a possible explanation for the trichophytin sensitivity found in atopic patients many of whom are allergic to Aspergillus antigens (Longbottom & Pepys 1964). These glycopeptides, like other dermatophyte extracts, also combine with C-reactive protein (Longbottom & Pepys 1964; Longbottom 1964) (see also Plate 3.1, facing p. 96).

Immunofluorescence tests, by the sandwich method, have failed to demonstrate specific antibodies to *T. mentagrophytes* in the sera of infected patients since all sera reacted; these reactions being inhibited by absorption of the sera with related and unrelated organisms (Walzer & Einbinder 1962).

Antigenic studies by agar gel diffusion and immuno-electrophoresis have shown that much cross-antigenicity, as might be expected, exists between species of the dermatophyte genera (Biguet, Andrieu & Tran van Ky 1961).

Farmer's Lung

Farmer's lung is a pulmonary hypersensitivity disease caused by the inhalation of the dust of mouldy overheated hay or other vegetable produce, with symptoms and signs attributable to a reaction in the most peripheral part of the broncho-pulmonary tract (see Chapter 36). It was registered in the United Kingdom as an industrial disease in 1965 (H.M.S.O. Cmnd. 2403). In the acute stages miliary infiltrations are seen on X-ray of the lungs due to cellular, granulomatous infiltration of the alveoli, and diffuse fibrosis of the lungs may result (Campbell 1932; Fuller 1953; Staines & Forman 1961; Dickie & Rankin 1958; Rankin *et al* 1962; Emanuel *et al* 1964).

Earlier reports suggested that farmer's lung was a bronchomycosis and many fungi were suspected for experimental reasons or because they were cultured from the sputum of exposed subjects. These included species of *Penicillium*, *Aspergillus*, *Mucor* and *Candida* (Fawcitt 1936, 1938a and b; Williams & Mulhall 1956; Pickworth 1961; Tornell 1946; Bexall & Edfeld 1949; Zettergren 1950;

Mann & Miall 1952; Soucheray 1954). However, Studdert (1953) pointed out that the explosive onset, rapid improvement on avoidance of the dust and the radiological findings were unlike any true fungous disease. No evidence of invasion of lung tissue of affected subjects was found by Fuller (1953) and Bishop, Melnick & Raine (1963).

In 1962 Pepys *et al* demonstrated precipitins in the sera of patients with farmer's lung against extracts of mouldy hay, and showed that these antibodies were directed against both fungal and additional antigens. Absorption of the sera with fungal antigens and extracts of good hay showed that the additional antigens appeared in the hay that had become mouldy and which had been responsible for attacks of farmer's lung. These findings have been confirmed by other workers (Kobayashi *et al* 1963; Bishop, Melnick & Raine 1963) and by experiments on animals reared on mouldy litter (Parish 1961, 1963). These farmer's lung hay, or FLH, antigens were at first thought to be degradation products of hay resulting from fungal and bacterial growth, however, the most important source of the FLH antigens has since been found to be the thermophilic actinomycete, *Micropolyspora faeni* (*Thermopolyspora polyspora*) and to a lesser extent *Thermoactinomyces* (*Micromonospora*) *vulgaris*. These organisms grow well at the temperatures of 40–60°C resulting from the florid bacterial and fungal growth (Pepys *et al* 1963; Gregory *et al* 1964; Festenstein *et al* 1965; Pepys & Jenkins 1965; Rankin *et al* 1965; Davies & Yull 1966; LaBerge & Stahmann 1966a and b).

Wenzel *et al* (1964) have cultured *M. faeni* from lung biopsy specimens of affected subjects but, since these organisms have also been cultured from the sputum of exposed but unaffected subjects, this finding is not necessarily decisive, particularly in view of the vast numbers of spores of *Micropolyspora* present in the dust of mouldy hay (Lacey & Lacey 1964).

Double diffusion testing in agar gel has been the most commonly used serological test. Antigens have been prepared by extracting defatted mouldy hay and cultures of *M. faeni* in carbol saline (Pepys & Jenkins 1965). In 205 patients, positive reactions to mouldy hay extract were obtained in 91% and to *M. faeni* in 85%. Minor antigenic differences have been found in extracts prepared from cultures on nutrient media and on hay; extracts of the latter being more potent (Jenkins 1964). In previous studies limited precipitation reactions against extracts of mouldy hay were given by the sera of patients with sarcoidosis (32%) and by sera from asthmatic (12%) and apparently normal subjects (7%) (Pepys *et al* 1962). These reactions were not due to the FLH antigens of *M. faeni* since no precipitins were found against the latter in tests, on the above sera and on large numbers of subjects not exposed to mouldy hay. By contrast, of 122 farmers exposed to mouldy hay, and in whom a diagnosis of farmer's lung was not made, 17% had precipitins against *Micropolyspora antigens.*

Whilst the double diffusion test is slightly more sensitive than the immuno-

electrophoretic test, the latter is more discriminating, giving better evidence of the specific reactions to the FLH antigens (Pepys & Jenkins 1965). Characteristic precipitin arcs are given in three main regions, termed A, B and C, in tests of farmer's lung sera against extracts of *M. faeni* (see Plate 3.3, facing p. 97). Other arcs are frequently present, some in the C-region due to a *Mucor* sp. antigen, and further analysis of these reactions is needed.

Grading of the intensity of the precipitin reactions in terms of these arcs has shown that the higher the grade the more frequent and severe the attacks. FLH precipitin reactions have also been reported by Jenkins & Pepys (1965) in 71% of cattle suffering from a pulmonary disease of the fog-fever group, due to feeding on mouldy hay.

More recently a latex particle agglutination test has been developed which gives results comparable to those obtained by double-diffusion tests, the two tests together however giving positive results in about 95% of cases due to mouldy hay (Murray & Pepys 1967).

Treatment of extracts of cultures of *M. faeni* by the addition of trichloroacetic acid leads to the precipitation of a predominantly protein fraction, corresponding with antigens A and B, and leaves, in the supernatant, a predominantly polysaccharide fraction, corresponding with C antigen. The trichloroacetic acid extract of mouldy hay prepared by Kobayashi *et al* (1963) consisted predominantly of the C antigen, and gave positive reactions only with the sera of affected subjects and not of controls. Reactions to the C antigen were obtained by Pepys & Jenkins (1965) in only about one-half of affected cases, consisting chiefly of the most strongly reacting sera, which in turn tend to be obtained from the most severely affected patients. This would give an impression of apparently greater specificity of the trichloroacetic acid extract as reported by Kobayashi *et al* (1963) and LaBerge & Stahmann (1966a).

Further investigations by LaBerge & Stahmann (1966b) of antigens from cultures of *M. faeni* (*T. polyspora*) treated by trichloroacetic acid has shown the presence of antigens labelled A, B and C as well as others. These antigenic glycopeptide fractions were found to be composed primarily of polysaccharides containing galactose, arabinose and glucosamine. The 'A' and 'B' components of the trichloroacetic acid soluble C-antigen of LaBerge & Stahmann (1966) and Kobayashi *et al* (1963) do not correspond to the predominantly protein and faster-moving A and B antigens initially described by Pepys & Jenkins (1965), which in immuno-electrophoresis tests give more crescentic and well defined arcs which lie nearer to the anode.

In patients with farmer's lung due to other mouldy vegetable dusts rather than hay, FLH precipitin reactions were obtained in one-half (Pepys & Jenkins 1965).

Consideration must be given to the possibility that other thermophilic actinomycetes, other micro-organismal flora, and indeed any of the sources of organic antigen in mouldy hay, may be the sources of antigen in those cases

where the FLH antigens of *M. faeni* do not appear to be the main or sole cause.

In the disease called maple-bark pneumonitis, resembling farmer's lung and due to the inhalation of the spores of *Cryptostroma* (*Coniosporium*) *corticale*, precipitins have been reported and also positive immediate and more slowly developing skin test reactions were obtained (Emanuel, Wenzel & Lawton 1966).

Miscellaneous Fungal Infections

Systemic infections in man with the Zygomycete order of the Phycomycetes are relatively rare, and little is known of the immunological findings. Positive skin and complement fixation tests to extracts of autoclaved mycelium of *Rhizopus arrhizus* have been reported (Blank *et al* 1962). It should, however, be noted that precipitins against extracts of the *Mucor* sp. are common in farmers suffering from farmer's lung or exposed mouldy hay, in whom there is no evidence of infection (Pepys *et al* 1962). There is no serological information on subcutaneous phycomycosis which occurs quite commonly.

Infections with *Geotrichum candidum* are also rare, and here too in one patient suffering from late onset asthma, positive skin and agglutinin tests with rising titres were obtained and the fungus was cultured from the sputum (Ross, Reid & Speirs 1966).

REFERENCES

Abernathy R.S. & Heiner D.C. (1961) Precipitation reactions in agar in North American Blastomycosis. *J. lab. clin. Med.* **57,** 604.

Abrahams I. & Gilleran T.G. (1960) Studies on actively acquired immunity to experimental cryptococcosis in mice. *J. Immunol.* **85,** 629.

Akiba T., Iwata K. & Inouye S. (1957) Studies on the serological diagnosis of deep-seated candidiasis. *Jap. J. Microbiol.* **1,** 11.

Al-Doory Y. & Gordon M.A. (1963) Application of fluorescent antibody procedures to the study of pathogenic dematiaceous fungi. I. Differentiation of *Cladosporium carrionii* and *Cladosporium bantianum*. *J. Bact.* **86,** 332.

Anderson K. & Beech M. (1958) Serological tests for the early diagnosis of cryptococcal infection. *Med. J. Aust.* **2,** 601.

Austwick P.E.C. (1965) Pathogenicity. In *The Genus Aspergillus*, ch. VII. Ed. Raper K.B. & Fennell D.I. Baltimore: Williams & Wilkins.

Baliña P., Bosq P., Negroni P. & Quiroga M. (1932) Un caso de chromoblastomicosis, autoctono de Argentina. *Rev. argent. Dermato-sif.* **16,** 369.

Bank H., Shibolet S., Gilat T., Altmann G. & Heller H. (1962) Mucormycosis of head and neck structures. *Brit. med. J.* **i,** 766.

Barker S.A. & Trotter M.D. (1960) Isolation of purified trichophytin. *Nature* (*Lond.*) **188,** 232.

Barker S.A., Cruickshank C.N.D., Morris J.H. & Wood S.R. (1962) The isolation of trichophytin glycopeptide and its structure in relation to the immediate and delayed reactions. *Immunology* **5,** 627.

Baiows A. (1963) Comparison of *Blastomyces dermatitidis* vaccine and blastomycin as skin testing agents for the diagnosis of blastomycosis. Abstract VIIIth Int. Cong. Microbiol.

BAXTER M., MURRAY I.G. & TAYLOR J.J. (1966) A case of mycetoma with serological diagnosis of *Allescheria boydii*. *Sabouraudia* **5**, 138.

BENNETT J.E., HASENCLEVER H.F. & BAUM G.L. (1965) Evaluation of a skin test for cryptococcosis. *Amer. Rev. resp. Dis.* **91**, 616.

BENNETT D.E. (1966) The histoplasmin latex agglutination test: Clinical evaluation and a review of the literature. *Amer. J. med. Sci.* **251**, 175.

BEXALL E. & EDFELT O. (1949) Monilia infection during threshing work. *Svenska Läkartidn.* **46**, 2433.

BIGUET J., ANDRIEU S. & TRAN VAN KY P. (1961) Application des methodes électrophorétiques et immuno-chimiques à l'étude des fractions antigéniques des Dermatophytes. Premiers résultats concernant *Ctenomyces mentagrophytes*. *C. R. Acad. Sci. (Paris)*, **253**, 2167.

BIGUET J., TRAN VAN KY P., ANDRIEU S. & DEGAEY R. (1965) Étude immunoélectrophorétique de la nature et de l'ordre d'apparition des anticorps précipitants du sérum de lapins en fonction de leur mode d'immunisation contre *Candida albicans*. *Sabouraudia* **4**, 148.

BIGUET J. & TRAN VAN KY P. (1966) Radioimmunoélectrophorèse et localisation des immunoglobulines sériques au cours des affections parasitaires essai préliminaire. A propos de plusiers cas d'aspergillomes bronchopulmonaires. *Revue Immunol.* **30**, 165.

BISHOP J.M., MELNICK S.C. & RAINE J.L. (1963) Farmer's lung: studies of pulmonary function and aetiology. *Quart. J. Med.* **32**, 257.

BOJALIL L.F. & ZAMORA A. (1963) Precipitin and skin tests in the diagnosis of mycetoma due to *Nocardia*. *Proc. Soc. exp. Biol. (N.Y.)*, **113**, 40.

BLOOMFIELD N., GORDON M.A. & ELMENDORF D.F. (1963) Detection of *Cryptococcus neoformans* antigen in body fluids by latex particle agglutination. *Proc. Soc. exp. Biol. (N.Y.)* **114**, 64.

BRODY J.I. & FINCH S.L. (1960) Candida-reacting antibody in the serum of patients with lymphomas and related disorders. *Blood* **15**, 830.

BUCKLEY H.R. & MURRAY I.G. (1966) Precipitating antibodies in chromomycosis. *Sabouraudia* **5**, 78.

BUSEY J.F. (1964) Blastomycosis cooperative study of the Veterans Administration. Blastomycosis I. A review of 198 collected cases in Veterans Administration Hospitals. *Amer. Rev. resp. Dis.* **89**, 659.

CAMPBELL C.C. & BINKLEY G.E. (1953) Serologic diagnosis with respect to histoplasmosis, coccidioidomycosis and blastomycosis and the problem of cross reactions. *J. Lab. clin. Med.* **42**, 896.

CAMPBELL C.C. (1960) The accuracy of serologic methods in diagnosis. *Ann. N.Y. Acad. Sci.* **89**, 163.

CAMPBELL C.C. & HILL G.B. (1964) Further studies on the development of complement fixing antibodies and precipitins in healthy histoplasmin sensitive persons following a single histoplasmin skin test. *Amer. Rev. resp. Dis.* **90**, 927.

CAMPBELL C.C. (1965) Problems associated with antigenic analysis of *Histoplasma capsulatum* and other mycotic agents. *Amer. Rev. resp. Dis.* **92**, 113.

CAMPBELL J.M. (1932) Acute symptoms following work with hay. *Brit. med. J.* **ii**, 1143.

CAMPBELL M.J. & CLAYTON Y.M. (1964) Bronchopulmonary aspergillosis. *Amer. Rev. resp. Dis.* **89**, 186.

CARSKI T.R., COZAD G.C. & LARSE H.W. (1962) Detection of *Histoplasma capsulatum* in sputum by means of fluorescent antibody staining. *Amer. J. clin. Path.* **37**, 465.

CHEW W.H. & THEUS T.L. (1967). Candida precipitins. *J. Immunol.* **98**, 220.

CODNER R.C., CRUICKSHANK C.N.D., TROTTER M.D. & WOOD S.R. (1961) The production

of Trichophytin antigen in submerged culture of *Trichophyton mentagrophytes*. *Sabouraudia* **1**, 116.

COMAISH J.S., GIBSON B. & GREEN C.A. (1963) Candidiasis—serology and diagnosis. *J. invest. Derm.* **40**, 139.

CONVERSE J.L., CASTLEBERRY M.W., BESEMER A.R. & SNYDER E.M. (1962) Immunization of mice against *Coccidioides immitis*. *J. Bact.* **84**, 46.

CONVERSE J.L. (1965) The effect of nonviable and viable vaccines in experimental coccidioidomycosis. *Amer. Rev. resp. Dis.* **92**, 159.

COSTELLO M.J., DE FEO C.P. (Jr.) & LITTMAN M.L. (1959) Chromoblastomycosis treated with local infiltration of Amphotericin-B solution. *Archs Derm.* **79**, 184.

CUMMINS C.S. (1962) Chemical composition and antigenic structure of cell walls of *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Actinomyces* and *Arthrobacter*. *J. gen. Microbiol.* **28**, 35.

DAVIES D.G. & YULL A.J. (1966) A comparison of the precipitin test in asymptomatic and symptomatic cases of farmer's lung. *Mon. Bull. Minist. Hlth* **25**, 75.

DE BRITO T., RAPHAEL A., FAVA NETTO C. & SAMPAIO S. DE A.P. (1961) Histopathology of the skin test using a polysaccharide antigen of the *Paracoccidioides brasiliensis*. *J. invest. Derm.* **37**, 29.

DICKIE H.A. & RANKIN J. (1958) Farmer's Lung: an acute granulomatous interstitial pneumonitis occurring in agricultural workers. *J. Amer. med. Ass.* **167**, 1069.

DROUHET E. & SEGRETAIN G. (1950) Biologie et pouvoir pathogène de *Torulopsis neoformans* (*Torula histolytica*). *Rev. Path. comp.* **50**, 37.

DROUHET E., SEGRETAIN G., PESLE G. & BIDET L. (1963) Étude des précipitines sériques en milieu gélosé pour le diagnostic des aspergilloses bronchopulmonaires. *Ann. Inst. Pasteur* **105**, 597.

EINBINDER J.M., BENHAM R.W. & NELSON C.T. (1954) Chemical analysis of the capsular substances of *Cryptococcus neoformans*. *J. invest. Derm.* **22**, 279.

ELINOV N.P. & ZAIKINA N.A. (1959) The precipitation test in the serological diagnosis of moniliasis. *Zh. Mikrobiol., Moscou* **30**, 42.

EMANUEL D.A., WENZEL F.J., BOWERMAN C.I. & LAWTON B.R. (1964) Farmer's Lung. Clinical, pathologic, and immunologic study of twenty-four patients. *Amer. J. Med.* **37**, 392.

EMANUEL D.A., WENZEL F.J. & LAWTON B.R. (1966) Pneumonitis due to *Cryptostroma corticale* (Maple-bark disease). *New Engl. J. Med.* **274**, 1413.

ESTRADA-PARRA S., ZAMORA A. & BOJALIL L.F. (1965) Immuno-chemistry of the group-specific polysaccharide of *Nocardia brasiliensis*. *J. Bact.* **90**, 571.

EVANS E.E. (1949) An immunologic comparison of twelve strains of *Cryptococcus neoformans* (*Torula histolytica*). *Proc. Soc. exp. Biol. (N.Y.)* **71**, 644.

EVANS E.E. (1950) The antigenic composition of *Cryptococcus neoformans*. I. A serologic classification by means of the capsular and agglutination reactions. *J. Immunol.* **64**, 423.

EVANS E.E. & KESSEL J.F. (1951) The antigenic composition of *Cryptococcus neoformans*. II. Serologic studies with the capsular polysaccharide. *J. Immunol.* **67**, 109.

EVANS E.E., SORENSEN L.J. & WALLS K.W. (1953) The antigenic composition of *Cryptococcus neoformans*. V. A survey of cross-reactions among strains of *Cryptococcus* and other antigens. *J. Bact.* **66**, 287.

EVANS E.E. & THERIAULT R.J. (1953) The antigenic composition of *Cryptococcus neoformans*. IV. The use of paper chromatography for following purification of the capsular polysaccharide. *J. Bact.* **65**, 571.

FAVA NETTO C. (1955) Estudos quantitativos sôbre a fixaçâo do complemento na blastomicose sul-americana com antígeno polisaciridíco. *Arnos Cirurg. clin. exp.* **18**, 197.

FAVA NETTO C., FERRI R.G. & LACAZ C. DA S. (1959). Proteinograma e algumas 'provas de fase aguda do sôro' na blastomicose sul-americana. Estudo comparativo com as reacçoes de fixação do complemento e de precipitação. *Med. Cirurg. Pharm.* **277,** 159.

FAVA NETTO C. & RAPHAEL A. (1961) A reação intradérmica com polisacaride do *Paracoccidioides brasiliensis* na blastomicose sul-americana. *Rev. Inst. Med. trop. S. Paulo.* **3,** 161.

FAVA NETTO C. (1961) Contribuição para o estudo immunológico de blastomicose de Lutz (blastomicose sul-americana). *Rev. Inst. Lutz* **21,** 99.

FAVA NETTO C. (1965) The immunology of South American blastomycosis. *Mycopathologia* **26,** 349.

FAWCITT R. (1936) Fungoid conditions of the lung I and II. *Brit. J. Radiol.* **9,** 172 and 354.

FAWCITT R. (1938a) Occupational diseases of the lungs in agricultural workers. *Brit. J. Radiol.* **11,** 378.

FAWCITT R. (1938b) The roentgenological recognition of certain broncho-mycoses involving occupational risks. *Amer. J. Roentgenol.* **39,** 19.

FEINBERG J.G. & TEMPLE A. (1963) An investigation on precipitins to moulds in asthmatic sera. *Int. Arch. Allergy, N.Y.* **22,** 274.

FESTENSTEIN G.N., LACEY J., SKINNER F.A., JENKINS P.A. & PEPYS J. (1965) Self-heating of hay and grain in Dewar flasks and the development of farmer's lung antigens. *J. gen. Microbiol.* **41,** 389.

FULLER C.J. (1953) Farmer's lung: a review of present knowledge. *Thorax* **8,** 59.

GADEBUSCH H. (1961) Natural host resistance to infection with *Cryptococcus neoformans. J. infect. Dis.* **109,** 147.

GERNEZ-RIEUX CH., BIGUET J., VOISIN, C., CAPRON A., BALGARIES E. & TRAN VAN KY P. (1963) Diagnostic sérologique des aspergillomes bronchopulmonaires par immunoélectrophorèse. *J. franç. Méd. Chir. thor.* **17,** 663.

GOLDIN M. (1957) Studies on haemagglutination by antisera to *Candida albicans* and related yeast-like fungi. *Bact. Proc.* p. 106.

GONZALEZ-OCHOA A. & SOTO FIGUEROA E. (1947) Polisacaridos del *Sporotrichum schenckii*; datos immunológicos; intradermo-reacción en el diagnóstico de la esporotrichosis. *Rev. Inst. Salubr. Enferm. trop, Méx.* **8,** 143.

GONZALEZ-OCHOA A. & BARANDA F. (1953) Una prueba cutánea para el diagnóstico del micetoma actinomicósico por *Nocardia brasiliensis. Rev. Inst. Salubr. Enferm. trop. Méx.* **13,** 189.

GOOS R.D. & SUMMERS D.F. (1964) Use of fluorescent antibody techniques in observations on the morphogenesis of fungi. *Mycologia* **56,** 701.

GORDON M.A. (1958a) Differentiation of yeasts by means of fluorescent antibody. *Proc. Soc. exp. Biol. (N.Y.),* **97,** 694.

GORDON M.A. (1958b) Rapid serological differentiation of *Candida albicans* from *Candida stellatoidea. J. invest. Derm.* **31,** 123.

GORDON M.A. & AL-DOORY Y. (1965) Application of fluorescent antibody procedures to the study of pathogenic dematiaceous fungi. II. Serological relationships of the genus *Fonsecaea. J. Bact.* **89,** 551.

GORDON M.A. & VEDDER D.K. (1966) Serologic tests in diagnosis and prognosis of cryptococcosis. *J. Amer. med. Ass.* **197,** 961.

GREGORY P.H., FESTENSTEIN G.N., LACEY M.E., SKINNER F.A., PEPYS J. & JENKINS P.A. (1964) Farmer's lung disease: the development of antigens in moulding hay. *J. gen. Microbiol.* **36,** 429.

GREEN C.H., DE LALLA L.S. & TOMPKINS V.N. 1960) Separation of specific antigens of

Histoplasma capsulatum by ion exchange chromatography. *Proc. Soc. exp. Biol. (N.Y.)* **105,** 140.

HASENCLEVER H.F. (1961) Antigenic studies of *Candida*. III. Comparative pathogenicity of *Candida albicans* group A, group B and *Candida stellatoidea*. *J. Bact.* **82,** 578.

HASENCLEVER H.F. & MITCHELL W.O. (1963a) Antigenic studies of *Candida*. IV. The relationship of the antigenic groups of *Candida albicans* to their isolation from various clinical specimens. *Sabouraudia* **2,** 201.

HASENCLEVER H.F. & MITCHELL W.O. (1963b) Acquired immunity to candidiasis in mice. *J. Bact.* **86,** 401.

HASENCLEVER H.F. & MITCHELL W.O. (1964a) A study of yeast surface antigens by agglutination inhibition. *Sabouraudia* **3,** 288.

HASENCLEVER H.F. & MITCHELL W.O. (1964b) Immunochemical studies on polysaccharides of yeasts. *J. Immunol.* **93,** 763.

HASENCLEVER H.F. (1965) The antigens of *Candida albicans*. *Amer. Rev. resp. Dis.* **92,** 150.

HAYWARD B.J., AUGUSTIN R. & LONGBOTTOM J.L. (1960) Separation and characterization of antibodies to moulds. *Acta Allerg.*, Kbh. **15,** (Suppl. VII), 87.

HEINER D.C. (1958) Diagnosis of histoplasmosis using precipitin reactions in agar gel. *Pediatrics* **22,** 616.

HELLUY J.R., LOCHARD J., BRIQUEL P., PERCEBOIS G. & GUÉDON (1963) A propos d'un cas d'aspergilloma bronchique à *Aspergillus nidulans*. *Ann. méd. Nancy* **2,** 1422.

HILL G.A. & MARCUS S. (1959) Resistance induced against *Histoplasma capsulatum*: Quantitative aspects. *J. infect. Dis.* **105,** 26.

HILL G.B. & CAMPBELL C.C. (1956) A further evaluation of histoplasmin and yeast phase antigen of *Histoplasma capsulatum* in the complement fixation test. *J. Lab. clin. Med.* **48,** 255.

HILL G.B. & CAMPBELL C.C. (1962) Commercially available histoplasmin sensitized latex particles in an agglutination test for histoplasmosis. *Mycopathologia* **18,** 169.

H.M.S.O. (1964) Cmnd. 2403 Ministry of Pensions and National Insurance. 'Farmer's Lung'. Report by the Industrial Injuries Advisory Council.

HOLTI G. (1966) Candida allergy. In *Symposium on Candida Infections*, p. 73. Ed. WINNER H.I. & HURLEY R. Edinburgh and London: E. & S. Livingstone.

HUPPERT M. & BAILEY J.W. (1963) Immunodiffusion as a screening test for coccidioidomycosis serology. *Sabouraudia* **2,** 284.

HUPPERT M. & BAILEY J.W. (1965a) The use of immunodiffusion tests in coccidioidomycosis. I. The accuracy and reproducibility of the immunodiffusion test which correlates with complement fixation. *Amer. J. Clin. Path.* **44,** 364.

HUPPERT M. & BAILEY J.W. (1965b) The use of immunodiffusion tests in coccidioidomycosis. II. An immunodiffusion test as a substitute for the tube precipitin test. *Amer. J. clin. Path.* **44,** 369.

ISENBERG H.D., ALLERHAND J., BERKMAN J.I. & GOLDBERG D. (1963) Immunological and toxic differences between mouse-virulent and mouse-avirulent *Candida albicans*. *J. Bact.* **86,** 1010.

ITO Y. (1965) On the immunologically active substances of the dermatophytes. I and II. *J. invest. Derm.* **45,** 275.

JENKINS P.A. (1964) Immunological studies in Farmer's Lung. Thesis for the Ph.D. examination. University of London.

JENKINS P.A. & PEPYS J. (1965) Fog fever. Precipitin (F.L.H.) reactions to mouldy hay. *Vet. Rec.* **77,** 464.

JILSON O. F. & HUPPERT M. (1949) The immediate weal and the 24–48 hour tuberculin type oedematous reactions to trichophytin. *J. Invest. Derm.* **12,** 179.

KADEN R.H. (1957) Precipitin studies for the identity of antigens in different species of *Sporotrichum*. *Mycopathologia* **8,** 260.

KAPLAN W. & IVENS M.S. (1960) Fluorescent antibody staining of *Sporotrichum schenckii* in cultures and clinical materials. *J. Invest. Derm.* **35,** 151.

KAPLAN W. & GONZALEZ-OCHOA A. (1963) Application of the fluorescent antibody technique to the rapid diagnosis of sporotrichosis. *J. Lab. clin. Med.* **62,** 835.

KAPLAN W. & KAUFMAN L. (1963) Specific fluorescent antiglobulins for the detection and identification of *Blastomyces dermatitidis* yeast phase cells. *Mycopathologia* **19,** 173.

KAPLAN W. & CLIFFORD M.K. (1964) Production of fluorescent antibody reagents specific for the tissue form of *Coccidioides immitis*. *Amer. Rev. resp. Dis.* **89,** 651.

KAPLAN W., HUPPERT M., KRAFT D.E. & BAILEY J. (1966) Fluorescent antibody inhibition test for *Coccidioides immitis* antibodies. *Sabouraudia* **5,** 1.

KASE A. & MARSHALL J.D. (1960) A study of *Cryptococcus neoformans* by means of the fluorescent antibody technic. *Amer. J. clin. Path.* **34,** 52.

KAUFMAN L. & KAPLAN W. (1961) Preparation of a fluorescent antibody specific for the yeast phase of *Histoplasma capsulatum*. *J. Bact.* **82,** 729.

KAUFMAN L., SCHUBERT J.H. & KAPLAN W. (1962) Fluorescent antibody inhibition test for histoplasmosis. *J. Lab. clin. Med.* **59,** 1033.

KAUFMAN L. & KAPLAN W. (1963) Serological characterization of pathogenic fungi by means of fluorescent antibodies. I. Antigenic relationships between yeast and mycelial forms of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. *J. Bact.* **85,** 986.

KAUFMAN L. & BRANDT B. (1964) Fluorescent antibody studies of the mycelial form of *Histoplasma capsulatum* and morphologically similar fungi. *J. Bact.* **87,** 120.

KAUFMAN L. & BLUMER S. (1965) Development and evaluation of agglutination and fluorescent antibody procedures for the identification of *Cryptococcosis neoformans*. *Sabouraudia* **4,** 57.

KAUFMAN L., BRANDT B. & MCLAUGHLIN D. (1965) Evaluation of the fluorescent antibody and agar-gel precipitin test for detecting *Histoplasma* antibodies in anti-complementary sera. *Amer. J. Hyg.* **79,** 181.

KAUFMAN L. (1966) Serology of systemic fungus diseases. *Publ. Hlth. Rep.* **81,** 177.

KEMP G. & SOLOTOROVSKY M. (1964) Localization of antigens in mechanically disrupted cells of certain species of the genera *Candida* and *Torulopsis*. *J. Immunol.* **93,** 305.

KLITE P.D. (1965) The interpretation of agar gel precipitin reactions in histoplasmosis. *J. Lab. clin. Med.* **66,** 770.

KNIGHT R.A., HILL G. & MARCUS S. (1959) Immunization of mice with polysaccharides of *Histoplasma capsulatum*. *Proc. Soc. exp. Biol. (N.Y.)* **100,** 356.

KOBAYASHI M., STAHMANN M.A., RANKIN J. & DICKIE H.A. (1963) Antigens in mouldy hay as the cause of farmer's lung. *Proc. Soc. exp. Biol. (N.Y.)* **113,** 472.

KUNZ C. (1959) Fluorescenz-serologische Untersuchungen an einen pathogenen Pilzstamm (*Sporotrichum schenckii*). *Arch. klin. exp. Derm.* **209,** 200.

LABERGE D.E. & STAHMANN M.A. (1966a) Antigens from mouldy hay involved in farmer's lung. *Proc. Soc. exp. Biol. (N.Y.)* **121,** 458.

LABERGE D.E. & STAHMANN M.A. (1966b) Antigens from *Thermopolyspora polyspora* involved in farmer's lung. *Proc. Soc. exp. Biol. (N.Y.)* **121,** 463.

LACAZ C. DA S., PASSOS FILHO M.C.R., FAVA NETTO C. & MACARRON B. (1959) Contribuição para o estudo da blastomicose-infecção: inquérito com a paracoccidioidina. (Estudo serológico e clínico-radiológico dos paracoccidioidino positivos) *Rev. Inst. Med. trop. S. Paulo* **1,** 245.

LACEY J. & LACEY M.E. (1964) Spore concentrations in the air of farm buildings. *Trans. Brit. mycol. Soc.* **47,** 547.

LEHNER T. (1965) Immunofluorescent investigation of *Candida albicans* antibodies in human saliva. *Arch. oral Biol.* **10,** 975.

LEHNER T. (1966) Immunofluorescence study of *Candida albicans* in candidiasis, carriers and controls. *J. Path. Bact.* **91,** 97.

LEVINE H.B., MILLER R.L. & SMITH C.E. (1962) Influence of vaccination on respiratory coccidioidal disease in cynomolgous monkeys. *J. Immunol.* **89,** 242.

LEVINE H.B. & KONG Y.M. (1965) Immunity development in mice receiving killed *Coccidioides immitis* spherules: effect of removing residual vaccine. *Sabouraudia* **4,** 164.

LEVINE H.B., KONG Y.M. & SMITH C.E. (1965) Immunization of mice to *Coccidioides immitis*: dose, regimen and spherulation stage of killed spherule vaccines. *J. Immunol.* **94,** 132.

LONGBOTTOM J.L., AUGUSTIN R. & HAYWARD B.J. (1960) Antigenic relationships in moulds. *Acta Allerg., Kbh.* **15** (suppl. VII), 94.

LONGBOTTOM J.L. (1964) Immunological investigation of *Aspergillus fumigatus* in relation to disease in man. Thesis for Ph.D. examination, University of London.

LONGBOTTOM J.L. & PEPYS J. (1964) Pulmonary aspergillosis. Diagnostic and immunological significance of antigens and C-substance in *Aspergillus fumigatus*. *J. Path. Bact.* **88,** 141.

LONGBOTTOM J.L., PEPYS J. & TEMPLE-CLIVE F. (1964) Diagnostic precipitin test in *Aspergillus* pulmonary mycetoma. *Lancet* **i,** 588.

LURIE H.I. (1948) A common antigenic factor in different species of *Sporotrichum*. *Mycologia* **40,** 106.

LYNCH H.J. & PLEXICO K.L. (1962) A rapid method for screening sputums for *Histoplasma capsulatum* employing the fluorescent-antibody technic. *New. Engl. J. Med.* **226,** 811.

MCDEARMAN S.C. & YOUNG J.M. (1960) The development of positive serologic tests with *Histoplasma capsulatum* antigens following single histoplasmin skin tests. *Amer. J. clin. Path.* **34,** 434.

MAEKELT G.A. (1960) Estudio sobre el volor de antigenos de *Histoplasma capsulatum* y de *Paracoccidioides brasiliensis* para el serodiagnositico de estos micosa. *Arch. venez. Med. trop.* **3,** 149.

MAHGOUB E.S. (1964) The value of gel diffusions in the diagnosis of mycetoma. *Trans. R. Soc. trop. Med. Hyg.* **58,** 560.

MAHGOUB E.S. (1965) Mycoses of the Sudan. Thesis for Ph.D. examination, University of London.

MANN B. & MIALL W. (1952) A case of farmer's lung. *Tubercle, Lond.* **33,** 48.

MARSHALL J.D., IVERSON L., EVELAND W.C. & KASE A. (1961) Applications and limitation of the fluorescent antibody stain in the specific diagnosis of cryptococcosis. *Lab. Invest.* **10,** 719.

MARTIN D.S., BAKER R.D. & CONANT N.F. (1936) A case of verrucous dermatitis caused by *Hormodendrum pedrosoi* (Chromoblastomycosis) in North Carolina. *Amer. J. trop. Med.* **16,** 594.

MARTIN D.S. (1953) Serologic studies on North American blastomycosis. Studies with soluble antigens from untreated and sonic treated yeast phase cells of *Blastomyces dermatitidis*. *J. Immunol.* **71,** 192.

MAYS E.E., HAWKINS J.A. & KUHN L.R. (1964) The clinical usefulness of fungal serologic testing. *Dis. Chest,* **46,** 205.

MERIN J.A. (1930) Zür Mykologie der Chromoblastomykose. *Arch. Derm. Syph., Berl.* **162,** 300.

MERIN J.A. (1932) Weiters Beobachtungen über den Erreger der europaischen Chromoblastomykose. *Arch. Derm. Syph., Berl.* **166,** 722.

MONTPELLIER J. & CATANEI A. (1927) Mycose humaine due à un champignon du genre '*Hormodendron*: *H. algeriensis* nov. sp.' *Ann. Derm. Syph. (Paris)* **8,** 626.

MOSES A. (1916) Faxacio de complemento au blastomicose. *Mem. Inst. Osw. Cruz* **8,** (Part 2), 68.

MOURAD S. & FRIEDMAN L. (1961) Active immunization of mice against *Candida albicans*. *Proc. Soc. exp. Biol. (N.Y.)* **106,** 570.

MURRAY I.G. & MOGHRABY I.M.EL (1964) The value of skin tests in distinguishing between maduromycetoma and actinomycetoma. *Trans. R. Soc. trop. Med. Hyg.* **58,** 557.

MURRAY I.G. & BUCKLEY H.R. (1966) Serological study of *Candida* species. In *Symposium on Candida Infections*, p. 44, ed. WINNER H.I. & HURLEY R. Edinburgh and London: E. & S. Livingstone.

MURRAY I.G. & PEPYS J. (1967) Latex agglutination test in farmer's lung. *Mon. Bull. Min. Alth.* **26,** 96.

NEILL J.M., ABRAHAMS I. & KAPROS C.E. (1950) A comparison of the immunogenicity of weakly encapsulated and of strongly encapsulated strains of *Cryptococcus neoformans* (*Torula histolytica*). *J. Bact.* **59,** 263.

NEILL J.M., SUGG J.Y. & MCCAULEY D.W. (1951) Serologically reactive material in spinal fluid, blood and urine from a human case of cryptococcosis. *Proc. Soc. exp. Biol., N.Y.* **77,** 775.

NEILL J.M., CASTILLO C.G. & PINKES A.H. (1955) Serological relationships between fungi and bacteria. I. Cross-reactions of *Sporotrichum schenckii* with pneumococci. *J. Immunol.* **74,** 120.

NEVES H. (1962) Trichophytin reaction in natural and experimental dermatophytosis and in tuberculosis. *Sabouraudia* **1,** 197.

NICHOLAS W.M., WEIR J.A., KUHN L.R., CAMPBELL C.C., NOLTE L.B. & HILL G.B. (1961) Serologic effects of histoplasmin skin testing. *Amer. Rev. resp. Dis.* **83,** 276.

NORDEN A. (1951) Sporotrichosis. Clinical and laboratory features and a serologic study in experimental animals and humans. *Acta path. microbiol. scand.* Supplement 89.

PAPPAGIANSIS D., SMITH C.E., KOBAYASHI G.S. & SAITO M.T. (1961) Studies of antigen from young mycelia of *Coccidioides immitis*. *J. infect. Dis.* **108,** 35.

PAPPAGIANSIS D., LINDSEY N.J., SMITH C.E. & SAITO M.T. (1965) Antibodies in human coccidioidomycosis: immunoelectrophoretic properties. *Proc. Soc. exp. Biol. (N.Y.).* **118,** 118.

PARISH W.E. (1961) The response of normal and sensitized animals to products of mouldy hay. *Acta allerg., Kbh.* **16,** 78.

PARISH W.E. (1963) Farmer's lung. I. An immunological study of some antigenic components of mouldy foodstuffs. *Thorax* **18,** 83.

PECK S.M., BERGAMIN R., KELLEC L. & REIN C.R. (1955) The sero-diagnosis of moniliasis—its value and limitation. *J. invest Derm.* **25,** 301.

PEPYS J., FAUX J.A., LONGBOTTOM J.L., MCCARTHY D.S. & HARGREAVE F.E. (1967) Allergic reactions to *Candida albicans* in respiratory disease. To be published.

PEPYS J. & JENKINS P.A. (1965) Precipitin (F.L.H.) test in farmer's lung. *Thorax* **20,** 21.

PEPYS J., JENKINS P.A., FESTENSTEIN G.N., GREGORY P.H., LACEY M.E. & SKINNER F.A (1963) Farmer's Lung. Thermophilic actinomycetes as a source of 'farmer's lung hay' antigen. *Lancet* **ii,** 607.

PEPYS J. & LONGBOTTOM J.L. (1967) Immunological methods in mycology. In *Handbook of Experimental Immunology*, Ch. 25, ed. WEIR D.M. Oxford: Blackwell.

PEPYS J., RIDDELL R.W., CITRON K.M. & CLAYTON Y.M. (1962) Precipitins against extracts of hay and moulds in the serum of patients with farmer's lung, aspergillosis, asthma and sarcoidosis. *Thorax* **17,** 366.

PEPYS J., RIDDELL R.W. & CLAYTON Y.M. (1959) Human precipitins against common pathogenic and non-pathogenic fungi. *Nature (Lond.)* **184,** 1328.

PEPYS J., RIDDELL R.W., CITRON K.M., CLAYTON Y.M. & SHORT E.I. (1959) Clinical and

immunologic significance of *Aspergillus fumigatus* in the sputum. *Amer. Rev. resp. Dis.* **80,** 167.

PEREIRA A.M., GONÇALVES A.P., LACAZ C. DA S., FAVA NETTO C. & CASTRO R.M. (1962) Immunologia de esporotricose. I. La prova da esporotriquina após a cura da esporotricose. II. A prova da esporotriquina em crianças sem esporotricose. *Rev. Inst. Med. trop. S. Paulo,* **4,** 383 and 386.

PICKWORTH K.H. (1961) Farmer's lung. *Lancet* **ii,** 660.

PINE L., KAUFMAN L. & BOONE C.J. (1964) Comparative fluorescent antibody staining of *Histoplasma capsulatum* and *H. duboisii* with a specific anti-yeast phase of *H. capsulatum* conjugate. *Mycopathologia* **24,** 315.

POLLOCK A.Q. & WARD L.M. (1962) A haemagglutination test for cryptococcosis. *Amer. J. Med.* **32,** 6.

POST G.W., JACKSON A., GARBER P.E. & VEACH G.E. (1958) Pulmonary sporotrichosis. *Dis Chest.* **34,** 455.

RANKIN J., JAESCKE W.H., CALLIES Q.C. & DICKIE H.A. (1962) Farmer's lung. Physiopathologic features of the acute interstitial granulomatous pneumonitis of agricultural workers. *Ann. intern. Med.* **57,** 606.

RANKIN J., KOBAYASHI M., BARBEE R.A. & DICKIE H.A. (1965) Agricultural dusts and diffuse pulmonary fibrosis. *Arch. envir. Hlth.* **10,** 278.

REBERS P.A., BARKER S.A., HEIDELBERGER M., DISCHE Z. & EVANS E.E. (1958) Precipitation of the specific polysaccharide of *Cryptococcus neoformans* A by types II and XIV antipneumococcal sera. *J. Amer. chem. Soc.* **80,** 1135.

REDMOND A., CARRE I.J., BIGGART J.D. & MACKENZIE D.W.R. (1965) Aspergillosis (*A. nidulans*) involving bone. *J. Path. Bact.* **89,** 391.

RESTREPO M.A. (1966) La prueba de immunodiffusion en el diagnostico de la paracoccidioidomicosis. *Sabouraudia* **4,** 223.

RIMBAUD P., RIOUX J.A., DUNTZE F. & BASTIDE J.M. (1960) Sérologie et allergie cutanée au *Candida albicans.* Étude comparative. *Bull. Soc. franç. Derm. Syph.* **67,** 689.

ROSS, J.D., REID K.D.G. & SPEIRS C.F. (1966) Bronchopulmonary geotrichosis with severe asthma. *Brit. med. J.* **i,** 1400.

ROTH F.J. & GOLDSTEIN M.L. (1961) Inhibition of growth of pathogenic yeasts by human serum. *J. invest. Derm.* **36,** 383.

ROWE J.R., NEWCOMER V.D. & WRIGHT E.T. (1963) Studies of the soluble antigens of *Coccidioides immitis* by immunodiffusion. *J. invest. Derm.* **41,** 225.

ROWE J.R., NEWCOMER V.D. & LANDOU J.W. (1963) Effects of cultural conditions on the development of antigens by *Coccidioides immitis.* I. Immunodiffusion studies. *J. invest. Derm.* **41,** 343.

ROWE J.R., LANDAU J.W. & NEWCOMER V.D. (1965) Effects of cultural conditions on the development of antigens by *Coccidioides immitis.* II. Complement fixation and immunodiffusion studies with human serum. *J. Invest. Derm.* **44,** 237.

SALVIN S.B. & SMITH R.F. (1961) An antigen for the detection of hypersensitivity to *Cryptococcus neoformans. Proc. Soc. exp. Biol. (N.Y.)* **108,** 498.

SASLAW S. & CARLISLE H.N. (1958) Histoplasmin-latex agglutination test. II. Results with human sera. *Proc. Soc. exp. Biol. (N.Y.)* **97,** 700.

SAWAKI Y., HUPPERT M., BAILEY J.W. & YAGI Y. (1966) Patterns of human antibody reactions in coccidioidomycosis. *J. Bact.* **91,** 422.

SCLAFER J. & HEWITT J. (1960) Valeur des tests cutanes dans l'allergie a *Candida albicans. Path. Biol.* **8,** 323.

SCHNEIDAU J.D., LAMAR L.M. & HAIRSTON M.A. (1964) Cutaneous hypersensitivity to sporotrichin in Louisiana. *J. Amer. med. Ass.* **188,** 371.

SCHUBERT J.H. & AJELLO L. (1957) Variation in complement fixation antigenicity of different yeast phase strains of *Histoplasma capsulatum*. *J. Lab. clin. Med.* **50,** 304.

SCHUBERT J.H. & HAMPSON R.C. (1962) An appraisal of serologic tests for coccidioidomycosis. *Amer. J. Hyg.* **76,** 144.

SCHUBERT J.H. & WIGGINS G.L. (1963) The evaluation of serologic tests for histoplasmosis in relation to the clinical diagnosis. *Amer. J. Hyg.* **77,** 240.

SCOTT S.M., PEASLEY E.D. & CRYMES T.P. (1961) Pulmonary sporotrichosis. *New Eng. J. Med.* **265,** 453.

SEELIGER H.P.R. (1956) A serologic study of hyphomycetes causing mycetoma in man. *J. invest. Derm.* **26,** 81.

SEELIGER H.P.R., LACAZ C. DA S. & ULSON C.M. (1959) Identification of fungi by serologic tests. Further serologic studies with dematiaceous fungi. *Proc. 6th Congr. trop. Med. Malor.* **4,** 636.

SEELIGER H.P.R. (1960) Les phénomènes d'immunité dans les Cryptococcoses. *Path. Biol.* **8,** 297.

SHIPE E.L., WILLIAMS M. & VANN J. (1963) Comparison of a fluorescent antibody inhibition test with histoplasmin-complement-fixation and histoplasmin latex agglutination tests for histoplasmosis. *Publ. Hlth Lab.* **21,** 169.

SILVA M.F.N., NEVES H., PEREIRA A.M., GONÇALVES A.P., LACAZ C. DA S., FAVA NETTO C. & CASTRO R.M. (1963) Immunologia da esporotricose. III. A prova da esporotriquina em Portugal, em pessoas sem esporotricose. *Rev. Inst. Med. trop. S. Paulo* **5,** 12.

SILVA M.E. & KAPLAN W. (1965) Specific fluorescein labelled anti-globulin for the yeast form of *Paracoccidioides brasiliensis*. *Amer. J. trop. Med. Hyg.* **14,** 290.

SJÖBERG K.H. (1966) Moniliasis—an internal disease? Three cases of idiopathic hypoparathyroidism with moniliasis, steatorrhea, primary amenorrhea and pernicious anaemia. *Acta med. scand.* **179,** 157.

SLACK J.M., WINGER A. & MOORE D.W. (1961) Serological grouping of Actinomyces by means of fluorescent antibodies. *J. Bact.* **82,** 54.

SMITH C.E., WHITING E.G., BAKER E.E., ROSENBERGER H.G., BEARD R.R. & SAITO M.T. (1948) The use of coccidioidin. *Amer. Rev. Tuberc.* **57,** 330.

SMITH C.E., SAITO M.T., BEARD R.R., KEPP R.M., CLARK R.W. & EDDIE B.U. (1950) Serological tests in the diagnosis and prognosis of coccidioidomycosis. *Amer. J. Hyg.* **52,** 1.

SMITH C.E., SAITO M.T. & SIMONS S.A. (1956) Pattern of 39,500 serologic tests in coccidioidomycosis. *J. Amer. med. Ass.* **160,** 546.

SMITH C.E., SAITO M.T., CAMPBELL C.C., HILL G., SASLAW S., SALVIN S.B., FENTON J.E. & KRUPP M.A. (1957) Comparison of five methods of quantitative complement fixation tests for coccidioidomycosis. *Publ. Hlth Rep., Wash.* **72,** 888.

SOUCHERAY P.H. (1954) Farmer's lung—a form of broncho-pulmonary moniliasis. *Minn. Med.* **37,** 251.

STAINES F.H. & FORMAN J.A.S. (1961) A survey of farmer's lung. *J. Coll. gen. Practnrs.* **4,** 351.

STALLYBRASS F.C. (1964a) Candida precipitins. *J. Path. Bact.* **87,** 89.

STALLYBRASS F.C. (1964b) The incidence of the serological groups of *Candida albicans* in southern England. *J. Hyg., Camb.* **62,** 395.

STALLYBRASS F.C. (1965) The antibody response to some antigens of *Candida albicans*. *J. Path. Bact.* **90,** 205.

STUDDERT T.C. (1953) Farmer's lung. *Brit. med. J.* **i,** 1305.

SUMMERS D.F., GROLLMAN A.P. & HASENCLEVER H.F. (1964) Polysaccharide antigens of *Candida* cell wall. *J. Immunol.* **92,** 491.

TASCHDJIAN C.L., DOBKIN G.B., CAROLINE L. & KOZINN P.J. (1964) Immune studies relating

to candidiasis. II. Experimental and preliminary studies on antibody formation in systemic candidiasis. *Sabouraudia* **3,** 129.

TASCHDJIAN C.L., KOZINN P.J. & CAROLINE L. (1964) Immune studies in candidiasis. III. Precipitating antibodies in systemic candidiasis. *Sabouraudia* **3,** 312.

THOMPSON K.W. (1942) The skin reacting antigen of *Trichophyton purpureum*. *J. invest. Derm.* **5,** 475.

TODD R.L. (1937) Studies on the yeast-like organisms isolated from the mouths and throats of normal persons. *Amer. J. Hyg.* **25,** 212.

TOMPKINS V.N. (1965) Soluble antigenic constituents of yeast phase *Histoplasma capsulatum* *Amer. Rev. resp. Dis.* **92,** 126.

TORNELL E. (1946) Thresher's lung. A fungoid disease resembling tuberculosis or morbus Schaumann. *Acta med. scand.* **125,** 191.

TRAN VAN KY P., BIGUET J. & ANDRIEU S. (1963) Étude par l'électrophorése et la double diffusion en gélose des substances antigéniques excrétées dans le milieu de culture de *Candida albicans*. *Sabouraudia* **2,** 164.

TRAN VAN KY P., HAVEZ R., BIGUET J. & LEYS C. (1966) Localization comparée des precipitines anti *Aspergillus fumigatus* dans les globulines immunes du sérum des lapin hyperimmunisés et des malades atteints d'aspergillome pulmonaire. *Ann. Inst. Pasteur* **110,** 86.

TSUCHIYA T., KAWAKITA S. & UDAGAWA M. (1963) The rapid identification of Cryptococcus neoformans by serology. *Sabouraudia* **2,** 209.

VALLERY-RADOT P. & GIROUD P. (1929) Sporomycose des pelleteurs de grains. *Bull. Soc. méd. Hôp. Paris* **52,** 1632.

VOGEL R.A. & PADULA J.F. (1958) Indirect staining reaction with fluorescent antibody for detection of antibodies to pathogenic fungi. *Proc. Soc. exp. Biol. (N.Y.)* **98,** 135.

VOGEL R.A., SELLERS T.F. & WOODWARD P. (1961) Fluorescent antibody techniques applied to the study of human cryptococcosis. *J. Amer. med. Ass.* **178,** 921.

WALZER R.A. & EINBINDER J. (1962) Immunofluorescent studies in dermatophyte infection. *J. Invest Derm.* **39,** 165.

WALRAFF E.B., SNOW I. & WILSON S. (1965) Coccidioidin induction of skin sensitivity and positive serology. *Proc. Soc. exp. Biol. (N.Y.)* **119,** 914.

WALRAFF E.B. & WILSON S.A. (1965) Skin-test activity of dialysed coccidioidins. *Amer. Rev. resp. Dis.* **92,** 121.

WENZEL F.J., EMANUEL D.A., LAWTON B.R. & MAGNIN G.E. (1964) Isolation of the causative agent of farmer's lung. *Ann. Allergy* **22,** 533.

WERNSDÖRFER R., PEREIRA A.M., GONÇALVES A.P., LACAZ C. DA S., FAVA NETTO C., CASTRO R.M. & DE BRITO A. (1963) Immunologia da esporotricose. IV. A prova da esporotriquina na Alemanha e no Brasil, em pessoas sem esporotricose. *Rev. Inst. Med. trop. S. Paulo* **5,** 217.

WIGGINS G.L. & SCHUBERT J.H. (1965) Relationship of histoplasmin agar gel bands and complement fixation titres in histoplasmosis. *J. Bact.* **89,** 589.

WILLIAMS D.I. & MULHALL P.P. (1956) Farmer's lung in Radnor and North Breconshire. *Brit. med. J.* **ii,** 1216.

WINNER H.I. (1955) A study of *Candida albicans* agglutinins in human sera. *J. Hyg., Camb.* **53,** 509.

WINNER H.I. (1956) Immunity in experimental moniliasis. *J. Path. Bact.* **71,** 234.

WOOD S.R. & CRUICKSHANK C.N.D. (1962) The relation between trichophytin sensitivity and fungal infection. *Brit. J. Derm.* **74,** 329.

ZAMORA A., BOJALIL L.F. & BASTARRACHEA F. (1963) Immunologically active polysaccharides from *Nocardia asteroides* and *Nocardia brasiliensis*. *J. Bact.* **85,** 549.

ZETTERGREN L. (1950) Thresher's lung. *Acta Soc. Med. upsal.* **55,** 257.

CHAPTER 4

DIAGNOSIS OF VIRUS DISEASES

F.O.MacCallum

INTRODUCTION

The immunological methods that may be used for the diagnosis of virus infections are the same as those used in tests for infections with other organisms, but the relative 'importance' of the different types of diagnostic test varies with different viruses. The bedsonia group of agents, psittacosis/ornithosis, lymphogranuloma venereum (LGV) and trachoma/inclusion conjunctivitis, and *Mycoplasma pneumoniae* are included with the viruses in this section. The tests, arranged in order of general usefulness, are complement fixation (direct and indirect), haemagglutination and haemagglutination inhibition, haemadsorption and haemadsorption inhibition, neutralization of infection, agglutination and precipitation (gel diffusion), immunofluorescent staining and skin tests. In most of the serological tests performed in virology one is testing for the appearance of a rise in antibody some days after the onset of illness but when a high titre of antigen is present in blood or tissues at an early stage as in variola, trachoma and influenza, one may test for the presence of antigen in blood or tissues of the patient using specific antibody-containing serum, with complement

fixation, gel diffusion or immunofluorescence. Similar use of antibody is made to detect the presence of virus (antigen) in tissues of infected animals and fertile eggs or in tissue cultures.

DIAGNOSTIC METHODS IN GENERAL

Complement Fixation Test (CFT)

Complement-fixing antibodies are probably formed in all virus infections and may be of two kinds, either reacting with the antigens in the virus particle itself or with so-called 'soluble' (S) antigens which are usually a non-infectious form of virus in the early stage of its development, and may be obtained free of virus. Antibodies to soluble antigen are usually only detectable for a relatively short period after active infection and are not produced by injection of a preparation of non-replicating (inactivated) virus unless it contains a large amount of 'S' antigen. In the case of a virus in which there are several types or subtypes the virus particle antigen and its antibody are type or subtype specific, whereas the soluble antigen is only group specific, for example there is a soluble antigen common to all viruses of the influenza A group (A, A1, A2) and a virus particle CF antigen specific for the subtypes found in 1933–46, 1946–55 and 1957 onwards. However, the type specificity of the virus antigens can usually be altered or degraded by chemical or heat treatment, for example type-specific CF antibody can be detected in patients with poliovirus infection if a live virus antigen is used but if this is heated or irradiated with ultraviolet light to kill the virus the antigen becomes degraded and cross-reactions between types occur.

There are also common complement-fixing antigens among certain groups of viruses which may be otherwise differentiated by neutralizing antibody, or other tests, for example between the polioviruses and certain coxsackie and echoviruses, groups of arthropod-borne viruses, and members of the psittacosis-LGV group. The strength or titre of the antibody produced and detected varies from virus to virus, as a result of the system of the body which is affected, the mechanism of replication of the virus and of course the titre and structure of the antigen used to detect the antibody. The strength and duration of response will, to some extent, depend on the duration of the infection, being of very high titre and persistent (9 months to several years) in certain presumed latent infections as with the psittacosis-LGV group, in chronic Q fever and for the duration of life in herpes simplex, and low and of short duration (up to 9 months) in localizing skin infections, as in intradermal inoculation of vaccinia virus or in upper respiratory tract infections. The specificity, among members of certain inter-related groups such as the arthropod-borne viruses, will also be dependent on the number of infections with members of the group which the patient has had, e.g. an attack of yellow fever produces only yellow fever antibody, but

later infection with another member of the same group B, for example St Louis encephalitis virus, will produce antibody to this and also stimulate a rise in antibody to yellow fever, and possibly other members of the group. In certain patients and species of animals and birds, with psittacosis-ornithosis infections, in which antibiotics may have been used in the treatment, the formation of antibody may be interfered with or delayed. In fact in the psittacosis-ornithosis group, particularly in avian infections, ordinary or direct CF-antibody is frequently not detectable, but a non-complement-fixing antibody is present which is capable of interfering with the combination of the antigen and a specific complement-fixing antibody in the serum of some other mammalian species. This is measured by the indirect CF or CF inhibition test (Rice 1948) (see below).

The antigens used to detect virus antibodies may be prepared from tissues of human patients, infected animals or chick embryos, or from fluid and cells of infected tissue cultures. The tendency for antibodies to normal or damaged tissues to be present in human and animal sera following certain diseases and the relatively low titre of most antigens prepared from the infected tissues of these two hosts limits their use as antigens. Those viruses which grow well in chick embryo produce antigens of higher titre though the problem of reaction between purely tissue antigens and antibodies to them in the serum still has to be contended with, but less frequently in sera which have been recently collected under sterile conditions and heated at 56–60°C for 20–30 min before use. Tissue culture antigens have a tendency to fewer non-specific reactions and are available for a large number of viruses which will not grow in animals or chick embryos.

The basic principles of the test (see Chapter 1) are those described originally by Bordet but various modifications have been introduced mainly to increase the sensitivity of the test because of the weak antigens which have been available. It is of historical interest that the first tests for complement fixation with a pox virus were reported by Jobling in 1906 using vaccinia at much the same time that Wassermann and his colleagues were developing the basic technique of the CF test for syphilis. Some of the methods introduced are quite satisfactory for routine clinical diagnosis of virus infections where the aim is to demonstrate a rising titre during convalescence by comparing a serum taken in the acute stage of the illness with one taken usually 10–21 days after the onset of illness—the interval depending on the particular virus and type of antibody being investigated. Most of the tests were designed originally to use 0.2 ml amounts each of serum, antigen and complement, and 0.4 ml of sensitized sheep r.b.c. in small test tubes in a water-bath, but these extravagant quantities were reduced by many laboratories some years ago by the introduction of unit volumes of 0.1 ml amounts in the cups of plastic trays first devised for haemagglutination tests for influenza and of 0.02 ml in drops on flat plastic plates (Fulton & Dumbell 1949) placed in special chambers or boxes during incubation to prevent evaporation.

The test in cups of plastic trays is less refined than that of Fulton and Dumbell but is capable of being used very rapidly on a large scale in routine diagnostic laboratories. A unit volume of 0.02 ml in cups of trays has recently been used more widely by the application of the Takatsy technique with platinum loops instead of pipettes to make dilutions *in situ*.

In routine diagnosis either of two methods of the first stage of the reaction between antigen, antibody and complement may be used. In the usual test for antibodies in a patient's serum against a number of virus antigens many virus antigens combine with their antibody to bind complement adequately after incubation for 1–2 hr at 37°C but this is not true for all antigens or the antibodies in many children's sera where it is necessary for the first stage of the reaction to be allowed to occur slowly at 4°C for 16–18 hr (overnight). The main difficulty arising out of this test is that any anticomplementary activity of the patient's serum is also increased during this prolonged fixation. Where alternative periods are possible, a laboratory will use whichever method fits most readily into its administration.

The amount or dose of antigen used to test against the 'unknown' serum is determined by tests against known positive human or animal sera. Where human sera are being examined this dose is best determined against a human serum because the optimum dose to use may be different from that obtained against an immune or hyperimmune animal serum. However, not all human sera have the same optimum with a given antigen and more detailed tests may be necessary in special cases.

Blood should be collected with a clean, dry, sterile syringe and needle, but if a syringe freshly sterilized by boiling is used it should be rinsed with sterile normal saline. The blood should be delivered into a clean, dry, sterile bottle (no anticoagulant), with a screw-on lid and washer, and not into tubes with cork bungs or cotton wool plugs. If these precautions are not taken the serum is likely to be rendered anticomplementary and useless. Occasionally the serum of an individual possesses anticomplementary activity due to some unknown inherent factor; this may be related to certain auto-allergic reactions. It may be possible to convert anticomplementary sera to a usable state by heating at 56°C for 30 min or 60°C for 20 min, or by absorption with complement, or with mouse liver powder.

The test consists of five variables and is subject to error if not performed carefully. Even when a single antigen and ostensibly the same technique are used two-fold differences in titre on either side of the expected titre of the standard serum may be obtained by different workers. Even the same individual may obtain two-fold differences with the same materials on different days. Thus in practically every instance one must be able to show at least a four-fold rise in antibody titre between the serum taken in the acute stage of the disease (preferably not later than the 5th day after onset) and that collected in

convalesence (12th–42nd day) before one can consider the result 'positive'. The time of the first appearance of CF antibody varies with different viruses and also depends on whether or not the illness is a primary infection in some group where there are a number of antigenically related viruses. In the latter a more rapid rise may be expected in second infections. CF antibody will seldom be detected before the 10th day (it is found early in influenza and mumps) but is usually present by the 21st day. The time at which a peak titre is reached and maintained also varies in infection with different viruses and in different patients but a serum taken before the 6th week will almost certainly show a four-fold or greater rise. Most CF antibodies to soluble antigen will have fallen to a low level or have disappeared by 6–9 months but antibodies to some virus particle antigens may be detected for 12–18 months or longer. Examination of a single specimen of serum in convalescence is rarely justified unless (1) one suspects that infection has occurred with some virus, rickettsia or bedsonia not normally present in the area or (2) the patient, already convalescent, is possibly the first case preceding a wave of similar illness in the community. In the latter situation the result of a CF test on the index case or cases may indicate the virus possibly responsible for the current cases, and be a guide to the methods of isolation and serological tests to be used on them.

The deficiency in the value of these tests from the standpoint of the clinician is that as a rule not even a presumptive or speculative diagnosis can be hazarded from the result of a CF test on blood taken less than 10 days from the onset of illness, and a pair of sera are really essential. By the time these are collected and examined the patient has usually recovered or died.

However, in the case of variola in the viraemic stage up to and inclusive of the 1st day of rash, particularly in a previously unvaccinated patient, there may be sufficient virus (antigen) in the blood to act as antigen which may be detected in a CF test with high titre positive serum. The development of high titre positive sera against other viruses may make diagnosis in the viraemic stage a possibility, particularly in known contacts, and needs further exploration.

The unusual circumstances often attending a variola infection as compared with other virus infections also introduces another possible method of early serological presumptive diagnosis. A number of cases of mild variola major, in which skin lesions are absent or scant but pneumonia may be present, are difficult to diagnose but capable of transmitting severe disease to others. In an infected unvaccinated person CF antibody would not be detectable before the 10th day of illness. However, in a person vaccinated 6 months to 10 years previously, an accelerated response may occur and the detection of CF antibody within the 1st week of illness would be diagnostic even without a rash. The same type of accelerated response may be seen in zoster or possibly in successive infections with different types in a group of related viruses which have a common CF antigen but do not give cross-protection, e.g. in adenoviruses.

Complement Fixation Inhibition Test

Rice (1948) observed that the sera of certain avian and mammalian species exert a specific inhibitory effect in complement fixation test systems. The indirect CF or complement fixation inhibition test was developed to detect and measure this inhibitory or blocking antibody which is unable to fix guinea-pig complement but can combine with the specific antigen. This combination or union can be detected by the addition of a specific indicator antibody which will fix complement in the presence of specific antigen. If the serum being tested contains inhibiting antibody it will combine with the antigen so that the specific antibody in the indicator (positive) serum has no antigen with which to combine and, as complement will not then be fixed, the red blood cells will be haemolysed. This is a positive result in contrast to the direct complement fixation test where a positive is shown by no haemolysis. The only human infection in which this test has been of much value is psittacosis-ornithosis although Terzin (1958) has indicated additional possibilities. Further investigations may show it to be of value in infections with some entero and other recently isolated viruses. In ornithosis the indicator antibody is obtained from naturally infected human patients or pigeons.

Sera which are anticomplementary even after inactivation by heat (58–59°C for 30 min) or other means cannot be used in this test. As in all other diagnostic serological tests in virology, paired sera must be examined. If a suspected case of psittacosis-ornithosis does not react in the direct test it should be tested in the indirect test.

Conglutinating Complement Absorption Test

As discussed in Chapter 1, this test is comparable with the haemolytic complement fixation test but is based on another property of complement, namely conglutination, instead of haemolysis. The value of the test lies in the fact that complements other than that of the guinea-pig may be used and these may, in certain circumstances, be more sensitive in demonstrating antibody-antigen interaction.

Despite this greater sensitivity the test is more involved and more prone to the influence of anticomplementary factors which puts it at a disadvantage.

The test has been used in Q fever, psittacosis, vaccinia and influenza, but has not been found to have any practical advantage over other tests which are simpler.

Agglutination and Precipitation Reactions

These tests have received relatively little attention from virologists because of physical difficulties due to the small size of the viruses and lack of virus suspensions of sufficiently high titre.

Agglutination refers to the aggregation produced by the clumping of relatively large particles such as virus elementary bodies, which can be seen under the ordinary light microscope, as with psittacosis and vaccinia. On the other hand precipitation usually describes those reactions in which the aggregating material comes out of solution, for example the soluble antigens of vaccinia, varicella, herpes simplex and influenza.

The soluble antigens of a rickettsia or large virus may be as large as a small virus elementary body so that absolute differentiation of the type of reaction on the bases of particle size is not always possible.

A third term, flocculation, is used to describe aggregation of large particles and soluble antigens together in a single reaction. This reaction is exemplified by what happens when specific immune serum is mixed with vaccinia, influenza or polio virus under the appropriate conditions. These viruses also fix complement but as complement fixation requires less antigen, not all complement-fixing viruses necessarily cause flocculation.

Agglutination reactions may be used with rickettsia and some of the large viruses but in practice are only used in typhus and Q fever. However, considerable use has been made of this test in the past in studies of the antigenic structure of vaccinia. The same can be said of precipitin tests in the liquid state. Detection of precipitation in solid agar is described below.

Coating of collodion particles with small viruses has been investigated in agglutination tests with one or two viruses but the method has not come into general use.

Double Diffusion Agar Tests

In this type of reaction (see Chapter 1) antigen in suspension and antibody in serum are allowed to diffuse slowly through semi-solid agar from separate wells and if specific for each other they precipitate (forming a white line) at the point of meeting. This test was first used in virology by Jensen & Francis (1953) who described the reaction using influenza virus and antiserum in tubes rather like a ring test for brucella antibody. Later the plate method developed by Ouchterlony (1948) for the study of the reaction between diphtheria toxin and antitoxin was applied to the study of antigens and antibodies associated with many viruses. For quantitative studies Ouchterlony's method in which the reagents are placed in cups in agar in a Petri dish is used, but for rapid diagnostic tests using small amounts of antigen and serum a microtest in agar on a glass microscope slide may be used. The antigen or serum to be tested is placed in a cup in the centre of the area of agar and equidistant around it are placed the corresponding sera or antigens and the whole is incubated in a moist atmosphere in a Petri dish or other container, usually at 37°C. For example in a case of suspected smallpox, the material from the lesion (vesicle fluid, etc.) is placed in the centre and vaccinia and zoster positive sera and negative control sera are placed

around the periphery as described by Dumbell & Nizamuddin (1959). A positive result may be available in an hour or two if it is a case of smallpox, vaccinia or cowpox, but if it is a case of varicella the reaction, which only takes place if the vesicle fluid is used, is slower and an answer may not be available for 18 hr. This test may be used also for routine antibody tests on sera.

Neutralization Tests

In the tests described up to this point one is usually measuring some antibody in serum which, if present and rising, is indicative of recent infection. (The exception is of course the smallpox group where, as stated previously, there is sufficient virus and antigen in visible lesions that one can test for antigen.) The presence of such antibodies infers that infection is current or has occurred recently and usually means that the host is in a protected state as far as disease from the particular virus is concerned. This protected state or immunity may, however, persist for several years or even a lifetime, long after the complement-fixing or other transient antibodies have disappeared. The presence, and to some extent the degree, of protection is indicated by the level of neutralizing antibodies, so-called because of their capacity to prevent the virus infecting animals, chick embryos or tissue culture or prevent its participation in certain *in vitro* tests such as haemagglutination. The antibody participating in the latter reaction appears to be closely related to the neutralizing or protective antibody in the case of some viruses but does not always show complete correlation.

In vivo neutralization tests are inevitably more cumbersome, time-consuming and expensive than CFTS and other *in vitro* tests and will seldom be used for diagnostic purposes except in those rare instances where no other suitable test is available or where they may give a specific answer concerning a member of a group of viruses in which CF or other tests are less specific (e.g. arboviruses).

In vitro neutralization tests are now mostly carried out in tissue cultures in which the affected cells show specific changes, but mouse protection tests are still used in some virus infections and pock neutralization tests on the chorioallantoic membrane of fertile hens' eggs may be convenient with the variola-vaccinia group. In the usual test, dilutions of serum (two- or four-fold as a rule) are mixed with a constant dose of virus and incubated at 37°, 22° or 4°C for varying periods of time, depending upon the stability of the virus at one or other of these temperatures, and the mixture is then inoculated into an adequate number of hosts or tissue cultures. The result is compared with that produced by the virus alone and when mixed with a known positive serum. Occasionally a constant amount of undiluted serum is mixed with various doses of virus and compared as before with negative and positive controls.

Haemagglutination and Haemagglutination Inhibition (HI) Tests

The independent observations of McClelland & Hare (1941) and Hirst (1941) that influenza virus would agglutinate the red blood cells of infected and normal chick embryos and adult fowl opened up a whole new field for study of the immunology of viruses and the mode of action of viruses on cells. Later workers found haemagglutinins associated with all three influenza viruses, A, B and C, and the viruses related to them (all known as myxo viruses); the pox viruses (vaccinia, variola, cowpox and mousepox) but *not* chickenpox, with arthropod-borne viruses, some entero- and adeno-viruses, and the psittacosis-ornithosis group of agents. The species of bird or animal from which the red blood cells are obtained, the temperature at which the reaction takes place and the deleterious effect of certain non-specific inhibitors in the serum on the haemagglutinin varies with different viruses and groups of viruses. The haemagglutinins may be inseparable from the virus or obtained as a separate substance.

A haemagglutination-inhibiting antibody is found as the result of infection with or injection of some virus preparation, for example influenza virus vaccine, which contains the haemagglutinin, so that a test for a rise in titre of HI antibody in convalescent sera may be used in diagnosis and a similar rise after inoculation of influenza vaccine is indicative, by inference, of the immune state. Antisera may be used to identify freshly isolated haemagglutinating viruses.

Although the HI test at first appeared to be much simpler than complement-fixation it was soon found that there were drawbacks, particularly normal serum inhibitors which limited its use as a routine diagnostic method in influenza.

The application of HA and HI tests is described briefly below under the different groups of viruses.

A number of viruses, which do not produce obvious degeneration of tissue culture cells, haemagglutinins or CF antigen in tissue culture or infect fertile hens' eggs or animals, may produce antigens at the surface of cells in tissue culture so that red blood cells of various species, particularly the guinea-pig, will adsorb to the tissue cells (haemadsorption) and this phenomenon can be specifically prevented by the related antibody. This phenomenon was first observed by Vogel & Shelokov (1957) working with Asian influenza virus in monkey kidney tissue cultures and most of the other viruses subsequently detected by this test have been found responsible for respiratory tract disease.

Immunofluorescent Staining

The application of this technique (see Chapter 1) has developed from the work of Coons and his colleagues which was first reported in 1942. It consists of labelling antibodies with a fluorescent dye, allowing the labelled antibodies to react with their specific antigen and observing the preparation illuminated by

ultraviolet light in a fluorescence microscope. Antibody reactions may be examined in smears of cells or tissue sections from man, animals, birds, chick embryos or tissue cultures and may be applied in three ways.

1. In the direct method the fluorescein-labelled antibody is added to a preparation of antigen.

2. In the indirect method, the antigen is first treated with unlabelled specific antiserum and this complex is then treated with fluorescein-labelled antibody to the specific globulin, for example specific human antibodies may be localized with conjugates of rabbit or goat anti-human γ-globulin. This is more sensitive than method 1.

3. The complement-staining procedure whereby a mixture of a specific antiserum and whole fresh guinea-pig serum (complement) are applied to a section or smear containing the test antigen. This complex is visualized by adding fluorescein-labelled antibodies to guinea-pig complement. It has been suggested that this technique is more sensitive than indirect method 2.

These methods have been used particularly in virus research to study the site of virus multiplication in cells but their practical application in routine diagnosis is being extended. Suitable reagents are not always readily available; there has been particular difficulty with non-specific staining. If specific sera can be provided any slight increase in cost will be justified for there is no doubt that if any newly discovered antiviral drugs are to be used satisfactorily they must be applied early in the disease and it is likely that a relatively precise diagnosis of the responsible virus will be required. To date, the indirect test has found its most valuable application in the rapid diagnosis of rabies (Goldwasser *et al* 1959) in the central nervous system and salivary gland of suspected vectors. It has been used in the diagnosis of influenza from smears of nasal mucosa (Liu 1956), the more rapid identification of viruses in tissue cultures and diagnosis of trachoma in smears of conjunctival cells. It is possible that specific staining of infected cells in other body fluids such as CSF may be found practicable.

Skin Tests

Intradermal inoculation of virus to detect hypersensitivity is of little practical importance in virus infections, but is valuable in diagnosis of infection with the psittacosis-LGV group of agents, particularly the latter. The test has also been studied in mumps infections and of course the reaction may be seen in the process of vaccination against smallpox.

Most antibodies to microbial infections are γ-globulins and are of the 7S or 19S variety. In numerous virus infections the early appearing neutralizing and haemagglutination-inhibiting antibodies are associated with 19S globulins but these are later superseded by antibodies associated with 7S which are more avid. Virus complement-fixing antibodies which usually appear slightly later

have generally been found to be 7S. Thus measurement of the relative amounts of 19S and 7S antibody may provide a valuable index for recognizing recent virus infection and differentiating between primary and secondary responses.

DIAGNOSTIC METHODS FOR INDIVIDUAL VIRUS DISEASES

A brief note of the application of the various procedures in infections with *Rickettsia burneti*, bedsoniae, *Mycoplasma pneumoniae* and different viruses is given below.

Q Fever

Infection with *R. burneti* may cause illness, particularly pneumonitis, which is indistinguishable from numerous virus infections. Complement fixation is the test of choice though an agglutination reaction is sometimes used. Growth of the rickettsia and hence antibody production may be inhibited by the use of tetracycline drugs so that tests on several samples of serum collected at 2, 3 and even 4 weeks, in addition to the acute stage sample may be necessary before a diagnostic rise in antibody can be detected.

The usual antigen for routine diagnosis is made from infected yolk sacs of fertile hens' eggs and is known as 'phase 2' antigen but in chronic cases not only does high titre antibody appear to this but also an antibody appears to what is known as 'phase 1' antigen. This is not found in the ordinary case which makes an uneventful recovery.

Psittacosis—LGV—Trachoma

Complement fixation is the test of choice in this group.

There are two CF antigens, a group and a type-specific, in these agents. For general purposes a heated suspension of the virus is used for the group test. Absorption of the serum with the group antigen removes the group antibody and leaves the specific one. The serum can then be tested with fresh, untreated virus containing type specific antigen. This test is seldom carried out because the type specific antigen is heat labile and there is considerable difficulty in preserving it in the active state. As the difference between psittacosis and lymphogranuloma venereum is usually obvious clinically no attempt is made to carry out the more laborious type-specific test. Occasional patients, particularly those treated early with tetracyclines, may give negative results in routine CF test, at least for the first few weeks. However some of these patients may develop an antibody which can be detected in the CF inhibition test. This latter antibody is also more commonly found than that giving direct CF in sera of birds infected with ornithosis virus.

The agent of trachoma contains the dominant group antigen which reacts fully with human psittacosis or LGV positive sera or with hyperimmune animal

heterologous bedsonia serum. However, the conventional test for group antibody is of no value as positive results are obtained in only a minority of well-established cases and in these only low-titre reactions are found. It remains to be seen whether a test for specific antibody will be of any value.

The agent of psittacosis possesses a haemagglutinin separable from the infectious particle and active against mouse red blood cells but the HI test has no advantage over the CF test for diagnosis. The same applies to the agglutination test.

These agents produce a toxin, the effect of which is tested by intravenous inoculation of mice, but this is not suitable nor are other animal tests for the routine demonstration of neutralizing antibody.

In addition to the CF test, a skin test for hypersensitivity is possible in infections with this group. It was first described by Frei (1925) for the diagnosis of lymphogranuloma venereum, and herein lies its main value but positive reactions have been detected in psittacosis. A suspension of the organism inactivated by heat or phenol is injected intradermally, usually on the skin of the forearm; a suspension of the same type of tissue (usually yolk sac of fertile hens' egg) as that in which the agent has grown is injected at a separate site as a control. In a positive reaction a nodule appears at the site of the specific antigen on about the second day and reaches a maximum in about 4 days when it should measure about 6–8 mm in diameter. The nodule may persist for several weeks. No nodule should be seen at the site of inoculation of the control antigen unless the patient is sensitive to egg protein. It may not be possible to interpret the reaction in such a patient. The test usually becomes positive 2–6 weeks after infection and probably more than 95% of patients with LGV give a positive reaction. In these patients a positive reaction usually remains throughout life even if treatment has been successful and CF antibody may have fallen to an undetectable level, so a positive reaction by itself is not indicative of active infection.

Heated trachoma antigen reacts as a skin test antigen in patients with LGV but the test is negative or equivocal in trachoma patients presumably because the nature of the infection does not usually lead to 'dermal sensitivity'.

Mycoplasma Pneumoniae (Eaton Agent)

Antibody to this agent can be detected by various procedures using an antigen prepared from broth cultures.

The complement fixation test is simple and sufficiently sensitive for routine diagnostic purposes. For the determination of antibody levels in a population a more sensitive method is required, such as metabolic inhibition, immunofluorescence or indirect haemagglutination. Of these the metabolic inhibition test is probably the most suitable for large-scale surveys.

Immunofluorescence using infected chick-embryo lung sections or mycoplasma colonies transferred to a glass slide as antigen is probably the most

reliable and specific test. It is not technically suitable for large scale surveys.

Cold agglutinins frequently appear in the serum of patients with Eaton agent pneumonia, usually during the second or third week of illness. The peak is often short-lived and may be missed. These are auto-antibodies with a specificity anti-I and can occasionally cause haemolytic anaemia.

Agglutinins to streptococcus MG sometimes develop during the course of atypical pneumonia, but are no longer so important in diagnosis now that specific tests for antibodies to the causative agent have been developed.

Pox Viruses (Vaccinia, Variola, Cowpox)

No differentiation is possible by CF test as only a group-reacting antigen and antibody are produced. Other laboratory methods of diagnosis such as virus isolation are usually more satisfactory where there is clinical uncertainty. The rare occasion when a complement fixation test for antibody is of value is described in the section on general application of the test. However, the CF test for the detection of antigen in blood or in material from lesions using positive serum is of great value in making a presumptive early diagnosis, when the Ouchterlony test is not possible. The latter is much more rapid than CFT.

The red blood cells of only about 50% of adult chickens are agglutinated by members of this group of viruses. The haemagglutinin is produced in the chorioallantoic membranes of chick embryos or in tissue cultures and in contrast to that of influenza it can be obtained free from active virus. It is heat-stable and distinct from the soluble CF antigen. Haemagglutination takes place at 37°C and elution and serum inhibitors play virtually no part. The haemagglutinin from variola, vaccinia and cowpox are indistinguishable. The antibody produced is consequently the same and the test cannot be used in differential diagnosis. In an unvaccinated patient or one vaccinated some years previously, with possible recent exposure to variola (and from whom no specimens from skin lesions are available) the presence of this antibody in a titre of 1/80 or higher after the 5th day of illness is highly significant. Immunofluorescence may be used to detect virus antigen but considerable trouble has arisen from non-specific reactions.

Varicella-Zoster

Complement fixation and gel-diffusion tests are the diagnostic tests of choice and may be used to detect antigen in vesicle fluid or antibodies in serum.

Although the use of varicella crusts as a suitable source of antigen has been reported in the past this has not been the experience in recent years; only vesicle fluid can be relied on, in marked contrast to what occurs in pox virus infections. The soluble antigen in the fluid has a higher titre than the sedimentable (virus particle) fraction. CF antigens are now commonly prepared from infected tissue culture cells. Immunofluorescence may be used to identify virus antigen in tissue

cultures. The titre of antibody is usually higher in zoster cases than in varicella and even the acute serum in zoster may be positive. Thus a zoster convalescent serum is more suitable as a positive control to test a new batch of antigen or identify an unknown vesicle fluid.

Herpes Simplex

This virus contains a virus particle (V) and soluble CF (S) antigen and corresponding antibodies are found in patients' sera. The antigens used have been prepared from infected mouse brain, chorioallantoic membrane of fertile hens' eggs and from tissue cultures. Limited studies have suggested slight differences in antigenic structure may occur in different strains of virus and further investigations seem warranted.

In primary infection CF antibodies appear about the 10th–14th day, reach a maximum titre in several weeks and after a gradual fall of varying degree to a base line of 1/8 to 1/64, occasionally 1/256, the titre usually remains stable for the remainder of the patient's life. It is generally assumed that once infection with this virus has occurred the virus remains at the primary site, usually in the latent phase, throughout life. 'Attacks' or relapses occur when the state of the tissue, of the virus or of antibody is such that virus replication reaches a stage where it causes cell damage which is manifest by vesicle formation. This latter occurs in the presence of neutralizing antibody which can be demonstrated in eggs or tissue culture and although there is some difference of opinion, whether 'attacks' are preceded by a fall and accompanied or followed by a rise in antibody each time, the changes in titre, if any, have been found to be slight. Hence it can be seen that the mere presence of antibody is not indicative of the aetiology of a 'herpetic' lesion nor any illness without typical lesions (encephalitis). Numerous antigens have been detected in the virus by using gel-diffusion test but this has not yet been shown to be of practical value. If large amounts of antigen are present its identification in tissues or cells by the immunofluorescence technique may be more rapid than by tissue culture.

Influenza A, B and C

Complement fixation tests are the common method of serological diagnosis in infections with these viruses. There are two antigens, a virus antigen (V) which is specific for the subtype of virus, for example A, A1, A2, and a soluble antigen (S) which is obtained separate from the virus particle and is group specific, i.e. for all A viruses, or all B viruses. The 'S' antigen is the one most commonly used in routine diagnosis as the antibody to it will be detected even when a new subtype of virus appears within the groups as did A2 (Asian A) in 1957. Once the virus responsible for an epidemic has been isolated the V antigen may give a slightly higher percentage of positive reactions because some individuals who have a residual 'S' antibody titre may not show an increase in

convalescence when re-infected. The 'S' antibody may also be in very low titre or undetectable in children undergoing a primary infection and here the test for 'V' antibody will be of value. The 'V' antibody is probably also a good indicator of immunity.

Anti-V serum made in guinea-pigs may also be used in CF test for typing new viruses isolated in eggs or tissue culture though haemadsorption-inhibition may be preferable for the latter. CF antibody may appear in humans in low titre for a short time after injection of inactivated virus vaccine.

The viruses of influenza, mumps and Newcastle disease of fowl agglutinate red blood cells of a wide variety of mammals and fowl because the virus particles are able to attach themselves to two or more red blood cells probably by combining with a mucoprotein site. It seems likely that a virus particle has many haemagglutinin sites. These viruses can also elute from the red cells by a process which is enzymatic in nature, but the virus haemagglutinin and virus enzyme are not identical.

At 22°C and higher temperatures the virus gradually makes the cell refractory to subsequent agglutination by the same virus but the latter remains essentially unchanged by the reaction. Near 0°C agglutination occurs without elution. The action of the virus is imitated by a soluble enzyme from *Vibrio cholerae*, known as RDE (receptor destroying enzyme). The virus can adsorb on to and destroy receptors on animal host cells such as mouse and ferret lung. The action of the virus on the red blood corpuscles can be prevented by certain soluble mucoproteins which may be present in human and animal sera and these must be destroyed by treatment of the serum with RDE, periodate or trypsin before a serum can be tested for antibody.

The HI antibody test for A or B viruses is specific for viruses in each subtype within the type, for example in A, there are viruses of 1930–46, 1946–57 and 1957 onwards, and the test is thus of less practical use for routine diagnosis than is the CF test using soluble antigen. Once the antigenic composition of the prevalent type or types is known, then the HI test (or CF test) using virus antigen can be used routinely. A pair of sera are essential from each patient, one taken before the 5th day and one after the 10th day and as in CF tests a fourfold or greater rise in the titre of the convalescent sample is required for the result to be considered positive.

The test has been used for the typing of influenza viruses but is not always satisfactory due to alterations in the virus structure in some strains making them refractory and haemadsorption-inhibition in monkey tissue cultures or complement fixation with anti V-serum will be necessary and may supersede the HI test.

Both complement-fixing and haemagglutination-inhibiting antibodies develop in most patients in convalescence but one or other may be absent in occasional patients.

Immunofluorescence with specific antiserum may be used to identify the virus in cells from the mucosa of the respiratory tract.

Mumps

All mumps viruses isolated so far have been sufficiently closely related to allow the use of a single CF antigen, although it is possible that serum from occasional cases may give a negative reaction.

There are a virus particle (V) and a soluble (S) CF antigen which may be used for two purposes. In an acute attack the antibody to the 'S' antigen usually appears quite early, possibly before that to 'V' antigen, and sometimes reaches a peak earlier and then gradually falls to a low level or disappears within 6–12 months. The antibody to 'V' antigen may be detectable for a year or two and is a useful test for immunity. Even when it has disappeared it may reappear rapidly after an intradermal skin test or subcutaneous injection of inactivated virus vaccine. Haemagglutination inhibition tests may also be used.

Heated virus has been used as a skin test antigen in mumps virus infections but the reaction is of little or no value in diagnosis since dermal hypersensitivity develops at such widely varying intervals in different persons after the onset of illness. However, such an injection may stimulate production of CF antibody, particularly in those infected some years previously and give a better idea of the immune state of the individual.

Para-Influenza Viruses

Four viruses are at present included in this group; so-called because of some of their characters and the types of disease which they produce. Complement fixation tests are the serological test of choice, although there may be some difficulty in interpretation of the results due to presence of antigenic overlap to two or more of the viruses. Neutralization of the haemadsorption effect of the viruses in tissue culture is also possible and is used for the identification of recently isolated viruses.

Adenoviruses

This group of viruses, of which more than twenty immunological types have been identified, has been associated with diseases of the respiratory tract, particularly, but also with conjunctivitis, aseptic meningitis and possibly inflammation of the mesenteric glands. Certain types (e.g. 1, 2 and 5) may colonize the adenoids and tonsils at a very early age without necessarily producing obvious disease. Antibodies may also appear at an early age as a result of inapparent infection at this or other sites.

The complement fixation test using an antigen common to all members of the group is the serological test of choice. However, the CF antibody response may be interfered with because of recent previous infection with other members

of the group. Virus neutralization tests are required for an exact serological diagnosis of the type involved but the large number of adenoviruses precludes such tests being carried out routinely on individual patients unless one has knowledge of the probable aetiological type. These tests may be performed in tissue cultures. Precipitin tests using the double diffusion through agar technique have also been used and give somewhat comparable results. A haemagglutinin for rat and other erythrocytes and hence a haemagglutination-inhibition test, have also been described associated with some types of adenoviruses.

Measles

Laboratory tests for measles are, of course, rarely required because a satisfactory clinical diagnosis can be made in nearly 100% of cases. However, complement fixation tests using tissue culture antigen and neutralization of the cytopathic effect of the virus in tissue culture are possible. The virus contains a haemagglutinin for simian erythrocytes so that haemagglutination-inhibition tests are possible.

Rubella

A neutralization test in tissue culture using sera which have not been inactivated has been the test of choice, but complement fixation and haemagglutination inhibition tests are now possible.

Arthropod-Borne Viruses (Arboviruses)

The complement-fixing antigen in these viruses is usually associated with the virus particle and the source of virus has been suckling mice brain. However, improved methods of tissue culture of a large number of these viruses may result in better antigen production in the future. The CF antibody usually appears before the neutralizing antibody in this group of infections, but there are exceptions, e.g. Western and Eastern equine encephalitis. CF antibody persists for a relatively short time, at least in high titre so that the test may be used even in surveys and when positive indicates relatively recent infection in the area being sampled. It is useful for diagnosis of specific infection but difficulties may arise particularly in areas where one or more of the viruses may be endemic because the antibody will react with more than one antigen of the group if repeated infection with the same virus occurs, or the patient is subsequently reinfected with another member of the same group or after passage of time following the primary infection. Cases have been described where the CF test has been negative.

A haemagglutinin associated with these viruses may be found in the brain or blood of mice infected within the first few days of life and in tissue cultures, and is most active against red blood cells of day-old chicks or of geese. Mouse brain contains non-specific haemagglutinins for erythrocytes from a variety of animal

species so the tissue must be treated with chemicals to remove these. Several different haemagglutinins reacting at different pH and temperatures are present in different arboviruses and have been used to classify the viruses into groups, A, B, C, etc. This classification is supported by the results of CF and neutralization tests.

HI antibody to one of these viruses usually appears before the corresponding CF antibody but usually later than neutralizing antibody. HI antibody rarely fails to develop. (Non-specific inhibitors may be present in serum of man and animals but can be removed by filtration through Seitz pads or other methods.) The antibody usually persists a long time and may be detectable for even the life time of the vertebrate host, particularly in the B group. At the same time the HI antibodies are more broadly reacting than either CF or neutralizing antibodies and the immunological overlap may prevent an accurate interpretation of the results of a serological survey if only this test is used.

Confusing HI antibody patterns may arise for the same reason as they do with CF antibody tests (see above). The test may be particularly useful in surveys with several antigens of a group in previously unexplored or incompletely studied areas where the pattern of results may indicate the specific infecting virus.

Because of the large number of viruses in some of the different groups and the requirement of large numbers of suckling mice for the neutralization test this has only been used in special laboratories. When tissue culture neutralization tests can be used in place of mice more widespread application of the test may be possible.

POLIOMYELITIS

The high rate of isolation of poliovirus from faeces of suspected patients by inoculation of tissue cultures, plus the widespread use of vaccine to reduce illness has obviated much of the need for the CF or other serological test although experience has shown that the CF test may be a useful tool to determine antibody response to attenuated live virus oral vaccine, particularly in primary infection of infants and children. Adults who may have had previous silent infection or inactivated virus vaccine and possess neutralizing antibodies may excrete virus and show a rise in neutralizing antibodies but little or no CF antibody. CF antibody may be detected for a short time after three or four doses of inactivated virus vaccine.

The most suitable antigen for detecting a specific rise in CF antibodies to poliomyelitis is a live virus antigen prepared from the fluid of tissue cultures infected with attenuated poliovirus. Heated antigens may give a broad response even in a serum collected in the acute stage of the illness and little or no rise in antibody may be detectable in convalescence, both sera being positive. Thus one cannot even say whether the patient's illness is due to poliomyelitis. Antibodies usually appear about the 14th day of illness in a primary infection but a

rise may not be detected until 3 weeks after onset. In patients who have had a previous exposure to one of the polioviruses CF antibody may appear about the 7th to 10th day after the onset of illness.

On the whole the test appears to be specific although there have been occasional reports of rise in antibody to this antigen when a coxsackie- or echovirus has been isolated and considered to be the aetiological agent responsible for the patient's illness. Double or rapidly successive infections may of course occur.

The possibility of the application of a new principle in virus serology arose in connection with the flocculation test for poliomyelitis (Smith *et al* 1956). In CF and neutralization tests it has always been agreed that one must be able to demonstrate a four-fold or greater rise in antibodies for a result to be significant (positive) in relation to the present illness because of the persistence of these antibodies for many months or years after the occurrence of infection, which may have been symptomless. Smith and his colleagues (1959) found that antibody to flocculating antigen appeared very early in the illness and then rapidly disappeared so that a positive result, with a maximum titre which might already be present when the patient is first seen (7th to 10th day) would likely be of significance as a 'presumptive positive'. It seemed possible that this test would be of considerable practical value as the inactivated virus CF antigens available at that time often failed to give a result that could be readily interpreted—however, other developments in prevention and diagnosis of poliovirus infection resulted in a diminished interest in the practical value of this test. In special cases neutralization tests in tissue culture may be carried out but this procedure should be reserved for investigation of the immune state and not requested as a diagnostic procedure by physicians.

Coxsackieviruses

These viruses, of which there are twenty-three in group A, and six in group B, are characterized by their pathogenicity for newborn mice. Unfortunately complement fixation has not been a very useful test in the serological diagnosis of these infections in man because of the heterotypic response when mouse tissue or tissue culture fluid are used as antigen. An anamnestic response is seen when second and subsequent infections occur with different types so that a rise in titre may occur in convalescence to more than one type, and may be greater to a heterologous virus. Some workers have reported cross-reactions with polio virus CF antigen and some echovirus antigens in sera of patients with infections with coxsackieviruses. A satisfactory CF test is not in use for diagnosis in human infections.

Complement fixation has, however, been used effectively as a method of typing freshly isolated coxsackieviruses, particularly of group A, using hyperimmune mouse serum or ascitic fluid, or guinea-pig serum and mouse torso as antigen.

Haemagglutinins have been found associated with a few coxsackieviruses but routine HI tests are not of practical value at present.

It is obvious from the above that the neutralization test is the only reliable specific serological test with these viruses except for a few types. The large number of virus types and the fact that large numbers of litters of newborn mice would be required for tests with many members of the A group, rule out this test as a routine procedure on sporadic cases of undifferentiated illness such as aseptic meningitis where a coxsackievirus may be suspected to be the aetiological agent. However, in outbreaks of disease, such as herpangina, pleurodynia (Bornholm disease), myocarditis and pericarditis where only a few types of a particular group may be suspected, neutralization tests on selected sera may be possible.

ECHOVIRUSES

Viruses are usually classed in this heterogeneous group at present if they grow only in primary human or other mammalian tissue cultures and do not infect newborn mice or other small laboratory animals but some may produce mild illness in primates and some strains of echo 9 cause myositis in newborn mice. There are about thirty types at present. There has been a general failure to produce monospecific CF antigens and patients infected with an echovirus have been reported to have developed four-fold rises in antibody against poliovirus when live poliovirus antigen was used. Thus the CF test is of limited value in diagnosis.

Haemagglutinins for human O cells have been found associated with some strains of some echoviruses (types 3, 6, 7, 11, 12, 13, 19, 20, 21, 24, 29) and once a particular type has been identified in an outbreak an HI test may be useful.

Because a very large number of echovirus types may cause syndromes such as aseptic meningitis, neutralization tests are only practical when a particular type has first been isolated from several patients. The same applies to other types of illness such as upper respiratory tract infection or diarrhoea.

REFERENCES

COONS A.H., CREECH H.J., JONES R.N. & BERLINER E. (1942) The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J. Immunol.* **45**, 159.

DUMBELL K.R. & NIZAMUDDIN M. (1959) An agar-gel precipitation test for the laboratory diagnosis of smallpox. *Lancet* **i**, 916.

FREI W. (1925) Eine neue Hartreaktion bei, 'Lymphogranuloma inguinale'. *Klin. Wschr.* **4**, 2148.

FULTON F. & DUMBELL K.R. (1949) The serological comparison of strains of influenza virus. *J. Gen. Microbiol.* **3**, 97.

GOLDWASSER R.A., KISSLING R.E., CARSKI T.R. & HOSTY T.S. (1959) Fluorescent antibody staining of rabies virus antigens in the salivary glands of rabid animals. *Bull. World Health Organization, Geneva.* **20**, 579.

HIRST G.K. (1941) Agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science* **94,** 22.

JENSEN K.E. & FRANCIS T. (1953) Antigen-antibody precipitates in solid medium with influenza virus. *J. Immunol.* **70,** 321.

JOBLING J.W. (1906) The occurrence of specific immunity principles in the blood of vaccinated calves. *J. exp. Med.* **8,** 707.

LIU, C. (1956) Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein-labelled antibody. *Proc. Soc. exp. Biol. (N.Y.)* **92,** 883.

MCCLELLAND L. & HARE R. (1941) The adsorption of influenza virus by red cells and a new *in vitro* method of measuring antibodies for influenza virus. *Canad. J. Publ. Hlth.* **32,** 530.

OUCHTERLONY O. (1948) *In vitro* method for testing the toxin producing capacity of diphtheria bacilli. *Acta path. microbiol. Scand.* **25,** 186.

RICE C.E. (1948) The inhibitory effects of certain avian and mammalian antisera in specific complement fixation systems. *J. Immunol.* **59,** 365.

SMITH W., BOISSARD G.P.B., CHURCHER G.M. & PARKER L. (1959) Poliomyelitis virus flocculation reaction. *Lancet* **ii,** 586.

SMITH W., SHEFFIELD F.W., LEE L.H. & CHURCHER G. (1956) A specific virus antibody flocculation reaction with poliomyelitis virus. *Lancet* **i,** 710.

TERZIN A.L. (1958) The complement-fixation-inhibition tests as a serological tool in the virus laboratory. *J. Hyg. Epidem. Microbiol. and Immunol.* **2,** 36.

VOGEL J. & SHELOKOV A. (1957) Adsorption-haemagglutination test for influenza virus in monkey kidney tissue culture. *Science* **126,** 358.

CHAPTER 5

THE DIAGNOSIS OF PROTOZOAL DISEASES

J.D.FULTON

INTRODUCTION

The medical importance of Protozoan parasites to man has long been recognized on account of the manifold deaths and suffering which they have caused. The extent of the morbidity and economic losses for which they have been responsible in domestic stock is equally alarming. Surveys have shown that a cosmopolitan disease like amoebiasis has an incidence of carriers in this country and North America of approximately 10–20% of the population and may reach a figure of 50% in those countries with a lower standard of hygiene. The economic importance of trypanosomiasis has been recognized since 1880 when it was shown that the disease of horses and camels known as surra was due to *Trypanosoma evansi*. It has been pointed out that trypanosomiasis is a unique disease in so far as it has, alone, denied vast areas of land in Africa to all domestic animals other than poultry. Near the turn of the century *T. gambiense* was incriminated as the cause of sleeping sickness. A few years later *T. rhodesiense* was shown to be the cause of a similar but more acute disease. Ravages of human trypanosomiasis have been brought about by these two parasites in East and West Africa for centuries as well as by *T. cruzi* in South America. Visceral leishmaniasis (kala-azar) which occurs in all continents with the exception of Australia, still presents a serious problem in India and other countries where untold suffering and death has been the fate of millions. The cutaneous and mucocutaneous form

of this disease has been responsible for much human deformity in the Old and New World. Malaria has had a strong influence on man's destiny throughout the ages. In spite of the success of many eradication schemes it has recently been estimated that two or three hundred million attacks of malaria are suffered each year with resultant two million deaths in the same period. The economic losses from the ill-health which follows in afflicted countries are incalculable and progress towards a better standard of living thereby stifled. Piroplasmosis comprises a group of diseases of great economic importance which affects a wide range of wild and domesticated animals. One of the best known among them is East Coast fever which has existed for centuries in Africa, and is the cause of a high mortality among bovines. Until recently man was considered immune to infection by *Babesia* but recent work has indicated that this is not the case. *Toxoplasma* was first described in 1908 as a cause of death of rabbits in Brazil. Interest in this parasite remained largely zoological until its association with human disease was discovered in 1939. Two reference laboratories have now been set up in this country for its detection, thus indicating the interest of the Public Health Authorities in this infection. Coccidiosis has also proved of great economic importance and the cause of serious losses among domestic stock. The remaining protozoal diseases dealt with in this chapter are, relatively, of minor importance. However, as a group they have impoverished the world to such an extent that their recognition and control is now a matter of some urgency, with complete eradication as the final goal.

At the start of the century immunological methods proved helpful in the classification of trypanosomes, at the time when many new species were being discovered. Probably the main aim of the immunologist has been to discover the means of preventing disease and early work in the field of parasitic diseases was largely concerned with the development of protective immunity as the result of previous infection. Basic phenomena in the process of immunity are now being intensively studied. The publications of Taliaferro (1930) and of Culbertson (1941) gave a stimulus to studies of this type, and to the application of immunological methods to diagnosis. The limited progress made may be a reflection of the fact that some protozoa such as the commensal forms of *Entamoeba histolytica*, which do not invade tissues but live in the lumen of the gut, give rise to a poor antibody response. A similar poor response has frequently been observed when killed protozoal parasites are used as antigens. Moreover protozoa are often difficult to culture axenically and antigenic material is correspondingly hard to obtain in quantity. In general it can be said that clinicians are averse to diagnosis of parasitic infections by immunological methods and prefer the direct method of approach by microscopical examination. The use of immunological methods has on occasion been helpful indirectly in aiding the completion of life cycles of certain parasites. Thus, by accepting the fact that different developmental stages in a parasite have similar antigenic constitution, it was shown that

Herpetomonas papatasii is synonymous with *Leishmania tropica* and proved at the same time that the sandfly is a natural carrier of cutaneous leishmaniasis.

Classification

The phylum Protozoa comprises a varied collection of single-celled organisms which are somewhat difficult to classify on account of their varied life cycles. It was customary in the past to divide Protozoa into four classes which contain parasites important to man. This classification, although unsatisfactory in some respects, has for simplicity been retained.

Phylum Protozoa

Class I	*Rhizopoda* Amoebiasis
Class II	*Mastigophora* (Flagellata) A. African trypanosomiasis (sleeping sickness, nagana) B. South American trypanosomiasis (Chagas's disease) C. Leishmaniasis i. Visceral (kala-azar) ii. Cutaneous and mucocutaneous D. Trichomoniasis (vaginitis)
Class III	*Sporozoa* Malaria Babesiasis (piroplasmosis) Toxoplasmosis Sarcosporidiosis Coccidiosis
Class IV	*Ciliata* Balantidiasis

RHIZOPODA

Amoebiasis

In the class Rhizopoda the only parasite pathogenic to man is *Entamoeba histolytica*, normally a parasite of large intestine or lower ileum. The life cycle involves a trophozoite and cystic stage. It may complete this cycle in the lumen of the gut as a commensal or invade tissues with resultant ulcer formation and dysentery. The parasite lends itself to study in laboratory animals such as rhesus monkeys or rodents. Besides the normal gut infection in man parasites may be found in skin, liver, spleen, lung, brain and other tissues.

It was Lösch in St Petersburg who first demonstrated the pathogenic nature of this amoeba, later named *E. histolytica*. Dobell (1919) in his monograph on

The Amoebae Living in Man did much to clarify the existing confusion and Craig discussed the epidemiology, pathology and clinical features of the disease in *Amoebiasis and Amoebic Dysentery* and also Deschiens (1965) in *L'Amibiase et L'Amibe Dysentérique*. It is one of cosmopolitan character with a higher incidence and more severe clinical features in tropical and subtropical countries and spread is favoured by unhygienic conditions.

Until recently serological diagnosis has not been widely employed and microscopical detection of the parasite was generally attempted but may prove difficult. The development of immuno-diagnostic methods therefore appears desirable.

Complement Fixation

The method was first employed by Izar (1914) but his antigens were very impure and gave rise to conflicting results in human patients and experimentally infected cats. Craig (1929) indicated the practical results which could be obtained by this method, which has subsequently been employed by a number of other workers, e.g. Rees *et al* (1942), whose antigen consisted of saline extracts of the parasite cultured with a single species of bacterium. The method of carrying out the test was outlined by Bozicevich *et al* (1946) and it proved of considerable value in detecting extra intestinal forms of the disease. Fulton *et al* (1951) prepared a similar antigen grown with a single bacterium and also one from *E. coli* as well as the corresponding antisera. It was found that cross reactions occurred to a limited extent between these two amoeba the titre being higher in the homologous reaction as later verified by Goldman (1954) who employed fluorescent antibody techniques. Antigenic strain differences appear to exist in *E. histolytica* and the use of a polyvalent antigen has been suggested by CF tests. Since the antibody titre in this test decreases rapidly after successful treatment it seems that the complement fixation test might be useful in prognosis as well as diagnosis. The specificity of the test has however been seriously questioned and low titres are generally obtained in absence of tissue invasion. False negatives and false positive results are encountered. The test at present is regarded as of supplemental value only to other direct methods of diagnosis. The use of purified antigen is most desirable.

Precipitin Test

Wagener (1924) used this test in studying experimental amoebic dysentery of cats. After numerous passages of the organism in these animals antiserum was obtained from a moribund animal. Antigen consisted of extracts in Coca's fluid of scrapings from ulcerated bowel. A strongly positive precipitin reaction was obtained during acute infections. Moan (1957) devised a precipitin test in which a water-clear antigen was prepared from extracts of *E. histolytica* grown in culture with bacteria from *E. terrapinae* and human bacteria were absent.

Inactivated sera were used and specific results appeared to be obtained, while false positive results were virtually absent and false negative were few. The test was however not positive in early asymptomatic cases of amoebiasis but only at the dysenteric stage. The test became negative after successful treatment. It is a simple and rapid procedure for detection of significant clinical infection but results are not uniform and further elaboration and more experience with it is required before final assessment of its value. The precipitin reaction has also been studied by means of double diffusion in agar gel by Siddiqui (1961) and Atchley *et al* (1963) as well as by Powell *et al* (1965) and Maddison (1965) who also used immunoelectrophoretic techniques. The antigen was prepared from *E. histolytica* grown with a single bacterium and broken up by ultrasonic treatment, using that from the bacterium alone as control. Some fractionation of the antigen was also attempted. Antibodies against specific amoebic components were detected in 96% of cases of proved invasive amoebiasis, a smaller percentage in cyst passers and relatively few in other diseases. Anti-amoebic precipitins were present after treatment as Kessel *et al* (1965) had found and appeared to play no part in protective immunity. Their presence was regarded as of diagnostic and epidemiological significance. Negative results were indicative of absence of tissue invasion by *E. histolytica*. Maddison *et al* (1965a) made a comparison of haemagglutinins and precipitins in amoebiasis. A high percentage of antibodies was detected by both methods in invasive amoebiasis. Absorption of haemagglutinins without affecting the precipitin pattern indicated that more than one type of antibody was involved. The simplicity of the precipitin test should be of value in routine work. Maddison *et al* (1965b) point out that serology provides a more objective approach than faecal examination to indicate the significance of *E. histolytica* as an agent of disease. The presence of antibodies in reinfected or relapsed patients indicate that they are non-protective in character.

Immobilization Test

This type of test was first described by Cole & Kent (1953) who were able to immobilize *E. histolytica* trophozoites by immune sera prepared in rabbits. By similar means Zaman (1960) showed that a number of amoebae including *E. coli* were antigenically distinct from *E. histolytica*. Invasive forms of the latter could be distinguished from those which were non-invasive. The test is performed by incubating inactivated test sera with a 24-hr culture of the organism and recording the percentage immobilized. A value greater than 70% indicates a positive result. The test gives results comparable with those obtained in the CF test and is regarded as more sensitive. Biagi and colleagues (1961, 1966) obtained 84% of positives in confirmed cases of amoebiasis and about 18% of false positives. The test is generally regarded as being complex and because of its subjective nature is not likely to find wide application. Remobilization of affected parasites was studied by immunofluorescent techniques.

Agglutination Tests

Earlier workers had used the agglutination reaction for free-living amoebae. Greif (1947) used the method to show the presence of agglutinins in the sera of *E. histolytica* carriers, using cultured cysts of this organism. There appeared to be no correlation between agglutination and infection or with the results of the CF test.

Kessel *et al* (1965) have described an indirect haemagglutination test which shows promise of wide acceptance in diagnosis of *E. histolytica* infection. The antigen is prepared as an extract from washed cultured trophozoites and is used to sensitize tanned human type O erythrocytes. The test lends itself to quantitative interpretation and is regarded by the authors as sensitive and reasonably specific. Complement fixation reactions were carried out with the same antigen concurrently and the results for the two methods agreed very well. The antigen was prepared from *E. histolytica* grown with *T. cruzi* or *Mycoplasma* and the axenic method of culture of this organism described by Diamond (1961) should prove very useful in work of this nature for which pure antigens are required. In cases of liver abscess 100% positives were obtained, 98% in amoebic dysentery and 66% in asymptomatic carriers. Raised titres persisted in the HA test after treatment as in toxoplasmosis, but fell more rapidly in the CF test. The use of both tests concurrently has proved of great value to the clinician. Kagan (1965) in evaluating routine serological tests for the American Public Health Service for diagnosis of parasitic infection is concentrating on the HA test which he found reliable and reproducible and gave rise to high titres. The test requires further evaluation in chronic amoebic colitis and in the case of vague abdominal symptoms.

Skin Tests

A test of this nature has attractions in survey work but it is essential that pure antigens be used. Leal (1954) described an intradermal reaction in amoebiasis. Antigens were prepared as saline extracts from *E. histolytica* and the free-living *E. moshkovskii* and 0.1 ml of the Seitzed material was given intradermally and results were read 24 hr later. There was positive correlation between microscopical results and those obtained in this test. Somewhat similar results were reported by Kum (1958) but his antigens were very impure.

Fluorescent Antibody Tests

The early tests of Goldman (1953, 1954) provided a promising field for the detection and recognition of *E. histolytica* by immunological methods. He used fluorescein-tagged antibody for staining the above organism and also the non-pathogenic *E. coli*, with which it is not infrequently confused during faecal examinations. There was some cross reaction between them but the homologous anti-serum gave rise to the most intense staining. Goldman (1960), Goldman *et*

al (1960, 1962) carried out an antigenic analysis of *E. histolytica* by the same means. Jeanes (1964, 1966) also employed the indirect FA test successfully in the sero-diagnosis of hepatic amoebiasis. Toussaint & Anderson (1965) have used the fluorescent antibody technique with soluble antigen for the sero-diagnosis of infectious disease, by employing impregnated discs.

MASTIGOPHORA (FLAGELLATA)

Trypanosomiasis

There are three important diseases of man caused by parasites of the genus *Trypanosoma*, namely African sleeping sickness due to *T. gambiense* and *T. rhodesiense* which occur predominantly in West and East Africa respectively, the causative agents of which cannot be distinguished morphologically. The third known as Chagas's disease is caused by *T. cruzi* and occurs in South and Central America. The clinical pictures presented by infection with these parasites are characteristic. There is also an economically important disease of equines, of cosmopolitan nature, known as dourine which is caused by *T. equiperdum* and is spread during coitus by the infected male. The presence of protective antibodies in the blood of animals recovered from trypanosome infections has been recognized since the beginning of the century. Strain differences, however, exist and the behaviour to immune serum has at times made the results of cross-immunity experiments difficult to interpret.

African Sleeping Sickness

Complement Fixation

Although it had been shown early in this century that the complement-fixation test could be used to detect trypanosomiasis of man and animals the test lacked specificity because of the poor quality of the antigen which also did not keep well as a rule. Van Goidsenhoven & Schoenaers (1944) prepared one from blood of animals heavily infected with *Trypanosoma equiperdum* which they froze, distributed to ampoules and dried in vacuo at 75°C and the ampoules were sealed. Antigenic potency was unimpaired over a period of many months, and gave rise to a group reaction by which infection with a number of other trypanosomes could be detected. Rodhain *et al* (1941, 1944) used a similar antigen and found complement fixing antibodies in the blood and CSF of sleeping sickness patients. The reaction was positive after apparent cure. Schoenaers *et al* (1953) used the method widely in the diagnosis of Gambian sleeping sickness. In one series of 125 proven cases the sera of 95% were positive and negative results were obtained with 65 control patients. Evens *et al* (1953) continued the investigation and found it superior to any other in diagnosis, and it became negative on cure. Pautrizel *et al* (1959, 1960) have confirmed these results and improved the preparation of the antigen. Depoux *et al* (1956) also found the reaction was

specific and was of great service in diagnosis and can be used also in the detection of *T. rhodesiense*.

The test is also used for diagnosis of dourine in horses, and thus lacks specificity but is of value in group diagnosis. While it can be used to detect clinical cure the titres obtained do not correlate well with the clinical condition. The test can be used to detect infection when, as the result of pentamidine prophylaxis, trypanosomes cannot be found.

Agglutination Test

The power of immune serum to agglutinate trypanosomes was recognized early in this century and the reaction is non-specific. An investigation of this phenomenon by Soltys (1957) was made using live African trypanosomes of the *T. brucei* group. Sera were obtained from infected human patients or animals and from the latter after vaccination with dead parasites. It allowed the study of antigenic relationships of strains of the parasite. Titres were maximal after 2 weeks and persisted for life in the animals or for 3 months after drug treatment. Species specificity was observed and sera from *T. brucei* infections did not agglutinate the other morphologically indistinguishable members of the group. It was suggested that *T. brucei* and *T. rhodesiense* could be distinguished in this way which is otherwise only possible by tests in man. The method may also be used for diagnosis of low-grade infections. Cunningham & Vickerman (1961, 1962) have applied the method to an antigenic analysis of a number of strains of the *T. brucei* group. As antigen they used whole blood of heavily infected rodents. Agglutinins were detectable within a few days after infection. At least six antigens were present but if a clone was used for infection only a single antigen was detectable. The method was applied to detection of agglutinins in finger-prick blood which had been dried on filter paper and extracted. Strains from man and animals had antigens in common. Pautrizel *et al* (1962a) have studied this reaction in animals and in sleeping sickness patients. They believe the test is specific and reproducible provided a satisfactory antigen is used. In rabbits agglutination results indicated strain specificity.

Precipitin Test

The earliest results of this test were obtained in the trypanosome infection of horses known as dourine. By the method of double diffusion in agar gel Gray (1961) showed that the precipitative antibodies were present in the serum of animals immunized by infection or with dead *T. vivax*, *T. brucei* and *T. gambiense* trypanosomes. An antigen for the *Congolense* group of trypanosomes has not yet been obtained. Cross reactions occurred with the different trypanosomes and there is a common group antigen. In a few human patients with *T. gambiense* infection antibodies were also present. Detection of antigenic differences in trypanosomes morphologically similar may now be possible by this method.

Brown & Williamson (1962) have shown that in the *T. brucei* group at least three precipitinogens are present.

Adhesion Test

Rieckenberg (1917) described a new immunity test in experimental trypanosomiasis. When the blood of animals cured of infection by these parasites is mixed with citrated bouillon and the parent trypanosomes are added, they become laden with attached blood platelets in a few minutes. This reaction was obtained with five different strains and proved highly specific, so that a parent strain of trypanosomes could be distinguished. The phenomenon was extensively investigated by Duke & Wallace (1930) and by Wallace & Wormall (1931) mainly in monkeys with trypanosomes recently isolated from man and animals. The test which is closely related to that above was named 'The Red Cell Adhesion Test'. To carry out the test primate red cells, serum from suspected infection containing adhesin—believed to be of antibody nature—produced by trypanosomes, parasites the same as or related to those causing infection and a complement-like substance in the serum of normal animals, were required. When the test was positive red cells adhered to the trypanosomes. The authors believe that the phenomenon they observed is essentially the same as that of the original author. Results were variable however even when conditions were standardized, and proved group, but not species specific in character. The test has been used in epidemiological studies of wild animals and in the diagnosis of human disease, but is no longer employed.

Immuno-Conglutinin Levels

It has been suggested that a serological method to indicate the disappearance of trypanosomes from the blood would be of value. A long series of experiments conducted by Coombs and colleagues on immuno-conglutinin in the sera of man and animals has shown that after disappearance of the antigenic stimulation which gives rise to it there is a fall in level. Infection with *T. brucei* caused a notable rise in level and Ingram and Soltys (1960) made use of this fact to study the variation in levels in the sera of animals and find how it was affected by the use of chemotherapeutic agents. The substance appears in serum of rabbits within 7 days after infection and reaches a peak in 30 days. Therapeutic agents caused a drop in amount to the usual level when normal parasites were present but if drug-resistant parasites were used a fall did not occur. The authors suggest that the method could be used to indicate the progress of treatment. Pautrizel *et al* (1962b) applied the conglutination reaction to the serological diagnosis of trypanosomiasis in both human and animal infections. Good correlation was obtained with CF and agglutination techniques and the method is suggested for the diagnosis of human infection and for studying therapeutic activity of drugs but appears to have been very little used.

Fluorescent Antibody Test

Williams *et al* (1963) have used the fluorescent antibody test (FA) in animals infected with *T. rhodesiense* and *T. gambiense* for diagnostic purposes. The antibodies appear 2 weeks after infection and give rise to cross-reactions with other trypanosomes. Since there is a need for rapid and reliable method of diagnosis in the non-acute stage of trypanosome infection in man Sadun *et al* (1963) have employed the method in African sleeping sickness and in South American trypanosomiasis. Again cross reactions were met with in *T. gambiense*, *T. rhodesiense* and *T. cruzi* infections but not in other diseases. It was possible to use finger-prick blood dried on blotting paper and then extracted for the test. The value of the method is still *sub judice*.

Lucasse (1964) was the first person to apply this test to the CSF to indicate the degree of involvement of the CNS in sleeping sickness of man. The author noted that although antibodies are present in serum they may be absent in the CSF. All CSF specimens with pathogenic changes such as increase of white cells or alterations in protein showed the presence of antibodies. The method was sensitive and specific and of value for early indication of CNS involvement and for the progress of the disease. The maximum titre was 1/160 and titres correlated well with the pathological features of the CSF. Mattern *et al* (1965) used the test and considered it was an excellent method of diagnosing trypanosomiasis of the CNS.

Other Tests

Desowitz (1956) used the inhibition of oxygen uptake by *T. vivax* as a test for the quantitative estimation of antibodies in the serum of cattle infected with this organism. Lysis also occurred. Thurston (1958) showed that with *T. brucei* only a concentration of antiserum which rendered the parasites non-mobile and produced lysis was able to cause reduction in oxygen uptake.

Gill (1964) described an indirect haemagglutination test for the diagnosis of *T. evansi* infections. Very high dilutions of anti-*T. evansi* antibody were detected. The test is claimed to be sensitive and specific and easily performed and gives reproducible results but has had only a very limited trial.

A non-specific but promising method described by Mattern *et al* (1961, 1964, 1965), Nicoli *et al* (1961) employs electrophoresis. They found that in patients with *T. gambiense* infection there is a notable increase in the level of the IgM (β_2-macroglobulin) class of immunoglobulins in serum and CSF. The amount present may be sixteen times that in normal individuals and occurs in maximal amount 15 days after infection. Increases of this order are not pathognomic but occur so constantly in sleeping sickness that absence almost excludes the disease. This is accompanied by rise in IgM of the CSF even when cell and protein content is normal. IgM levels of serum fall rapidly when specific drug therapy is given in absence of CNS involvement but may still be raised in

CSF in chronic treated cases. These findings are of basic immunological importance. Quantitative examination of this class of immunoglobulin in serum and CSF could serve for the diagnosis of human cases of infection. An investigation has been carried out (Lumsden 1966) on various aspects of this interesting subject including that of a suitable means of measuring IgM. It confirmed the findings of Mattern in infections with *T. gambiense* and *T. rhodesiense*. Estimation of increases in the other immunoglobulin class IgG or IgA did not appear to offer diagnostic possibilities. Increases in IgM may occur in other diseases such as tuberculosis, leprosy, syphilis, kala azar and some forms of malaria. The methods described for IgM estimation apply to animals including bovines and other domestic types as well as to wild animals.

Chagas' Disease

This is an acute, subacute or chronic condition caused by infection with *Trypanosoma cruzi*, and occurs principally in South and Central America. A very small number of similar infections have been reported from the southern States of the U.S.A. In chronic cases trypanosomes are very hard to find and diagnosis has been aided or made possible by a number of serological tests.

Complement Fixation

The CF test in this disease first described in 1913 is known as the Machado reaction. The antigen was very impure and consisted of an aqueous extract of the spleens of heavily infected puppies. Kelser (1936) used an antigen prepared from cultures of the parasite. That of Davis (1943) was prepared from similar material and is of good quality so that it has found wide acceptance and in the hands of Muniz & de Freitas (1944) has proved very useful in diagnosis of the chronic disease. Further improvements in the use of the antigen were made by de Freitas & de Almeida (1949) and by Chaffee *et al* (1956). In established cases of the disease 98% of patients showed the presence of antibodies, while the number of positive reactions in South American leishmaniasis was low and absent in other diseases. Montaño & Nerós (1965) compared the value of the haemagglutination test and CF test in the serological diagnosis of the disease. The former was regarded as more sensitive and detected antibodies earlier in the disease, but has not been widely used.

Agglutination Test

Micro-agglutination tests had been described in 1940 and 1943 with antigen from culture forms of *T. cruzi* and immune serum from laboratory animals. Muniz & de Freitas (1944) compared the results in this test and those of complement fixation. Although there was absence of correlation in the tests they considered the agglutination test of value. A haemagglutination in which a polysaccharide antigen extracted from *T. cruzi* was used to sensitize red cells

gave satisfactory results in the hands of Muniz (1950). Attention has also been drawn above to the results of Montaño & Nerós (1965).

Precipitin Tests

Muniz (1947) described this reaction for diagnosis of acute and subacute forms of Chagas' disease, and prepared a stable polysaccharide antigen from culture forms of *T. cruzi* for this purpose. The test could be rapidly carried out with 0.6 ml serum and results read in a matter of minutes. It proved of value in diagnosis during the early stages of the disease and there was 100% correlation with the presence of trypanosomes detected microscopically. The test appeared to be specific and unlike the CF test did not give rise to cross reactions in patients with leishmaniasis. These findings have been confirmed by Pellegrino & Brener (1952) and by Pellegrino, Brener & Jacomo (1956). They have emphasized the value of the test in screening suspected cases.

Intradermal Test

Washed cultures of *T. cruzi* suspended in dilute phenol in saline were used as antigen, by Mayer & Pifano (1941). The reaction gave rise to inflammation and swelling at site of injection and was maximal in 48 hr. Rapid diagnosis of infection was possible and there were no cross reactions with leishmanial infections. Amato *et al* (1964) used a tissue culture antigen of *T. cruzi* in this test but results were unsatisfactory.

Conditioned Haemolysis

Muniz & Santos (1950) described this test (passive haemolysis test—see Chapter 1) as a diagnostic aid in order to avoid pitfalls inherent in the use of a non-specific *T. cruzi* antigen. Sera for test were first absorbed with guinea-pig kidney and then with normal sheep cells. Guinea-pig complement was absorbed with the cells used for sensitization with a polysaccharide obtained from *T. cruzi*. After incubation at 37°C for 1–2 hr of serum, sensitized red cells and complement, readings were made. Specificity of the reaction is determined by the absorbed antigen and not by the cells. The results compared favourably with those obtained in haemagglutination and complement fixation reactions. The test is not generally used but the authors believe that it would be greatly simplified by use of a pure antigen, which enabled absorption to be dispensed with.

Fluorescent Antibody Test

The indirect test was first used by Fife & Muschel (1959) for diagnosis of *T. cruzi* infection, with washed formalized parasites from culture as antigen. The method proved somewhat more sensitive than the CF test but gave rise to some false positive results. Sadun *et al* (1963) used this test for diagnosis of

African and American trypanosomiasis in man. Cross reactions were encountered in the trypanosome infections but not with other diseases. Finger-prick blood dried on filter paper could be used after extraction but results are still *sub judice*. Essenfeld & Fennel (1964) made a study of the reaction in experimental infections with *T. cruzi* by infecting guinea-pigs with five different strains, and using blood and tissue forms as antigen. Direct and indirect staining methods were employed with moderate results. Biagi *et al* (1964) compared the usefulness of the test with the CF test in the chronic disease. Leishmanial forms of the parasite were used as antigen. There was a good agreement in both tests and the ease of performance as well as economy of the FA test favour its use in diagnosis of the chronic disease.

Leishmaniasis

Leishmania infections occur in two forms, visceral (kala-azar) and cutaneous or mucocutaneous. It is convenient to recognize only two main species of parasite, namely *Leishmania donovani* giving rise to the generalized disease (kala-azar) and *L. tropica* to the other forms. Different species of these parasites are, however, now recognized on the basis of their varied clinical and immunological behaviour. They are shown in the classification table below. All forms of the parasite are morphologically identical.

Classification of Leishmaniases

1. Visceral	*L. donovani* (India, China, etc.)
	L. infantum (Mediterranean littoral)
2. Cutaneous and Mucocutaneous	
Old World	*L. tropica major* (wet form, rural)
	L. tropica minor (dry form, urban)
New World	*L. brasiliensis* (Espundia—Brazil)
	L. pifanoi (Venezuela)
	L. mexicana (Chiclero's ulcer—Guatemala, Mexico, British Honduras)
	L. (peruviana) (Uta, pian bois—Peru, Guianas, Costa Rica, Panama)

Agglutination Test

The first clear-cut demonstration of the presence of agglutinins was made in 1911 in the sera of rabbits immunized with cultures of *L. donovani*. Similar bodies were later demonstrated in the serum of a child with leishmanial infection. Numerous workers confirmed the earlier observations. Senekjie & Lewis (1944) were able to diagnose leishmanial infection by a slide agglutination test using the sera of rabbits immunized first with dead then with live organisms

of *L. donovani*, *L. tropica* and *L. braziliensis*. They found that the homologous organisms were agglutinated at a higher titre than heterologous types. A group reaction occurs in low dilutions but at higher dilutions the various species may be differentiated. It appeared that a rapid diagnosis of visceral, cutaneous and mucocutaneous leishmaniasis could be made by this method. Chang & Negherbon (1947) in studying the specificity of serological reactions with the above three organisms found that the micro-slide agglutination reaction was the most useful. There is still no general agreement about the specificity of the test and it seems that discrepancies in results of different workers may be due to variation of techniques or differences in the nature of the antigens employed. Cascio *et al* (1962) used a 'conditioned haemagglutination' test for detection of agglutinins with antigen absorbed on tanned sheep red cells. Bray & Lainson (1966) have developed a haemagglutination test for detection of antibodies in which sheep and human cells or latex particles were sensitized with antigens from eight strains of *Leishmania* spp. They found that serological differences occurred between the strains as for example *L. donovani* from India and Kenya, as well as many similarities.

Complement Fixation Test

This test was also first used in 1911 on the sera of immunized rabbits. The method proved somewhat unsatisfactory in the hands of other workers till an antigen prepared from human tubercle bacilli, the well-known antigen of Witebsky, Klingenstein & Kuhn, was introduced and gave satisfactory results in the hands of Greval, Sen Gupta & Napier (1939). Later the so-called leprosy-bacillus of Kedrowsky was employed as a source of antigen in this test and gave equally good results. Other acid-fast organisms such as *Mycobacterium phlei*, *M. avium* and *M. butyricum* have also been used as antigens after treatment in different ways. These antigens have proved stable and generally lack anti-complementary activity and in some cases are of such specificity that other diseases can be excluded by the test. Alcohol extraction of Kedrowsky's bacillus provided a simplified method of antigen preparation. Ghosh, Ghosh & Ray (1945) reported good results with a specific antigen prepared from *Leishmania donovani* cultures. The test was positive in early cases of kala-azar when other serological tests were still negative. Chang & Negherbon (1947) also used specific antigens prepared from leishmania species with which they showed that species specific and common antigens were present, the former being predominant. The general consensus of opinion among responsible workers is that the complement-fixation test is the most reliable of all in leishmaniasis. Recently Torrealba & Chaves-Torrealba (1964) used BCG as a source of antigen for diagnosis of human and canine leishmaniasis. The method proved sensitive and because of its ready availability had advantages over other antigens. Khaleque (1965) described a new and simpler method for preparation of antigen from

Kedrowsky's bacillus. The author claims that it showed marked specificity and a close correlation of results with clinical findings was observed.

A further test of this nature has been described in which leishmanial antigen is detected in the host's serum with the help of antibody from a rabbit hyper-immunized with cultures of *L. donovani*.

Precipitin Test

In serological studies of parasitic infections the precipitation reaction has probably been least used. Such a test was described for leishmaniasis in 1913. In evaluating the specificity of serological reactions for distinguishing the various leishmania species Chang & Negherbon (1947) successfully employed a ring test for the presence of precipitins but found it less sensitive than the agglutination test.

Intradermal Test

Because of its value in the field this test has been widely used and some workers claim that it is more reliable than microscopic examination for parasites. Differences in the degree of reaction have been noted in old and more recent infections. The first careful study of this nature was made by Wagener (1923) who reported results of an intradermal test in immune rabbits when she used as antigen extracts of the culture forms of *L. donovani* and *L. tropica*. Other workers used dogs and the test was then applied to man with even more definite results. It remained for Montenegro (1926) to show the diagnostic possibilities of this test in human mucocutaneous leishmaniasis, in Brazil. With an antigen prepared from cultures of *L. braziliensis* he obtained a delayed type of reaction and found that an extract of *L. tropica* also gave a positive result in a Brazilian patient, thus showing that common antigens are present in the two organisms concerned. The test has been claimed as specific but some workers have reported cross reactions with *Trypanosoma* species. The test was widely adopted and the original findings repeatedly confirmed. Similar findings were recorded in *Leishmania tropica* infections and the test proved even more reliable than microscopical examination of lesions. The test has sometimes proved positive before the appearance of lesions and also for some years after cure. Various writers have discussed the specific allergic reactions in cutaneous leishmaniasis when the Montenegro test is performed and have suggested standardization of the antigen and of the time at which the results should be read, to give more consistent findings among different workers. Pellegrino (1951) used a polysaccharide antigen isolated from culture forms of *L. braziliensis* and obtained the same results as with a saline-merthiolate suspension of whole organisms. In Spain it has been found that Mediterranean types of cutaneous leishmaniasis can be diagnosed in nearly 100% of cases by an antigen prepared from *L. braziliensis*.

Manson-Bahr & Southgate (1964) in recent work on kala-azar in East Africa

have used the leishmanin skin test on a large scale, with antigen prepared from a gerbil strain of *Leishmania*. The test is negative during the active phase of the disease and becomes positive on recovery. The test proved of value to detect endemic foci in the Sudan. There was cross immunity between rodent and between rodent and human strains. Cahill (1965) has used this simple and effective test for determining attack rates and distribution of leishmaniasis and for delineating endemic foci.

Immunofluorescent Techniques

Oddo & Cascio (1963) used the fluorescent antibody test for demonstration of antibodies in cutaneous and visceral leishmaniasis. Shaw & Voller (1964) employed culture and tissue forms of different *Leishmania* species as slide antigens and found that all sera from infection with the different species as well as from two trypanosome infections stained, but there was no staining with sera from healthy individuals or from those with other diseases including cutaneous leishmaniasis. The test was group specific and is of limited value where trypanosomiasis and leishmaniasis are endemic. Duxbury & Sadun (1964) found that the test lacked sensitivity and some false positive results occurred. Herman (1965) used the intracellular forms of *Leishmania donovani* as antigen and obtained results similar to those above. More experience is necessary before the test is used routinely.

Adhesion Test

The Rieckenberg phenomenon or Adhesion Test employed in diagnosis of trypanosomiasis has met with limited success in diagnosis of leishmaniasis. Mills, MacHattie & Chadwick (1931) found that the test was easily carried out and proved of value in the field as an aid to diagnosis of chronic and aberrant types of oriental sore in endemic areas, when microscopic examination of old lesions was not easy to carry out. When the test was used to determine the incidence of cutaneous and visceral leishmaniasis in dogs it proved unsatisfactory.

Trichomoniasis

There are three species of the genus *Trichomonas* which infect man. *Trichomonas tenax* of the mouth, *T. hominis* of the intestine and *T. vaginalis* of the genital tract. They are distinguished morphologically, culturally and by absence of cross infection. Only *T. vaginalis* is of clinical importance and infections may be symptomless or associated with severe vaginitis. *T. gallinae* of birds and *T. foetus*, the cause of abortion in cattle, are definite pathogens. There have been relatively few serological studies on the human species of the organism. Robertson (1941) employed the first satisfactory serological test for *T. foetus*, that of agglutination. Trussel (1946) used a micro-agglutination test for *T. vaginalis* and showed that this organism stimulates the production of antibodies in

experimentally inoculated animals. Feinberg (1952) used a capillary agglutination test for *T. foetus* which could be read microscopically and might be of value in diagnosis of *T. vaginalis*. McEntegart (1956) made a serological comparison of a strain of *T. vaginalis* with two strains of the cattle parasite *T. foetus*, which are respectively of medical and veterinary interest. He observed that on the only two previous occasions on which this was done the results were contradictory. The author found that the human and cattle strains were serologically distinct by means of haemagglutination and cross absorption tests. It was shown by Weld & Kean (1958) that there is a factor in the serum of man and animals which destroys suspensions of *T. vaginalis* and can be removed by heating at 56°C for 30 min. The presence of such a factor makes serological studies difficult. Kott & Adler (1961) in their serological studies of *Trichomonas* sp. parasitic in man used agglutination and haemagglutination techniques. They found that a number of serotypes of *T. vaginalis* existed and could be distinguished by the above methods and by absorption tests. Robertson (1960) used agglutination and gel-diffusion methods to distinguish strains of *T. foetus* from one another and from porcine strains. Elder (1964) described a new serological strain of this organism. McEntegart *et al* (1958) used fluorescein-labelled antisera to detect serological varieties of *T. vaginalis* and found it could readily be distinguished from *T. foetus* by this method. The value of the test for routine diagnosis has not been established. Pierce (1947) observed that an agglutin in for *T. foetus* is present in the mucoid vaginal secretions of infected heifers apparently before it is present in the blood. An intradermal test was also positive before agglutination by sera.

So far as the writer is aware serological methods are not routinely employed in the diagnosis of human infections but rather those of culture and microscopic examination of suspected material. Korik (1966) in Moscow, however, describes two immunological methods for the diagnosis of trichomoniasis, namely the complement fixation test and detection of trichomonadantigen. The former gave only 10% of positive results in patients. The second method, in which three anti-trichomonad sera from man and immunized rabbits were used, gave 83% of positive results in patients known to be infected. The latter method is recommended by him for routine use.

SPOROZOA

Malaria

It is now well known that malaria infections confer some degree of immunity on the host following recovery from infection, and the search for specific antibodies has been carried out by many workers during attempts at elucidation of this immune mechanism. Some investigators are of the opinion that a good serological method of diagnosis would be of value in this disease. For a long

time the difficulty of obtaining a sufficient amount of pure antigen proved a drawback in research but was largely resolved when simian malarias could be regularly maintained in the laboratory. Complement fixation, precipitation and agglutination tests as well as staining with fluorescein-labelled antibody are now accepted as specific methods of diagnosis but their practical value is still in doubt.

Complement Fixation

Thomson (1918) and Kingsbury (1927) reported promising results with antigens prepared from infected human red cells and Manson-Bahr (1927) used extracts of oocysts from anopheline mosquitoes as well as those of culicines but without success. The test was put on a sound basis by Coggeshall & Eaton (1938) who studied the reaction in monkey malaria. From the blood or spleens of animals with intense infections of *Plasmodium knowlesi* antigens were prepared which fixed complement when allowed to react with the serum of the infected animal. The parasites were first freed from red cells by freezing and thawing or grinding, or by long autolysis and were then extracted with saline. Complement fixing antibodies appeared in the blood early during infection and persisted during chronic infection and the reaction was regarded as specific. After the acute phase there was a rise in titre then a fall, with further rises after relapses. The amount of protective antibody determined in separate experiments did not correlate with the titre obtained in the complement fixation test. The antigen appeared to be of protein nature since it was not extractable with lipid solvents and was destroyed by trypsin. These experiments were extended to studies on human malaria by Eaton & Coggeshall (1939) using the antigens prepared from *P. knowlesi*. Tests were made on normal human serum and syphilitic serum, on the serum of patients with naturally acquired malaria, and on that of paretics who were undergoing therapy with *P. knowlesi*, *P. vivax* or *P. falciparum*. Positive results with syphilitic sera were not common, but strong positive reactions were obtained in all malaria infections. The reaction was found to be group-specific rather than species-specific, in confirmation of the earlier work of Kingsbury (1927). Titres rose for 1 month after infection then fell but were raised for a period of 12 months.

These results have been repeatedly substantiated by other workers and improvements were made in technique especially regarding the preparation of antigen by Dulaney & Stratman-Thomas (1940); Dulaney, Stratman-Thomas & Warr (1941). They found good agreement between the results of complement fixation tests and those obtained by microscopical examination of blood. In later work Dulaney assessed the value of this test in relapsing *vivax* malaria using the same antigen and found that it was more sensitive than blood examination in detecting sub-clinical infection. Rein *et al* (1949) also believed that the test could detect more infected patients than could blood examination during recurrent attacks. These authors used human parasites as well as those of *P.*

knowlesi in the test. They confirmed that a group rather than a species-specific reaction took place in which cross reactions between *P. vivax*, *P. malariae* and *P. knowlesi* occurred. The presence of syphilis did not affect the results. In their studies on the complement fixation test in human malaria Mayer & Heidelberger (1946) found that the malaria antigens contained the Wasserman antigen, but the specific malaria antigen was distinct from it. These authors believe that the complement fixation test has only a limited value in latent malaria, but was of value in chronic relapsing *vivax* malaria when the tests were repeated at intervals. If syphilis is excluded a positive reaction with a malarial antigen is specific for that disease. Kligler & Yoeli (1941) studied the diagnostic and epidemiological significance of the complement fixation test in human malaria, using an antigen from *P. knowlesi* and they confirmed earlier work. They also found that an antigen prepared from the fowl malaria parasite *P. gallinaceum* was as good as that from monkeys, both reacting with human sera in the same way. The test was considered by them of value as an index of endemic malaria. Subsequent workers made a study on troops returning from malarious areas and believed the complement fixation test to be of little value when *P. gallinaceum* was the source of antigen.

Agglutination Test

Eaton (1938) described an agglutination test for *P. knowlesi* in which immune serum from monkeys was used. Red cells heavily parasitized with mature forms were obtained from monkeys by centrifugation of blood and a further separation from non-parasitized red cells was accomplished by the same means. A dilute suspension of these cells, at least 50% of which were parasitized with mature forms, was made in saline. Sera were obtained from normal animals, from those with acute and chronic infections and also after hyperimmunization, as well as from monkeys infected with a different malaria parasite *P. inui*. All sera were inactivated at 56°C for ½ hr. The test used was of macroscopic character. Agglutinins appeared in serum 15 to 45 days after infection and suggested that the relative immunity of those animals to re-infection was associated with the presence of sensitizing antibodies in the blood stream. Superinfected animals agglutinated at a dilution of 1/1000. The results obtained were specific in character since sera from normal animals or those infected with a different species of parasite gave negative results. Agglutinins persisted in the sera of chronically infected animals for a year or more. The value of the test as a diagnostic measure was lessened by the difficulty in preparing a suitable antigen. So far it has not been used in human malaria. Agglutination by immune serum of the bird malaria parasites *P. circumflexum*, *P. gallinaceum* and *P. lophurae* has been reported.

Brown & Brown (1965) in their studies on antigenic variation in chronic infections with the monkey parasite *P. knowlesi* used the original agglutination

test of Eaton (1938). They found marked specificity in each relapse type and antigenic heterogeneity was increased with repeated passage. It is possible that antigenic variation may be a feature of the human disease.

Precipitin Test

The earliest test of this nature was that described by Pewny (1918). He used as antigen a saline extract of human malarial blood clot. Positive reactions at a serum dilution of 1/100 were reported. Positive precipitin tests were also described by Taliaferro, Taliaferro & Fisher (1927). The best antigen was obtained from malarial placenta after preliminary treatment with ether, followed by extraction with Coca's fluid and filtration to give a clear product. One volume layered with an equal volume of serum under test was incubated at 37°C for 1 hr and then left some hours in the ice box. A ring precipitate was formed and the authors considered the result indicative of a specific antigen-antibody reaction, not merely a non-specific flocculation. The results were confirmed later by the same authors who then found that the antigen acted best at pH 7.8. Good results were also obtained using infected red cells as antigen especially when obtained as large schizonts from the placenta. Stratman-Thomas & Dulaney (1940) failed to demonstrate a specific precipitin reaction on testing 287 sera from malarial patients or a specific skin test in 268 similar patients. On the other hand, Dulaney & House (1941) demonstrated precipitation reactions in human malaria with antigens prepared by extraction of *P. knowlesi*, either by use of antigen-coated collodion particles or by a method in which collodion particles were added at the time of mixing antigen and antibody. Similar results were obtained in both cases and could be read macroscopically.

Henry (1929) described a melanin flocculation test in malaria based on the assumption that the pigment formed during the course of the disease was melanin and that it possessed antigenic characters. There is a voluminous literature on the subject of the test but it is no longer believed to be a true antigen-antibody reaction and false positive results occur in other diseases. Nevertheless the test has been widely used in malarial surveys in North Africa.

Fluorescent Antibody Test

Tobie & Coatney (1961) reported that the method could be used to detect antibody in the serum of patients with *P. vivax* infection. For this purpose the globulin fraction of the serum was labelled with fluorescein isothiocyanate. *P. cynomolgi* B a parasite of monkeys, which also infects man, was found to be stainable in the same way with labelled homologous serum from an accidentally infected laboratory worker. Cross reactions between these two parasites were observed but antibody titres were higher in homologous sera. Ingram *et al* (1961) applied similar methods to the study of natural infections of monkeys. Antibody production was followed in human patients and monkeys during the

course of infection produced by blood inoculations or through sporozoites. The appearance of antibodies correlated well with the appearance of parasites and antibodies persisted after drug treatment. Voller & Bray (1962) carried out similar studies and demonstrated that malaria antibodies were present in the cord blood of immune mothers. Voller (1962) made attempts to differentiate species by this staining method. Cross reactions indicated different species possessed common antigens, but avian species had different antigens from those of mammalian plasmodia. Ingram & Carver (1963) in studying staining of tissue stages observed cross reactions with blood forms, and concluded that the method may be of limited value in diagnosis. The method has the great advantage that little antigen is required and it has proved of value in epidemiological studies to indicate the antibody status of a group. It seems clear that the method does not measure functional immunity. An attempt has been made by Goldman (1960) to measure intensity of fluorescence in the slide antigen and applied here would increase the value of the method in immuno-diagnosis. Voller (1964) has summarized the use of fluorescent antibody methods in malaria research. Collins *et al* (1966) have concluded that simian antigens could be used profitably in human surveys. El-Nahal & Bray (1966) found that Evans blue was of value to lessen non-specific staining.

Haemagglutination Test

Stein & Desowitz (1963, 1964) described a test for measuring antibody in human malaria infections, using formolized tanned sheep cells sensitized with human, rodent or simian antigens. Very high titres were obtained but numerous false positive results were common. Cross reactions with different species of parasite were also observed. The test shows promise and can be carried out with minimal quantities of antigen. Further experience with the test is required, including evaluation of the cross reactions and many workers are investigating these problems.

Piroplasmosis

About twenty species of the genus *Babesia* are found throughout the world in cattle and other domestic animals. They occur like malaria parasites in the host red cells and do not form pigment. Like the genus *Theileria* and *Anaplasma* (whose taxonomic position is not clearly established) they are transmitted in nature by ticks. *Babesia bigemina*, the cause of Texas fever in cattle, was described in 1893 by Smith & Kilborne while *B. bovis* causes a similar disease in European cattle and both are of great economic importance. Parasites of the genus *Babesia* were not known to infect man till Škrabalo & Deanović (1957) reported the occurrence of a fatal infection, accompanied by haemoglobinuria, in a native of Yugoslavia. He was a farmer whose spleen had been removed some years earlier and was constantly subjected to tick bites in an area where *Babesia* infection is common in cattle. Garnham & Bray (1959, 1961) showed that

splenectomized chimpanzees and rhesus monkeys acquire fulminating infections with *B. divergens* and *B. bovis* but could not be infected with *Theileria parva*. In East Africa this organism, which undergoes schizogony in the reticulo-endothelial cells of the host, gives rise to East Coast fever, a major cause of morbidity and mortality among cattle in that area. *Anaplasma* which also occurs in red cells is likewise a source of morbidity in cattle and other animals and is not infrequently accompanied by *Babesia* and *Theileria*. Rees & Mohler (1934) devised a complement fixation test for anaplasmosis which proved useful, since these parasites are small and hard to detect microscopically. Blood from calves infected with *A. marginale* is employed as a source of antigen. The test which is highly specific has been reviewed by Gates & Roby (1956). Ristic *et al* (1957) used fluorescein-labelled antibody to detect this small parasite in fixed blood smears. Ristic *et al* (1964) also used fluorescein-labelled anti-piroplasma globulin in the examination of blood films from splenectomized infected horses for the presence of parasites. Schindler & Dennig (1962) demonstrated *Babesia* antibodies in dogs by the complement fixation test 11–14 days after infection, using as antigen a saponin-treated preparation from infected blood cells. Pilchard & Ristic (1963) evaluated serological techniques for the diagnosis of anaplasmosis and equine piroplasmosis. They found the complement fixation and capillary tube agglutination test of practical value in the diagnosis of acute and chronic anaplasmosis, whereas gel diffusion, indirect haemagglutination and the fluorescent antibody test are primarily useful as research tools. The only useful test for equine piroplasmosis appears to be that of precipitation in gel.

Toxoplasmosis

Although *Toxoplasma* was first described in 1908 it was not until 1939 that Wolf, Cowen & Paige showed that the parasite was associated with human disease. The distribution of toxoplasmosis is world wide and antibody surveys in man and animals have been carried out by standard serological methods. The disease is regarded as a zoonosis and the taxonomic position of the genus *Toxoplasma* is still uncertain.

The presence of antibodies in infected monkeys and in man was demonstrated by protection tests in animals by Sabin & Olitsky (1937). A complement fixation test was described by Nicolau & Ravelo (1937). The antigens in their experiments like those of subsequent workers were very impure and contained host cells and other debris. The result was that results from different laboratories could not be compared. Fulton & Fulton (1965) have described an antigen which consists of a pure suspension of formolized *Toxoplasma gondii* prepared from the peritoneal exudate of infected cotton-rats which can readily be standardized by parasite counts, by optical density or by parasite nitrogen estimations. After freeze-drying in presence of 6% salt-free dextran it can readily be reconsituted and remained fully potent for more than 1 year.

A dermal sensitivity test to toxoplasma antigens (toxoplasmins) was described by Frenkel (1948) and was of delayed tuberculin type. The allergic reaction is not positive for some months after infection. Robertson (1965) used the test in surveys of severely subnormal patients in Lincolnshire. The test is not very informative because of its delayed type but has been used in population surveys to indicate the antibody status of a group.

The most widely used test is that of Sabin & Feldman (1948) generally known as the dye test. It is carried out by exposing peritoneal exudate from infected mice containing toxoplasma to serum antibodies in presence of a heat labile substance named 'activator' and methylene blue at pH 11. Staining of toxoplasma cytoplasm was prevented by antibodies. The activator substance appears to be of properdin nature. The same phenomenon was studied by Lelong and Desmonts (1952) as a lytic phenomenon in which destruction of parasite cytoplasm could be observed by means of phase contrast microscopy. Correlation between results of the dye test and clinical status of the patient is frequently absent and technically the method has some serious disadvantages.

A pock counting technique has been used in the serological diagnosis of the disease by Macfarlane & Ruchman (1948) and MacDonald (1949) and resembled that of virus neutralization but had only a limited application.

An indirect haemagglutination test was described by Jacobs & Lunde (1957) who used sensitized sheep red cells coated with a soluble antigen from infected mouse peritoneal exudate. Chordi *et al* (1964a) made a careful study on the specificity of the test. The modification described by Lewis & Kessel (1961) has been widely used in America. Jennis (1966) has used a simplified haemagglutination test for toxoplasmosis in which the cells before sensitization are treated with pyruvic aldehyde. A flocculation test by Siim & Lind (1960) is of similar nature. Further evaluation is necessary before the test can be regarded as satisfactory for routine use.

A fluorescence inhibition test was described by Goldman (1957a and b) who showed its antigen-antibody nature. Parasites normally difficult to find in tissue smears were made readily visible. A quantitative test for measuring antibody titres was based on the inhibition of specific staining by non-labelled antibody. Subsequently a large number of papers on the indirect fluorescent antibody test for the serodiagnosis of toxoplasmosis has appeared, including that by Fulton & Voller (1964) and Fletcher (1965). Wéry (1965) does not think that the fluorescent antibody test has solved many problems, although he found it reasonably specific in character. Others have claimed that it is economical and easy to carry out. Kramar (1965) used dried blood on filter paper in the indirect fluorescent antibody test after extraction with saline.

An agar gel diffusion test was first employed by O'Connor (1957a and b) to demonstrate the presence of precipitins in the aqueous humour of the eye of patients with uveitis. Strannegård (1962) has also used the method and found

relatively poor correlation with the results of complement fixation and dye test. Chordi *et al* (1964b) have carried out an analysis of *T. gondii* antigens by this method.

A quantitative direct agglutination method was described by Fulton & Turk (1959) using the pure suspension of parasites mentioned in connection with the complement fixation test. Fulton (1965) has now adapted the method for use on a micro scale so that only 1/10 of the amount of antigen originally used is now necessary when the micro titration apparatus of Takatsy is employed. The test is specific as shown by absorption and other methods. Its advantage lies in ease of performance, repeatability, naked eye reading of results and the use of formolized parasites which can be kept for many months and are non-infective.

A quantitative respiratory inhibition test was described by Fulton & Spooner (1960) which measured the effect of immune sera of oxygen uptake by a pure suspension of parasites. The method was more of academic than practical interest.

Engelbrecht (1965) described a slide flocculation test for diagnosis. The sediment from infected mouse peritoneal exudate obtained by centrifugation was frozen and subjected to ultrasonic treatment. On thawing formalin and gelatin was added to stabilize and leucocyte debris allowed to settle out. Flocculation occurred 10 min after addition of antiserum and results could then be read. Good correlation with the dye and haemagglutination test was claimed.

Sarcosporidiosis

Sarcosporidia are parasitic organisms of uncertain relationship and as yet undetermined taxonomic position. About fifty species have been named. The genus *Sarcocystis* was established in 1882. Infections are cosmopolitan in distribution, occurring in birds, reptiles, fish and mammals, including man. Few human cases have been reported and the disease is chiefly of importance for the veterinarian. These organisms have not infrequently been confused with toxoplasma. Mühlpfordt (1951) could not distinguish between them by means of serological methods including the dye test described earlier for toxoplasmosis. Awad (1954a) described a new dye test for *Toxoplasma* and *Sarcocystis* infections using *S. tenella* spores. Awad & Lainson (1954) found that the dye test gave positive results with *Toxoplasma* and *Sarcocystis* sera whereas the complement fixation test gave specific results. Awad's finding that sarcospores can be used in the dye test for toxoplasma was not confirmed by subsequent work. Awad (1954b) also carried out intradermal tests with 'sarcocystin', an antigen prepared from oesophageal cysts of sheep. A delayed type of reaction occurred as with tuberculin. The test lacked specificity and proved of little diagnostic value, and is recommended for use only in screning.

During the evaluation of immunofluorescent tests for detecting toxoplasma antibodies Fulton & Voller (1964) were fortunate in obtaining serum from a

patient with *Sarcocystis* infection which had been verified by biopsy. The serum in question had a high titre in this test when the homologous antigen was used but was negative with *Toxoplasma* antigen. Also high titre *Toxoplasma* sera did not react with *Sarcocystis*. These findings confirm the general consensus of opinion that *Sarcocystis* and *Toxoplasma* are immunologically distinct.

Coccidiosis

According to recent observations only 835 infections have been recorded in man in the Western Hemisphere, with species of the genus *Isospora*, two of which, *I. hominis* and *I. belli*, are recognized. They parasitize the mucosa and submucosa of the intestinal wall. Infection is cosmopolitan and epidemics have been recorded in the Near East and South-west Pacific Islands. There is a moderate endemicity in South America, more commonly in poor economic and sanitary conditions. Essentially nothing is known of the immunology of human coccidiosis, and diagnosis by serological methods has not been seriously attempted. Although negligible as human parasites coccidia cause great economic losses among domestic and game animals, especially poultry, rabbits and cattle. Numerous species are involved and vary in the site of development and in details of their schizogonic cycles. Immunity can be demonstrated after infection and is absolute. Patterson (1923) devised a complement fixation test in rabbits using extracts of infected liver as antigen and believed it was useful in diagnosis. Similar experiments carried out later did not support this conclusion. Precipitins, lysins and agglutinins were stated to be absent during infection but skin tests showed some promise in diagnosis. Recent work by Rose (1959) has shown, by means of quantitative precipitation reactions in gel and liquid media, that circulating antibodies to *Eimeria stiedae* occur in the sera of infected rabbits. She confirmed also that complement fixing antibodies were present and was of the opinion that different species of coccidia might be distinguished by the double diffusion method in gel. Horton-Smith, Beattie & Long (1961) came to the conclusion that immunity in *E. tenella* of fowls is mediated through the circulation by humoral antibodies or lymphoid cells or both.

CILIATA

Balantidiosis

The parasitic ciliate *Balantidium* is widely distributed in nature. In many parts of the world pigs are heavily infected but do not suffer and are probably the main source of human infection. It is the only parasitic ciliate of man and may cause diarrhoea and ulceration of the intestine. Different species are morphologically similar and Zaman (1964, 1965) has studied their antigenic relationships by means of the immobilization and fluorescent antibody reaction. Few

serological investigations have been carried out on the organism. The author used antisera from rabbits immunized with cultures of the organism in both tests. He believes these tests have an advantage over others, in which soluble antigens are used, because neither is affected by bacterial or other extraneous antigens present in these cultures. The serum was inactivated at 56°C for 30 min to avoid lysis of the organisms. Immobilization was accompanied by the presence of a precipitate on the surface of the organism. The highest titre obtained was 1:64 whereas Beale obtained a titre of 1:1000 with *Paramoecium*, a free-living form. The immobilization effect was prevented by absorption of antibodies by the ciliate. It was found that five pig strains cross-reacted but there was no cross reaction with a human strain. In the fluorescent antibody test staining was specific in unfixed specimens but not in those fixed. Controls, however, also showed fluorescent staining and it does not appear that the two tests alone or together are of diagnostic value. Dzbenski (1966) has also carried out immunofluorescent studies on *Balantidium coli*, to find if antibodies could be detected in the sera of naturally infected pigs with a view to using the method in detecting human infections. For comparison rabbits were immunized by subcutaneous inoculation with cultured organisms accompanied by Freund's adjuvant. The antigen was of fresh or fixed trophozoites and fluorescein-labelled goat anti-rabbit-globulin serum was used for staining by the indirect method. The method was unable to detect antibodies in the sera of naturally infected pigs.

REFERENCES

AMATO NETO V., MAGALDI C. & PESSOA S.B. (1964) Intradermal reaction for the diagnosis of Chagas's disease with a tissue culture antigen of *T. cruzi*. *Rev. Goiana Med.* **10**, 121.

ATCHLEY F.O., AUERHEIMER A.H. & WASLEY M.A. (1963) Precipitate patterns in agar gel with sera from human amebiasis and *Endamoeba histolytica* antigen. *J. Parasitol.* **49**, 313.

AWAD F.I. (1954a) A new dye test for *Toxoplasma* and *Sarcocystis* infections by use of *Sarcocystis tenella* spores. *Trans. Roy. Soc. trop. Med. Hyg.* **48**, 337.

AWAD F.I. (1954b) Thesis, London. Studies on Sarcosporidiosis with particular reference to serological reactions, host specificity and mode of injection.

AWAD F.I. & LAINSON R. (1954) A note on the serology of Sarcosporidiosis and Toxoplasmosis. *J. clin. Path.* **7**, 152.

BIAGI F., BELTRAN F.H. & ORTEGA P.S. (1966) Remobilization of *Entamoeba histolytica* after exposure to immobilizing antibodies. *Exper. Parasitol.* **18**, 87.

BIAGI F. & BUENTELLO L. (1961). Immobilization reaction for the diagnosis of amebiasis. *Exper. Parasitol.* **11**, 188.

BIAGI F., JAY J. & MARTINEZ M.R. (1964) The immunofluorescence test in the diagnosis of Chagas's disease. *Bol. Oficina Sanitaria Pan Americana* **57**, 237.

BOZICEVICH J., HOYEM H.M. & WALSTON V.M. (1946) A method of conducting the 50 per cent hemolysis end-point complement-fixation test for parasitic diseases. *Pub. Health Rep. Wash.* **61**, 529.

BRAY R.S. & LAINSON R. (1967) Studies on Immunology and Serology of Leishmaniasis. V. The use of particles as vehicles in passive agglutination tests. *Trans. Roy. Soc. trop. Med. Hyg.* **61**, 490.

BROWN K.N. & BROWN I.N. (1965) Immunity in Malaria. Antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature (Lond.)* **208,** 1286.

BROWN K.N. & WILLIAMSON J. (1962) Antigens of *Brucei* trypanosomes. *Nature (Lond.)* **194,** 1253.

CAHILL K.M. (1965) Leishmanin skin testing in Africa and the Middle East. *East African Med. J.* **42,** 213.

CASCIO G., PURPURA R. & PRIOLISI A. (1962) *Giorn di malattie infettive e Parasit.* **14,** 669. (*Trop. Dis Bull.* 1963, **60,** 628.)

CHAFFEE E.F., FIFE E.H. & KENT J.F. (1956) Diagnosis of *Trypanosoma cruzi* infection by complement fixation. *Amer. J. trop. Med. Hyg.* **5,** 763.

CHANG S.L. & NEGHERBON W.O. (1947) Studies on hemoflagellates. III. The specificity of serological reactions of *Leishmania donovani, L. braziliensis, L. tropica* and *Trypanosoma cruzi*. *J. infect. Dis.* **81,** 209.

CHORDI A., WALLS K.W. & KAGAN I.G. (1964a) Studies on the specificity of the indirect haemagglutination test for toxoplasmosis. *J. Immunol.* **93,** 1024.

CHORDI A., WALLS K. W. & KAGAN I.G. (1964b) Analysis of *Toxoplasma gondii* antigens by agar diffusion methods. *J. Immunol.* **93,** 1034.

COGGESHALL L.T. & EATON M.D. (1938) The complement fixation reaction in monkey malaria. *J. exper. Med.* **67,** 871.

COLE B.A. & KENT J.F. (1953) Immobilization of *Entamoeba histolytica in vitro* by antiserum produced in the rabbit. *Proc. Soc. exp. Biol. (N.Y.)* **83,** 811.

COLLINS W.E., JEFFERY G.M., GUINN E. & SKINNER J.L. (1966) Fluorescent antibody studies in human malaria. IV. Cross reactions between human and simian malaria. *Amer. J. trop. Med. Hyg.* **15,** 11.

CRAIG C.F. (1929) The technique and results of a complement fixation test for the diagnosis of infections with *Endamoeba histolytica*. *Amer. J. trop. Med.* **9,** 277.

CRAIG C.F. (1934) *Amebiasis and Amebic Dysentery*. Springfield, Illinois: Charles C. Thomas.

CULBERTSON J.T. (1941) *Immunity Against Animal Parasites*. New York: Morningside Heights, Columbia University Press.

CUNNINGHAM M.P. & VICKERMAN K. (1961) An improved agglutination reaction for use in antigenic analysis of African trypanosomes. *Trans. Roy. Soc. Trop. Med. Hyg.* **55,** 12.

CUNNINGHAM M.P. & VICKERMAN K. (1962) Antigenic analysis in the *Trypanosoma brucei* group using the agglutination reaction. *Trans. Roy. Soc. trop. Med. Hyg.* **56,** 48.

DAVIS D.J. (1943) An improved antigen for complement fixation in American trypanosomiasis. *Pub. Health Rep. Wash.* **58,** 775.

DÉPOUX R., MERVEILLE P. & CECCALDI J. (1956) Étude de la réaction de fixation du complément dans la trypanosomiase humaine. *Ann. Inst. Pasteur (Paris)* **91,** 684.

DESCHIENS R. (1965) *L'Amibiase et L'Amibe Dysentérique*, Masson et Cie., France.

DESOWITZ R.S. (1956) Effect of antibody on the respiratory rate of *Trypanosoma vivax*. *Nature (Lond.)* **177,** 132.

DIAMOND L.S. (1961) Axenic cultivation of *Entamoeba histolytica*. *Science* **134,** 331.

DOBELL C. (1919) *The Amoebae Living in Man*. London: John Bale Sons & Danielsson Ltd.

DUKE H.L. & WALLACE J.M. (1930) 'Red cell adhesion' in trypanosomiasis of man and animals. *Parasitology*, **22,** 414.

DULANEY A.D. & HOUSE V. (1941) Precipitative tests in malaria. *Proc. Soc. exp. Biol. (N.Y.)*, **48,** 620.

DULANEY A.D. & STRATMAN-THOMAS W.K. (1940) Complement fixation in human malaria. I. Results obtained with various antigens. *J. Immunol.* **39,** 247.

DULANEY A.D., STRATMAN-THOMAS W.K. & WARR O.S. (1941) Complement fixation in

malaria with special reference to the diagnosis of naturally acquired infections. *J. Bact.* **41**, 66.

DUXBURY R.E. & SADUN E.H. (1964) Fluorescent antibody test for the serodiagnosis of visceral leishmaniasis. *Amer. J. trop. Med. Hyg.* **13**, 525.

DZBENSKI T.H. (1966) Immunofluorescent studies on *Balantidium coli*. *Trans. Roy. Soc. trop. Med. Hyg.* **60**, 387.

EATON M.D. (1938) The agglutination of *Plasmodium knowlesi* by immune serum. *J. exper. Med.* **67**, 857.

EATON M.D. & COGGESHALL L.T. (1939) Complement fixation in human malaria with an antigen prepared from the monkey parasite *Plasmodium knowlesi*. *J. exper. Med.* **69**, 379.

ELDER J.K. (1964) Examination of twelve strains of *Trichomonas foetus* (Riedmuller) isolated in Queensland and the description of a new serotype *Trichomonas foetus* var. Brisbane. *Queensland J. Agric. Science*, **21**, 193.

EL-NAHAL H.M.S. & BRAY R.S. (1966) The use of Evans blue in the fluorescent antibody staining of malaria parasites. *Trans. Roy. Soc. trop. Med. Hyg.* **60**, 564.

ENGLEBRECHT E. (1965) Nouvelle réaction pour le diagnostic de la toxoplasmose. La floculation sur lame. *Pathologia and Microbiol. (Basel)* **28**, 907.

ESSENFELD F. & FENNELL R.H. Jr (1964) Immunofluorescent study of experimental *Trypanosoma cruzi* infection. *Proc. Soc. exp. Biol. (N.Y.)* **116**, 128.

EVENS F., SCHOENAERS F., NEUJEAN G., KAECKENBEEK A. & STYNS J. (1953) Valeur pratique de la réaction de fixation du complément dans la maladie du sommeil à *T. gambiense*. II[e] Partie. La réaction de fixation du complément lors d'une rechute après traitement médicamenteux. *Ann. Soc. belge Med. trop.* **33**, 389.

FEINBERG J.G. (1952) A capillary agglutination test for *Trichomonas foetus*. *J. Path. Bact.* **64**, 645.

FIFE E.H. & MUSCHEL L.H. (1959) Fluorescent antibody technic for serodiagnosis of *Trypanosoma cruzi* infection. *Proc. Soc. exp. Biol. (N.Y.)* **101**, 540.

FLETCHER S. (1965) Indirect fluorescent antibody technique in the serology of *Toxoplasma gondii*. *J. Clin. Path.* **18**, 193.

DE FREITAS J.L.P. & DE ALMEIDA J.O. (1949) *Hospital, Rio de Janeiro* **35**, 787. (*Trop. Dis. Bull.* 1949, **46**, 1129.)

FRENKEL J.K. (1948) Dermal hypersensitivity to Toxoplasma antigens (Toxoplasmins). *Proc. Soc. exp. Biol. (N.Y.)* **68**, 634.

FULTON J.D. (1965) Micro-agglutination test for Toxoplasma antibodies. *Immunology* Oxford, **9**, 491.

FULTON J.D. & FULTON F. (1965) Complement fixation tests in toxoplasmosis with purified antigen. *Nature* **205**, 776.

FULTON J.D., JOYNER L.P. & PRICE I.N.O. (1951) Studies on Protozoa IV. A complement fixation test for amoebiasis. *J. trop. Med. Hyg.* **54**, 27.

FULTON J.D. & SPOONER D.F. (1960) Metabolic studies on *Toxoplasma gondii*. *Exper. Parasit.* **9**, 293.

FULTON J.D. & TURK J.L. (1959) Direct agglutination test for toxoplasmosis. *Lancet* **ii**, 1068.

FULTON J.D. & VOLLER A. (1964) Evaluation of immunofluorescent and direct agglutination methods for detection of specific *Toxoplasma* antibodies. *Brit. med. J.* **ii**, 1173.

GARNHAM P.C.C. & BRAY R.S. (1959) The susceptibility of the higher primates to piroplasms. *J. Protozool.* **6**, 352.

GARNHAM P.C.C. & BRAY R.S. (1961) Failure to infect splenectomized primates with *Theileria parva*. *J. Parasit.* **47**, 538.

GATES D.W. & ROBY T.O. (1956) The status of the complement fixation test for the diagnosis of anaplasmosis in 1955. *Ann. N.Y. Acad. Sci.* **64**, Art. 2, 31.

GILL B.S. (1964) A procedure for the indirect haemagglutination test for the study of experimental *Trypanosoma evansi* infections. *Ann. trop. Med. Parasit.* **58,** 473.

VAN GOIDSENHOVEN C. & SCHOENAERS F. (1944) Sur la stabilization des antigènes utilisés au serodiagnostic des trypanosomiases. *Ann. Soc. belge Med. trop.* **24,** 167.

GOLDMAN M. (1953) Cytochemical differentiation of *Endamoeba histolytica* and *Endamoeba coli* by means of fluorescent antibody. *Amer. J. Hyg.* **58,** 319.

GOLDMAN M. (1954) Use of fluorescein-tagged antibody to identify cultures of *Endamoeba histolytica* and *Endamoeba coli*. *Amer. J. Hyg.* **59,** 318.

GOLDMAN M. (1957a) Staining *Toxoplasma gondii* with fluorescein-labelled antibody. I. The reaction in smears of peritoneal exudate. *J. exper. Med.* **105,** 549.

GOLDMAN M. (1957b) II. A new serological test for antibodies to toxoplasma based upon inhibition of specific staining. *J. exper. Med.* **105,** 557.

GOLDMAN M. (1960) Antigenic analysis of *Entamoeba histolytica* by means of fluorescent antibody. I. Instrumentation for microfluorimetry of stained amoebae. *Exper. Parasitol.* **9,** 25.

GOLDMAN M., CARVER R.K. & GLEASON N.N. (1960) II. *E. histolytica* and *E. hartmanii*. *Exper. Parasitol.* **10,** 366.

GOLDMAN M., GLEASON N.N. & CARVER R.K. (1962) III. Reactions of the Laredo strain with five anti-*histolytica* sera. *Am. J. trop. Med. Hyg.* **11,** 341.

GREVAL S.D.S., SEN GUPTA P.C. & NAPIER L.E. (1939) Serological reactions in Kala-azar: Complement fixation, false Wassermann reaction, and high anti-complementary titre. *Ind. J. med. Res.* **27,** 181.

GHOSH G., GHOSH N.N. & RAY J.C. (1945) A preliminary note on the complement-fixation reaction in Kala-azar with a specific antigen as an aid to diagnosis. *Ann. Biochem. (Calcutta)* **5,** 153.

GRAY A.R. (1961) Soluble antigens of *Trypanosoma vivax* and of other trypanosomes. *Immunology* **4,** 253.

GREIF R.L. (1947) Agglutination of *Endamoeba histolytica* cysts. *Am. J. Trop. Med.* **27,** 131.

HENRY A.X.F. (1929) Nouvelle technique de ferrofloculation pour l'étude sérologique du paludisme. *C.R. Soc. Biol. (Paris),* **100,** 671.

HERMAN R. (1965) Fluorescent antibody studies on the intracellular form of *Leishmania donovani* grown in cell culture. *Exper. Parasitol.* **17,** 218.

HORTON-SMITH C., BEATTIE J. & LONG P.L. (1961) Resistance to *Eimeria tenella* and its transference from one caecum to the other in individual fowls. *Immunology* **4,** 111.

INGRAM D.G. & SOLTYS M.A. (1960) Immunity in trypanosomiasis. IV. Immuno-conglutinin in animals infected with *Trypanosoma brucei*. *Parasitology* **50,** 231.

INGRAM R.L. & CARVER R.K. (1963) Malaria parasites: Fluorescent antibody technique for tissue stage study. *Science* **139,** 405.

INGRAM R.L., OTKEN L.B. & JUMPER J.R. (1961) Staining of malaria parasites by the fluorescent antibody technique. *Proc. Soc. exp. Biol. (N.Y.)* **106,** 52.

IZAR G. (1914) Studien über amoebenenteritis. Beiheft 2. *Arch. f. Schiffs und Tropenhyg.* **18,** 5.

JACOBS L. & LUNDE M.N. (1957) A haemagglutination test for toxoplasmosis. *J. Parasit.* **43,** 308.

JEANES A.L. (1964) Immunofluorescent diagnosis of amoebiasis. *Brit. med. J.* **ii,** 1531 (correspondence).

JEANES A.L. (1966) Indirect fluorescent antibody test in diagnosis of hepatic amoebiasis. *Brit. med. J.* **i,** 1464.

JENNIS F. (1966) A simplified haemagglutination test for toxoplasmosis using pyruvic aldehyde treated cells. *Aust. J. exp. Biol. Med. Sci.* **44,** 317.

KAGAN I.G. (1965) Evaluation of Routine Serologic Testing for Parasitic Diseases. *Amer. J. Pub. Health,* **55,** 1820.

KELSER R.A. (1936) A complement-fixation test for Chagas' disease employing an artificial culture antigen. *Amer. J. trop. Med.* **16,** 405.

KESSEL J.F., LEWIS W.P., PASQUEL C.M. & TURNER J.A. (1965). Indirect haemagglutination and complement fixation tests in Amebiasis. *Am. J. trop. Med. Hyg.* **14,** 540.

KHALEQUE K.A. (1965) Complement fixation test for Kala-azar with an antigen prepared from acid fast bacillus. *Pakistan J. of Med. Res.* **4,** 234.

KINGSBURY A.N. (1927) Some investigations of malarial fevers. IV. The complement fixation reaction. *Trans. roy. Soc. trop. Med. Hyg.* **20,** 359.

KLIGLER I.J. & YOELI M. (1941) The diagnostic and epidemiological significance of the complement fixation test in human malaria. *Amer. J. trop. Med.* **21,** 531.

KORIK L.M. (1966) *Vestnik Dermat i Veneral (Moscow),* **40,** 50. (*Bull. Hyg.* 1966, v. **41,** 759.)

KOTT, H. & ADLER S. (1961) A serological study of *Trichomonas* sp. parasitic in man. *Trans. Roy. Soc. trop. Med. Hyg.* **55,** 333.

KRAMAR J. (1965) *Ceskoslav Epidemiol. Mikrobiol. Immunol. Prague.* **14,** 59. (*Trop. Dis. Bull.* 1965, v. **62,** 919.)

KUM Y.K. (1958) The Immunological Diagnosis of Amoebic Dysentery. *J. Microbiol. Epidemiol. & Immunobiol.* **29,** 88.

LEAL R.A. (1954) Intradermal reaction in amoebiasis (Thesis). (*Tropical Diseases Bulletin,* 1954, v. **51,** 923.)

LELONG M. & DESMONTS G. (1952) Sur la nature du phénomène de Sabin & Feldman. *C.R. Soc. Biol. Parasitol.* **146,** 207.

LEWIS W.P. & KESSEL J.F. (1961) Haemagglutination in the diagnosis of toxoplasmosis and amebiasis. *Arch. Ophth.* **66,** 471.

LUCASSE C. (1964) Fluorescent antibody test as applied to cerebrospinal fluid in human sleeping sickness. *Bull. Soc. Path. Exot.* **57,** 283.

LUMSDEN W.H.R. (1966) Estimation of serum IgM in the diagnosis of human trypanosomiasis. *Trans. R. Soc. trop. Med. Hyg.* **60,** 125.

MACDONALD A. (1949) Serological diagnosis of human toxoplasmosis. *Lancet* **i,** 950.

MCENTEGART M.G. (1956) Serological comparison of a strain of *Trichomonas vaginalis* with Belfast & Manley strains *Trichomonas foetus. J. Path. Bact.* **71,** 111.

MCENTEGART M.G., CHADWICK C.S. & NAIRN R.C. (1958) Fluorescent antisera in the detection of serological varieties of *Trichomonas vaginalis. Brit. J. Vener. Dis.* **34,** 1.

MACFARLANE J.O. & RUCHMAN I. (1948) Cultivation of toxoplasma in the developing chick embryo. *Proc. Soc. exp. Biol. (N.Y.)* **67,** 1.

MADDISON S.E. (1965) Characterization of *Entamoeba histolytica* antigen-antibody reaction by gel diffusion. *Exper. Parasitol.* **16,** 224.

MADDISON S.E., POWELL S.J. & ELSDON-DEW R. (1965a) Comparisou of haemagglutinins and precipitins in amebiasis. *Amer. J. trop. Med. & Hyg.* **14,** 551.

MADDISON S.E., POWELL S.J. & ELSDON-DEW R. (1965b) Application of serology to the epidemiology of amebiasis. *Amer. J. trop. Med. & Hyg.* **14,** 554.

MANSON-BAHR P. (1927) Researches upon the production of malaria antigen for a complement-deviation test. *Trans. Roy. Soc. trop. Med. Hyg.* **21,** 63.

MANSON-BAHR P.E.C. & SOUTHGATE B.A. (1964) Recent research on Kala-azar in East Africa. *J. trop. Med. Hyg.* **67,** 79.

MATTERN P. (1964) Techniques et interêt épidemiologique du diagnostic de la trypanosomiase humaine Africaine par la recherche de la β_2-macroglobuline dans le sang et dans le LCR. *Ann. Inst. Pasteur (Paris)* **107,** 415.

MATTERN P., MASSEYEFF R., MICHEL R. & PERETTI P. (1961) Étude immunochimique de la β_2-macroglobuline des sérums des malades atteinte de trypanosomiase Africaine à *T. gambiense. Ann. Inst. Pasteur (Paris),* **101,** 382.

MATTERN P., PILLOT J. & BONNARDOT R. (1965) Contribution à l'étude des immunoglobulines du liquide cephalorachidien responsable du phénomène d'immunofluorescence dans la neurosyphilis et dans la trypanosomiase nerveuse. *Med. Afrique Noire (Dakar)* **12,** 219.

MAYER M.M. & HEIDELBERGER M. (1946) Studies in human malaria. V. Complement fixation reactions. *J. Immunol.* **54,** 89.

MAYER M. & PIFANO F. (1941) O diagnostico da molestia de Chagas por intradermoreacção com cultura de *Schizotrypanum cruzi*. *Brasil Méd.* **55,** 317. (*Trop. Dis. Bull.* 1941, **38,** 640.)

MILLS E.A., MACHATTIE C. & CHADWICK C.R. (1931) The Rieckenberg reaction as an aid to diagnosis in chronic and aberrant types of oriental sore. *Trans. roy. Soc. trop. Med. Hyg.* **25,** 205.

MOAN J.C. (1957) The serological diagnosis of amoebiasis by means of the precipitin test. *Amer. J. trop. Med. & Hyg.* **6,** 499.

MONTAÑO G. & NERÓS H. (1965) Haemagglutination test and complement fixation test in the serological diagnosis of Chagas's disease. A comparative study. *Bol. Chileno de Parasit.* **20,** 62.

MONTENEGRO J. (1926) Cutaneous reaction in leishmaniasis. *Arch. Dermat. & Syph.* **13,** 187.

MÜHLPFORDT H. (1951) Das verhalten Sarcosporidien-infizierter Tiere im Sero-Farbtest auf Toxoplasmose nach Sabin-Feldman. *Z. Tropenmed Parasit.* **3,** 205.

MUNIZ J. (1947) Do valór de reação de precipitina no diagnóstico das formas agudas e subagudas da 'Doenca de Chagas'. *Brasil-Méd.* **61,** 261. (*Trop. Dis. Bull.* 1948, **45,** 54.)

MUNIZ J. (1950) On the value of 'conditioned haemolysis' for the diagnosis of American trypanosomiasis. A comparative study with complement fixation and sensitized erythrocytes agglutination tests. *Hospital, Rio de Janeiro* **38,** 685.

MUNIZ J. & DE FREITAS G. (1944) Contribuição para o diagnóstico da doenca de Chagas pelas reações de imunidade. I. Estudo comparativo entre as reações de aglutinação e de fixaçãso do complemento. *Mem. Inst. Oswaldo Cruz.* **41,** 303. (*Trop. Dis. Bull.* 1945, **42,** 975.)

MUNIZ J. & DOS SANTOS M.C.F. (1950) Technic of 'Conditioned Hemolysis' applied in the diagnosis of American trypanosomiasis. *Hospital, Rio de Janeiro,* **38,** 617.

NICOLAU S. & RAVELO A. (1937) La réaction de fixation du complément dans le sérum et dans des extraits d'organes d'animaux atteints de toxoplasmose expérimentale. *Bull. Soc. Path. Exot.* **30,** 855.

NICOLI J., BERGOT J. & DEMARCHI J. (1961) Étude de protéines sériques au cours de la trypanosomiase humaine Afrique. *Ann. Inst. Pasteur (Paris)* **101,** 596.

O'CONNOR G.R. (1957a) Anti-toxoplasma precipitins in aqueous humor. New application of agar diffusion technique. *Arch. Ophthl.* **57,** 52.

O'CONNOR G.R. (1957b) Precipitating antibody to toxoplasma. A follow-up study on findings in the blood and aqueous humor. *Amer. J. Ophthl.* **44,** 75.

ODDO F.G. & CASCIO G. (1963) Il test di immuno-fluorerenza nelle leishmaniose vixerale et cutanea. *Rev. Instituto Seroterap Ch. Ital.* **38,** 139. (*Trop. Dis. Bull.* 1964, **61,** 258.)

PATTERSON S.W. (1923) A complement fixation test in coccidioisis of the rabbit. *Brit. J. exper. Path.* **4,** 1.

PAUTRIZEL R., DURET J., TRIBOULEY J. & RIPERT C. (1962a) Étude de la spécificité de la réaction d'agglutination des trypanosomes au cours des trypanosomoses. *Bull. Soc. Path. Exot.* **55,** 383.

PAUTRIZEL R., DURET J., TRIBOULEY J. & RIPERT C. (1962b) Application de la réaction de conglutination au diagnostic sérologique des trypanosomoses. *Bull. Soc. Path. Exot.* **55,** 391.

PAUTRIZEL R., LAFAYE A. & DURET J. (1959) I. Diagnostic sérologique de la maladie du sommeil. Amélioration de l'antigène preparé à partir de *Trypanosoma equiperdum*. *Bull. Soc. Path. Exot.* **52,** 318.

PAUTRIZEL R., MATTERN P. & DURET R. (1960) II. Les anticorps fixant le complément au cours de l'infection. *Bull. Soc. Path. Exot.* **53,** 878.

PELLEGRINO J. (1951) Nota preliminar sôbre a reação intradérmica feita com a fração polissacarídea isolada de formas de cultura da *Leishmania brasiliensis* em casos de leishmaniose tegumentar Americana. *Hospital, Rio de Janeiro* **39,** 859. (*Trop. Dis. Bull.*, 1953, **50,** 696.)

PELLEGRINO J. & BRENER Z. (1952) A reação de fixação do complemento na doença de Chagas. III. Observações sôbre as propriedades de fração polissacarídea isolada de formas de cutura do *Schizotrypanum cruzi*. *Hospital, Rio de Janeiro* **42,** 553. (*Trop. Dis. Bull.* 1953, **50,** 493.)

PELLEGRINO J., BRENER Z. & JACOMO R. (1956) A reação de precipitina na fase aguda da doença de Chagas. *Rev. Brasileira Malariologia, Rio de Janeiro* **8,** 247. (*Trop. Dis. Bull.* 1957, **54,** 1300.)

PEWNY W. (1918) Präzipitinversuche bei Malaria. *Wein Klin. Wschr.* **31,** 205.

PIERCE A.E. (1947) Demonstration of an agglutinin to *Trichomonas foetus* in the vaginal discharge of infected heifers. *J. comp. Path.* **57,** 84.

PILCHARD E.I. & RISTIC M. (1963) An evaluation of serological techniques for the diagnosis of anaplasmosis and equine piroplasmosis. (*Vet. Bull.* 1964, **34,** 650.)

POWELL S.J., MADDISON S.E., WILMOT A.J. & ELSDON-DEW R. (1965) Amoebic gel-diffusion precipitin test. *Lancet* **ii,** 602.

REES C.W., BOZICEVICH J., REARDON L.V. & JONES F. (1942) A preliminary note on the complement fixation test for amoebiasis with antigens prepared from *Endamoeba histolytica* grown with a single species of bacteria. *Am. J. trop. Med.* **22,** 581.

REES C.W. & MOHLER W.M. (1934) Preliminary studies on the complement fixation test for the diagnosis of bovine anaplasmosis. *J. Amer. Vet. Med. Ass.* **38,** 669.

REIN C.R., BUKANTZ S.C., KENT J.F., COOPER W.C., RUHE D.S. & COATNEY G.R. (1949) Studies in human malaria XIX. The course of the complement fixation reaction in sporozoite-induced St. Elizabeth strain vivax malaria. *Amer. J. Hyg.* **49,** 374.

RIECKENBERG H. (1917) Eine neue Immunitätsreaktion bei experimenteller-Trypanosomen-Infektion: Die Blutplättchenprobe. *Z. Immun.-Forsch. I. Teil Orig.* **26,** 53.

RISTIC M., OPPERMANN I., SIBINOVIC S. & PHILLIPS T.N. (1964) Equine piroplasmosis—a mixed strain of *Piroplasma Cuballi* and *Piroplasma equi* isolated in Florida and studied by the fluorescent antibody technique. *Amer. J. Vet. Res.* **25,** 15.

RISTIC M., WHITE F.H. & SANDERS D.A. (1957) Detection of *Anaplasma marginale* by means of fluorescein-labelled antibody. *Am. J. Vet. Res.* **18,** 924.

ROBERTSON J.S. (1965) Toxoplasma skin and dye test surveys of severely subnormal patients in Lincolnshire. *J. Hygiene,* **63,** 89.

ROBERTSON M. (1941) Agglutination reactions of certain trichomonads in sera obtained from immunized rabbits, with particul ar reference to *Trichomonas foetus*. *J. Path. Bact.* **53,** 391.

ROBERTSON M. (1960) The antigens of *Trcho monas foetus* isolated from cows and pigs. *J. Hyg. (Camb.)* **58,** 207.

RODHAIN J. & VAN GOIDSENHOVEN C. (1944) Persistence prolongée de sensibilisatrice fixant l'alexine apres guérison de la trypanosomiase. *Ann. Soc. Belge Méd. trop.* **24,** 345.

RODHAIN J., VALCKE G. & VAN GOIDSENHOVEN C. (1941) Considérations sur le diagnostic et la thérapeutique de la méningo-encéphalite trypanosomique humaine d'après l'observation de quatre Européens atteints de la maladie du sommeil. Existence dans le

sang et le liquide encéphalorachidien des malades de sensibilisatrices fixant le complément. *Ann. Soc. Belge de Méd. Trop.* **21,** 195.

ROSE M.E. (1959) Serological reactions in *Eimeria stiedae* infection of the rabbit. *Immunology Oxford* **2,** 112.

SABIN A.B. & FELDMAN H.A. (1948) Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (Toxoplasma). *Science* **108,** 660.

SABIN A.B. & OLITSKY P.K. (1937) Toxoplasma and obligate intracellular parasitism. *Science* **85,** 336.

SADUN E.H., DUXBURY R.E., WILLIAMS J.S. & ANDERSON R.I. (1963) Fluorescent antibody test for the sero-diagnosis of African and American trypanosomiasis in man. *J. Parasitol.* **49,** 385.

SCHINDLER R. & DENNIG H.K. (1962) *Zeit Tropenmed. Parasit.* **13,** 480. (*Vet. Bull.* **1963,** v. **60,** 674.)

SCHOENAERS F., NEUJEAN G. & EVENS F. (1953) Valeur pratique de la réaction de fixation du complément dans la maladie du sommeil à *Trypanosoma gambiense* I^re^ Partie. Le diagnostic de la maladie. *Ann. Soc. belge Méd. Trop.* **33,** 141.

SENEKJIE H.A. & LEWIS R.A. (1944) Diagnosis of leishmaniasis by slide agglutination. *Proc. Soc. exp. Biol. (N.Y.)* **57,** 17.

SHAW J.J. & VOLLER A. (1964) The detection of circulating antibody to kala-azar by means of immunofluorescent techniques. *Trans. Roy. Soc. trop. Med. Hyg.* **58,** 349.

SIDDIQUI W.A. (1961) Demonstration of antigen-antibody reaction with a monobacterial culture of *Entamoeba histolytica* in agar-gel and on cellulose acetate membrane. *J. Parasitol.* **47,** 371.

SIIM J. CHR. & LIND K. (1960) A toxoplasma flocculation test. *Acta path. microbiol. Scand.* **50,** 445.

SKRABALO Z. & DEANOVIĆ Z. (1957) Piroplasmosis in man. Report of a case. *Docum. med. geogr. trop. (Amst.)* **9,** 11.

SOLTYS M.A. (1957) Immunity in Trypanosomiasis. II. Agglutination reaction with African Trypanosomes. *Parasitology* **47,** 390.

STEIN B. & DESOWITZ R.S. (1963) The measurement of antibody in human malaria by a formolized tanned sheep cell haemagglutination test. *World Health Organization.*/Mal./ **393,** 1.

STEIN B. & DESOWITZ R.S. (1964) The measurement of antibody in human malaria by a formolized tanned sheep cell haemagglutination test. *Bull. World Health Organization, Geneva* **30,** 45.

STRANNEGÅRD O. (1962) Studies of *Toxoplasma* precipitinogens and their corresponding antibodies by means of diffusion-in-gel methods. *Brit. J. exper. Path.* **43,** 600.

STRATMAN-THOMAS W.K. & DULANEY A.D. (1940) Complement fixation in human malaria. II. Diagnostic application. *J. Immunol.* **39,** 257.

TALIAFERRO W.H. (1930) *The Immunology of Parasitic Infections.* London: John Bale Sons & Danielsson Ltd.

TALIAFERRO W.H., TALIAFERRO L.G. & FISHER A.B. (1927) A precipitin test in malaria. *J. Preventive Med. (Baltimore)* **1,** 343.

THOMSON J.G. (1918) Preliminary note on the complement deviation in cases of malaria. A new aid to diagnosis. *Brit. med. J.* **ii,** 628.

THURSTON J.P. (1958) The effect of immune sera on the respiration of *Trypanosoma brucei in vitro. Parasitology* **48,** 463

TOBIE J.E. & COATNEY G.R. (1961) Fluorescent antibody staining of human malaria parasites. *Exper. Parasitol.* **11,** 128.

TORREALBA J.W. & CHAVES-TORREALBA J. (1964) *Rev. Inst. Med. Trop. São Paulo.* **6,** 252. (*Trop. Dis. Bull.*, 1965, **62,** 620.)

TOUSSAINT A.J. & ANDERSON R.I. (1965) Soluble antigen fluorescent-antibody technique. *Applied Microbiol.* **13,** 552.

TRUSSELL R.E. (1946) Microagglutination tests with *Trichomonas vaginalis. J. Parasit.* **32,** 563.

VOLLER A. (1962) Fluorescent antibody studies on malaria parasites. *Bull. World Health Organization, Geneva.* **27,** 283.

VOLLER A. (1964) Fluorescent antibody methods and their use in Malaria Research. *Bull. World Health Organ. Geneva,* **30,** 343.

VOLLER A. & BRAY R.S. (1962) Fluorescent antibody staining as a measure of malarial antibody. *Proc. Soc. exp. Biol. & Med.* **110,** 907.

WAGENER E.H. (1923) A skin reaction to extracts of *Leishmania tropica* and *Leishmania infantum. Univ. Calif. Publ. Zool.* **20,** 477.

WAGENER E.H. (1924) A precipitin test in experimental amoebic dysentery in cats. *Univ. Calif. Publ. Zool.* **26,** 15.

WALLACE J.M. & WORMALL A. (1931) *Red cell adhesion in trypanosomiasis of man and animals. Parasitology,* **23,** 346.

WELD J.T. & KEAN B.H. (1958) A factor in serum of human beings and animals that destroys *Trichomonas vaginalis. Proc. Soc. exp. Biol. Med.* **98,** 494.

WÉRY M. (1965) Indications d'immunofluorescence dans la toxoplasmose clinique et expérimentale. *Ann. Soc. Belge Méd. Trop.* **45,** 541.

WILLIAMS J.S., DUXBURY R.E., ANDERSON R.I. & SADUN E.H. (1963) Fluorescent antibody reactions in *Trypanosoma rhodesiense* and *T. gambiense* in experimental animals. *J. Parasitol.* **49,** 380.

WOLF A., COWEN D. & PAIGE B. (1939) Human toxoplasmosis: Occurrence in infants as an encephalomyelitis. Verification by transmission to animals. *Science,* **89,** 226.

ZAMAN V. (1960) Studies with the immobilization reaction in the genus *Entamoeba. Ann. Trop. Med. Parasitol.* **54,** 381.

ZAMAN V. (1964) Studies on the immobilization reaction in the genus *Balantidium. Trans. Roy. Soc. trop. Med. Hyg.* **58,** 255.

ZAMAN V. (1965) The application of fluorescent antibody test to *Balantidium coli. Trans. Roy. Soc. trop. Med. Hyg.* **59,** 80.

CHAPTER 6

DIAGNOSIS OF HELMINTH INFECTIONS

E.J.L. SOULSBY

INTRODUCTION

In many helminth infections the most satisfactory method of diagnosis is the detection of eggs or larvae in the stools, urine or blood of an infected individual or animal. However, in some diseases the cellular reaction around the helminth prevents the ready recovery of eggs (this is seen for instance in chronic schistosomiasis), and other helminth diseases are associated with larval stages of the life cycle (e.g. hydatid disease, trichinellosis and visceral larva migrans), there being no external evidence of infection in these cases and alternative diagnostic tests must be used. The serological tests used in the diagnosis of helminth infection fall into two main categories; those for individual diagnosis and those for the mass survey type of diagnosis. The latter type of test can allow for a certain degree of inaccuracy since the more specific and detailed tests can be applied to individuals at a later stage.

Immuno-diagnostic tests in helminth diseases usually show a wide range of cross reactions between parasites of various families and even various orders. Such cross reactions may be of diagnostic value since a more readily available parasite can be used as the source of antigen. Thus, the dog heartworm, *Dirofilaria immitis*, has been used to supply skin test antigen for the detection of human filarial infection. Nevertheless, the existence of such cross reactions may make it difficult to assess the significance of a positive serological reaction to a given antigen in areas where helminth disease is endemic. There is, therefore, a need for antigen preparations with a greater specificity. The provision of satisfactory materials for antigens is also another difficulty. Sometimes these

materials must be obtained from another host animal, thus material for use in hydatid diagnosis may be obtained from a domestic animal such as the pig. The stages of the parasite which are generally freely available may not correspond to those which are concerned in the disease process. For example, in visceral larva migrans in children, it is the early larval stage of the dog roundworm *Toxocara canis* which is responsible for disease, but usually only the adult worm from the dog is available as a source of antigen. Parasites can be obtained from other animals in which they normally occur or in which experimental infections can be induced, but such preparations may contain protein of the donor host and this may lead to anomalous results, especially in patients with chronic or malignant diseases.

A possible answer to these problems is the provision of antigenic material from parasites cultured in *in vitro* conditions where host protein does not occur. At the same time, the various developmental stages, which are concerned in the causation of disease in the host, may also be obtained by *in vitro* culture techniques.

The majority of the diagnostic tests used in helminth infections rely on the detection of antibody either by serological tests or by skin tests. They give no indication as to the severity of the infection or indeed whether the infection is still active. Attempts have been made to develop tests for the detection of antigen, particularly in the urine of infected persons, and such techniques might indicate more clearly the severity and activity of the infection. In addition to the diagnosis of infection, a serological test may be of value in assessing the efficiency of therapy. This is seen in schistosomiasis and hydatid disease.

Generally, immuno-diagnostic tests are used for human helminth infections and have only a minor role to play in the diagnosis of helminths in animals.

The serological tests which have been used include all the well-known methods such as complement fixation, precipitation, agglutination of particles or erythrocytes coated with antigen and fluorescent antibody techniques. Various 'micro' modifications of these have been developed to accommodate innovations such as the collection of small drops of blood on filter paper for subsequent extraction for serological tests and the collection of plasma in heparinized capillary tubes or on plastic coated cards. In addition, living adult or larval helminths have been used for *in vitro* tests, and many of these rely upon the formation of precipitates around the organisms or at the various natural orifices of larvae. Others depend on the agglutination or immobilization of infective stages. Skin tests are usually performed by the intradermal route and have their value in mass surveys rather than for individual diagnosis. The type of reaction is usually of the immediate type (Type I), but in some cases, particularly where the helminth infection is in the early stages of invasion, a delayed type (Type III) of reaction occurs.

In general, the incidence of parasitic disease in man is low in Great Britain,

and even when infection does occur stool examination usually offers a much better chance of a successful diagnosis than immunological diagnosis. Nevertheless, infections where stool diagnosis is of no value do occur. Hydatid infection occurs sporadically, and there is a prevalent need for immunological diagnosis of this infection. Trichinosis is considered to be generally rare, but several outbreaks have occurred in the last few decades and surveys at autopsy show a general incidence of about 10%, a figure as high as in many other countries. Visceral larva migrans, caused by *Toxocara* spp. of dogs and cats, is possibly more common in children than generally realized, and there is a pressing need for a reliable immuno-diagnostic test for this condition.

With the ease with which persons may travel from one part of the world to another, the hitherto exotic parasitic diseases may be frequently presented for diagnosis, and entities such as bilharziasis and filariasis may be encountered in persons from the tropics. In addition, these two diseases are of importance from a global point of view, and an understanding of the techniques available for their diagnosis is to be encouraged.

It is accepted that the antigens for the various tests described are not readily available, but they can usually be obtained from parasitological laboratories. In addition, a register of helminth antigens is maintained by the Parasitic Diseases Unit, World Health Organization, from which further information is available.

NEMATODES

Trichinosis (Trichinella Spiralis Infection)

This infection is usually acquired by eating raw or undercooked pork infected with the larval stages of the parasite. Clinical signs, which are associated with the migration of larvae to muscles, consist of muscular pain and difficulty in breathing, mastication and swallowing. Oedema is frequent around the eyes. Central nervous system involvement including headache or visual disturbance is usually transitory. Eosinophilia is often marked, sometimes reaching 70% of the total white count. Eosinophilia usually precedes the development of positive serological or skin tests.

Unless there is a recognized outbreak of trichinosis where the clinical signs are displayed and expected, a provisional clinical diagnosis is frequently made on the results of serological procedures. In symptomatic cases of trichinosis, whatever serological test is used, a correlation exists between the incidence of positive reactions and the incidence of clinical disease. In asymptomatic infections, however, especially when tests are used in surveys, considerable difficulty is often experienced in assessing the results, this being especially so with skin tests. In pigs the value of tests can be assessed by post mortem examination, but even in these anomalous reactions are frequently seen.

Sources of Antigen

A larval stage antigen is usually used for the various serological tests, and it is prepared from *T. spiralis* larvae harvested from an acid pepsin digest of the muscles of artificially infected animals such as mice. Less usually, adult stages of the parasites are utilized. A variety of antigenic preparations have been made over the years. These range from simple extracts of larvae with Coca's saline to an acid soluble protein fraction (Melcher 1943). The metabolic antigens from adult or larval worms are also used. It is likely that any one of the so-called antigens is in fact a complex mixture of antigens, and Tanner & Gregory (1961) have shown, by immunoelectrophoretic techniques, that eleven distinct antigens occur in a buffered saline extract of larvae. Details for the collection of larvae in large quantities and the preparation of antigens for the various serological tests are given by Kagan (1960).

Diagnostic Tests

Complement fixation test

This test was introduced by Strobel in 1911, and it has served for many years as the major serodiagnostic test for trichinosis. Using a boiled saline extract of lyophilized larvae, Witebsky, Wels & Heide (1942) found it superior to other tests in sensitivity and specificity. Of 700 sera, some of which were Wassermann positive, from persons free of trichinosis, all were negative to the complement fixation with *T. spiralis* antigen. Workers in Germany employed an extract of infected pork as antigen and found the complement fixation test to be the best method for the serological diagnosis of trichinosis in man (Gaase 1949). In an outbreak of trichinosis, the complement fixation test may detect antibodies as early as 2–3 weeks after infection, and they may persist for 10–13 months (Bozicevich 1951). Though it is still employed as a routine test in some laboratories, it has to a great extent been replaced by other, more simple, and equally efficacious, techniques such as the various flocculation tests.

Precipitin tests

The ring precipitin test (the overlaying of diluted *Trichinella* antigen on undiluted serum in small tubes) has been used extensively in the diagnosis of trichinosis. Its one advantage is that it is easy to perform; however, it is less sensitive than other tests and it may show false reaction following the use of certain chemotherapeutic agents, in certain neoplastic diseases or when the albumin:globulin ratio is markedly altered (Southam, Thomason & Burchenal 1949).

The micro-precipitin test which is a modification of the precipitin reaction, relies upon the use of living *T. spiralis* larvae. Larvae are incubated in the serum under examination, and, if antibody is present, after a number of hours

precipitates develop at the natural orifices of the larvae, i.e. the mouth, the excretory pore and the anus. Of necessity this test is one which can be carried out only in a specialized laboratory where a regular supply of *T. spiralis* infected animals exists. Roth (1946) considers it to be one of the most delicate tests available to the serologist for the detection of trichinosis. The reaction becomes positive in the 2nd or 3rd week of illness, and there appears to be some correlation between the strength of the reaction and the degree of infection since in heavy infections the precipitates on the larvae appear more quickly and are more pronounced. However, it is not a test which lends itself to routine diagnosis and it is not in general use.

Flocculation and agglutination tests

In 1944, Suessenguth & Kline adapted the Kline technique for the diagnosis of syphilis to the diagnosis of trichinosis. Later, Suessenguth (1947) reported that an alkaline extract of lyophilized whole trichina provided a satisfactory antigen for the slide flocculation test. Since then the test has been successfully used to detect infections in both man and pigs. For example, positive reactions were obtained in pigs as early as the 8th day after experimental infection, and the reaction persisted for at least 903 days. In a series of over 1,000 human sera from diagnosed cases of trichinosis, 97% were positive by this technique, and of 7000 sera from the general population, 9.7% gave positive reactions. In a further series of 1000 serum samples, 10% were found to be positive (Greene & Breazeale, 1952). These figures of positive reactions in the sera of the general population are in general accord with the incidence of trichinosis in the human population of the United States as judged by autopsy findings.

Following the adaptation of the Kline test for the diagnosis of trichinosis, other particles such as collodion, carmine and cholesterol were assessed as antigen carriers, but bentonite particles were found to be of most use (Bozicevich *et al* 1951). Norman, Donaldson & Sadun (1956) concluded that the bentonite flocculation technique could be substituted for the complement-fixation test since it is more sensitive than the latter. If a metabolic antigen, prepared from the excretions of trichina larvae incubated in balanced salt solutions, is used the technique is even more sensitive than when the Melcher antigen is employed (Norman & Sadun 1959). The bentonite flocculation test has been used for several years in the United States, and there it is considered to be one of the serological tests of choice for the diagnosis of trichinosis of man. Its ease and speed of performance, its simplicity, its ability to detect antibodies during the acute phase of the disease and the stability of the reagents all recommend it. Details for the preparation of reagents and antigens and the interpretation of the test are given by Kagan (1960).

A latex agglutination test has given results comparable to those reported for the bentonite tests (Innella & Redner 1959), and a further flocculation

technique has employed cholesterol–lecithin crystals coated with a buffered saline extract of ether-treated *Trichinella* larvae. The latter has been modified by the addition of charcoal to the suspension (Anderson, Sadun & Schoenbechler, 1963) and the test is performed on plastic coated cards. Known as the 'charcoal agglutination' test, it provides a simple, sensitive method which can be used in the field without a microscope. The test is also able to detect antibodies over an extended period of time. It has much to recommend it as a routine procedure and a test kit is available for use by relatively unskilled personnel.

A fluorescent antibody test has been developed by Sadun, Anderson & Williams (1962). This utilizes washed muscle larvae of *T. spiralis*, fixed in 10% formaldehyde in saline containing 0.5% bovine serum albumin. Test sera are reacted with the larvae and following adequate washing, any adsorbed globulin is detected by a fluorescein conjugated anti-species globulin serum. The test possesses a high degree of specificity and sensitivity and has given reliable quantitative results either with fresh serum or with serum extracted from dried blood specimens on absorbent filter paper. A recent innovation has been the substitution of the anti-species serum by a fluorescein conjugated anti-guinea-pig (or rat) complement serum (Crandall, Belkin & Saadallah, 1966). Preserved larvae are exposed to the test serum in the presence of a complement source and after adequate washing are stained with the anti-complement serum. The advantage of the latter technique is that only a single immunofluorescent reagent is required to demonstrate antibody in sera from different species. The disadvantage of the fluorescent antibody technique is that it can be performed only in laboratories with the necessary equipment and trained personnel.

Intradermal test

The intradermal test has been used extensively for the diagnosis of individual cases of trichinosis and also for the large-scale diagnosis of asymptomatic infections. A résumé of the earlier literature is given by Gould (1945). Because of the wide variety of antigens used, it is difficult to assess the significance of the intradermal test in the diagnosis of trichinosis. Skin sensitivity can persist for at least 10 years, and some reports suggest that a positive reaction may be present even 20 years after infection. Where the test is used for survey purposes it is extremely difficult to correlate positive results with the presence of infection. This has been attempted in pigs, but still the test has been unsatisfactory. Cross reactions can occur with other helminths, and a marked sensitivity to *Ascaris suum* may lead to positive reactions with *T. spiralis* antigen (Soulsby, 1957b). In a survey of 388 persons, Gould (1945) found that while eighty-nine were infected at autopsy, only nine of these had given a positive skin test when examined before death. Of the remaining 299 which were not infected, sixteen had given positive reactions. A higher incidence of positive skin reactions has been found in patients with active tuberculosis, but no cross reactions occur with

T. spiralis antigen in animals infected with *Mycobacterium tuberculosis*. Some suggestions have been made that the consumption of pork containing dead *Trichinella* larvae may be enough to sensitize an individual; however, the feeding of trichinous meat to animals did not induce skin sensitivity. A higher incidence of positive intradermal reactions is seen in institutions such as prisons, orphanages and hospitals than in the normal population, and no adequate explanation for this situation has yet been put forward. A significant incidence of positive intradermal reactions can be present in communities of orthodox Jews, for example, and such individuals presumably do not advertently consume pork; here again no adequate explanation is available. It is possible that such positive reactions may be due to cross reactions with other helminths, and these tend to be more abundant in institutional life than in normal everyday life.

Urine precipitin test

This test is based on the detection of antigens in the urine of man and animals infected with trichinosis, and is a modification of that originally described by Fleck (1946). The test serum is usually prepared in rabbits. Urine from infected persons is used, either as collected or after concentration by gentle heating, and is then overlaid on the immune serum in a Durham tube. Zapart (1961) states that specific diagnosis of infection can be made with the technique 2 weeks after the onset of illness. The pH of the urine appears to exert no influence on the test, and positive results were obtained from urine varying from pH 6–10. Polish workers have found the test positive in all proven cases of trichinosis. Studies by Machnicka-Roguska (1963) revealed antigen in the blood and urine of *T. spiralis* infected rabbits from the 3rd day after infection, which persisted for at least 30 days in the urine and 34 to 84 days in the blood. Despite the obvious advantage of being able to detect infection long before a detectable antibody response occurs, this technique has not gained wide acceptance. It does, however, warrant further investigation.

Immuno-Diagnostic Test for Swine Trichinosis

Though many tests are available to the clinician for the diagnosis of trichinosis, little has been done to devise a simple and reliable test for its detection in twine. These animals are the major source of human infection, and presens meat inspection procedures consist either of a naked eye examination of a carcase or trichinoscopy (a search for the parasite on the projected image of a small piece of muscle). Both methods, almost certainly, fail to detect a substantial number of infected carcases. Various tests have been shown to detect infection in experimentally infected swine; thus Norman *et al* (1955) found the bentonite flocculation test able to detect antibodies two weeks after larvae were fed, and recently Scholtens *et al* (1966) reported the charcoal agglutination and fluorescence antibody tests to be the most accurate indicators of infection. However, the need is

for a test which will detect low-level natural infections and which can be used as a very rapid diagnostic procedure on swine awaiting slaughter or preferably on their carcases after slaughter.

Filariasis

Filarial infections are generally acquired in tropical areas either through the bite of an insect vector as in the case of *Wuchereria bancrofti* (elephantiasis) and *Onchocerca volvulus* (blinding filariasis) or by the ingestion of an infected crustacean, as in *Dracunculus medinensis*, the guinea worm.

As well as the true filarial parasites of man, there is an increasing number of reports of the filarial parasites of dogs and wild mammals occurring in man. The dog heartworm has been found in nine instances in man, and *Dirofilaria tenuis* of the racoon has been incriminated as the cause of subcutaneous nodules and abscesses in man in the southern United States (Beaver 1966).

In filarial infections, serological tests have been used most frequently for the diagnosis of infections with *Wuchereria bancrofti* and *Onchocerca volvulus*, and they have yet to be applied to the diagnosis of aberrant infections of animal filarids in man. During the last war in the Far East, they were used for the differentiation of *W. bancrofti* from streptococcal lymphangitis, epidermophytal lymphadenitis or traumatic orchitis. Cross reactions occur between all the six species of filarids which occur in man, and consequently a positive intradermal test, though giving evidence of infection, will not indicate which species is present. It is difficult to obtain *W. bancrofti* specimens for the preparation of antigens, and related parasites such as the dog heartworm *Dirofilaria immitis* and *Setaria equina* from the horse have been used. Sawada *et al* (1962a and b) obtained satisfactory results in skin tests on infected humans with a trichloracetic acid precipitated antigen from *D. immitis*, and Tada & Kawashima (1964) obtained good results with comparable materials. Sawada *et al* (1965) have prepared a protein antigen from *D. immitis* which in 0.05 γ amounts gave good immediate skin reactions in patients with *Wuchereria bancrofti* infection. Reactions in patients with other helminth diseases, e.g. paragonimiasis, schistosomiasis, ascariasis and enterobiasis were weak.

A comparison of antigens prepared from *D. immitis*, *S. equina*, *Litomosoides carinii* from the mouse or *O. volvulus* from the human was made in an onchocerciasis endemic area in Guatemala. The skin test showed the *O. volvulus* antigen to be most sensitive and specific, but the *D. immitis* antigen came next in producing satisfactory results (Bozicevich *et al*, 1947). Since the dog parasite is more freely available than the human form, there seems to be no reason why it should not be used as a routine test antigen. However, an antigen from *Dipetalonema witei*, of the jird, has been shown to be a useful 'group' antigen for the immunoelectrophoretic diagnosis of onchocerciasis (Biguet *et al* 1964).

Ascarisis (Including Visceral Larva Migrans)

Specific antibodies have been detected in persons infected with *Ascaris lumbricoides* by several workers, and both the complement fixation and precipitin tests have been utilized for the detection of infection. Hypersensitivity to *A. lumbricoides* is well recognized, and cutaneous tests demonstrating this have also been used extensively in man as diagnostic aids. Invariably the antigen has been prepared from adult worms, either an extract of the whole worm or of one of its tissues, particularly the intestinal tissue. The almost universal finding has been that such tests show a lack of correlation between immunological reactions and the presence of infection as judged by the presence of eggs in the faeces. In intradermal tests this has been ascribed to the persistence of skin hypersensitivity for long periods. In a series of skin tests carried out on 273 pigs, Soulsby (1957a) showed that no correlation existed between the presence of positive reactions and the presence of adult worms in the bowel, but the incidence of positive reactions increased with the severity of the pathology produced by the migrating stages of the parasite. In man and in animals a delayed type of reaction may be present, and this is associated with the early phase of infection.

Visceral Larva Migrans

This occurs as a disease entity in children, usually under 4 years of age, and is caused principally by the migration in children of the larvae of the dog ascarid worm, *Toxocara canis*. Other nematodes such as *Toxocara cati* (of cats), *Ascaris suum* (of pigs), *Physaloptera* spp. (of monkeys), etc. occasionally may be the causal agents. Lesions are usually produced in the liver and lungs, and there is usually a high persistent eosinophilia. A more serious consequence is an eosinophilic granulomatous lesion in the retina which may cause impairment or loss of vision or enucleation of the eye on suspicion of malignancy.

The antigens which have usually been employed for diagnostic tests have been preparations of the adult dog ascarid, however, the larval stages responsible for the disease entity are the second stage larvae which may well have a different antigenic constitution from the adult stage. Antigens of *A. lumbricoides* from man or *A. suum* from pigs will produce positive reactions in children infected with *T. canis* larvae. Since such children may also be infected with intestinal *A. lumbricoides* or with both *Ascaris* and *Toxocara* larvae, the presence of a positive serological or skin reaction can give no more than an indication that the child is sensitive to ascaris antigens in general.

The serology of visceral larva migrans has been studied using haemagglutination and flocculation tests with various ascaris antigens such as polysaccharides, saline extracts and a protein antigen prepared by Melcher's method. Serological activity was demonstrated with all antigens, but no specificity as between the ascarid antigens was seen with sera from children presumed to be affected with

visceral larva migrans (Kagan, Norman & Allain 1959). Skin tests have been used by some investigators in the diagnosis of this condition.

Duguid (1961) found that skin tests with extracts of *T. canis* in twelve patients with ocular lesions produced a delayed response while no reaction occurred in normal individuals. Woodruff & Thacker (1964) utilized an extract of adult *T. canis* in skin tests on thirty-five patients with a history of past or present asthma, choroidoretinitis or other unexplained eosinophilia and concluded that eleven had been infected with *T. canis* or *T. cati* in the past. These authors extended their studies with the skin test for *Toxocara* infection in humans (Woodruff, Bisseru & Bowe 1966) and concluded that there was a statistically significant relationship between this infection (as determined by skin tests) and the occurrence of poliomyelitis and epilepsy.

Nevertheless, the antigens available from adult *Toxocara* for the immunodiagnosis of visceral larva migrans are still relatively unpurified, and liberal interpretation of reactions obtained with them should be guarded against.

Using agar gel and paper electrophoresis, Kent (1960) has separated various antigens from the water extract of adult females *A. lumbricoides*. Several antigens were obtained which appeared to be specific for *A. lumbricoides*, and when tested against sixteen sera from suspected cases of visceral larva migrans, only two antigens gave positive precipitin reaction in agar diffusion. The further chemical and physical analysis of *T. canis* by comparable methods may well result in specific antigens for this parasite and allow for the specific serological or allergic diagnosis of the condition.

Mention has been made of the fact that second stage larvae of *T. canis* are responsible for visceral larva migrans but that antigens from the adult parasite are normally used in diagnosis. To overcome this rather obvious potential source of difficulty, Olsen (1960) developed a test which utilized living second stage larvae of *T. canis*, which were incubated at 37°C in sealed hanging drop preparations of test serum and streptomycin. Precipitates developed at the mouth, excretory pore and anus of larvae in the presence of infected serum. A survey of seventy children, randomly selected at a clinic in Galveston, Texas, and not considered as clinical cases of visceral larva migrans, showed two strongly positive to the test, these also each having a high eosinophilia. Other children infected with *Trichuris* or *Ascaris* had negative and weak reactions respectively (Richards, Olsen & Box 1962).

A similar investigation to the above was carried out by Hogarth-Scott (1966), who used a fluorescent antibody technique. In two children, in whom ascaroid larvae were detected histologically in a liver biopsy and in an enucleated eye, respectively, antibodies were detected at the various pores of the second stage of *T. canis* larvae by the fluorescent antibody technique. Examination of the sera from two groups of suspect cases of visceral larva migrans showed eight of twenty-eight and three of ten to have antibodies to *T. canis* present. Sera from

healthy people chosen at random, laboratory workers allergic to nematodes and persons infected with *Ascaris* or *Ancylostoma* failed to react in the test (Hogarth-Scott, 1966).

Since Loeffler's syndrome may be associated with sensitization with various helminths, it is likely that positive reactions will be obtained with *Ascaris* antigens in skin tests, and this should be kept in mind in suspected cases of visceral larva migrans.

CESTODES

Hydatid Infection (Echinococcosis)

This is acquired by the ingestion of eggs of the dog tapeworm *Echinococcus granulosus* and occasionally of those of the closely related form of dogs and foxes, *Echinococcus multilocularis*. In man, who serves as an intermediate host, the larval stage, hydatid cyst, is usually found in the liver or the lungs and in these sites may reach a considerable size. The larval stage of *E. multilocularis* in man may show continual proliferation of tissue and a tendency to invade the organs, chiefly the liver.

In general, the presence of an active cyst is associated with the production of antibodies and the rupture of the cyst frequently produces a marked rise in antibody levels. Surgical removal, as well as suppuration, degeneration and calcification of a cyst results in a marked reduction of antibody levels.

The source of antigen is usually from a host species other than man, though occasionally human hydatid material has been used. The hosts most commonly used are the pig and the sheep, but Norman & Kagan (1961) have prepared antigens for skin tests from larval *E. multilocularis* rather than the hydatid fluid of *E. granulosus*. *E. multilocularis* is readily maintained by serial passage in the peritoneal cavity of gerbils, and uniform batches of antigen can be prepared from this material

Diagnostic Tests

Complement fixation test

The complement fixation test for hydatid disease was first introduced by Ghedini (1906), and usually unpreserved hydatid fluid from human or sheep cysts was used as antigen. Throughout the years this test has provided a valuable diagnostic test for living cysts in all locations of the body; where cerebral involvement is suspected cerebrospinal fluid can be substituted for serum. The complement fixation test can also be used as a criterion of successful operation. In the case of unsuccessful removal of all hydatid cysts, the antibody titre remains high.

Despite its long history of usefulness, the complement-fixation test has a lower sensitivity than other tests (e.g. passive haemagglutination), and non-

specific reactions may occur, especially in patients with cancer (Gräfe 1964). The success of the complement fixation tests depends on the provision of a satisfactory supply of antigens. In general, hydatid cysts which are fertile, that is, those which contain scolices, are more satisfactory for serological tests than those that are sterile. Consequently, hydatid material from sheep or from the pig is much more satisfactory than that from the bovine. Attempts to increase the sensitivity of the complement-fixation technique have included the use of a conglutination system (Pautrizel & Bailenger 1961) and a semi-quantitative micromethod (Pauluzzi 1964).

Passive haemagglutination test

The haemagglutination test is being increasingly used in the diagnosis of echinococcosis since its introduction by Garabedian, Matossian & Djanian (1957). It is more sensitive than the complement fixation test and has a high level of specificity. Kagan *et al* (1966) found no reaction with it in sixty-seven non-hydatid infected persons (including some with cancer, other miscellaneous diseases and *Taenia saginata* infection), and thirty-two of thirty-nine persons with liver hydatid gave positive reactions while six of eighteen with lung hydatid reacted. The difference in reactivity between hepatic and pulmonary hydatid infection has been noted by several workers (e.g. Abrabatzis & Papapanagiotou 1963; Cowling 1964), but the reason for this is not yet known.

Patients with active hydatid cysts may show passive haemagglutination titres up to 1/120,000, but between titres of 1/50 to 1/200 there is a proportion of non-specific reactions which has been traced to cross reactions between host proteins in the tissues and fluids of the hydatid parasite and antibodies in the sera of patients suffering from various diseases, especially hepatic disease (Kagan *et al* 1960). Kagan (1963) has suggested the use of a titre of 1/400 as a positive diagnostic reaction, and using this criterion only 5 of 175 sera of patients with other diseases were positive. Other workers have employed similar dilutions as significant positive reactions.

Sheep or pig hydatid fluid is normally used for the passive haemagglutination test. The use of formalinized red cells has greatly aided standardization of the procedure: such cells, tanned and coated with antigen, can be frozen or lyophilized and kept for at least 18 months with little or no loss of activity (Kagan 1963).

Flocculation tests

The bentonite flocculation test for hydatid disease was introduced by Norman, Sadun & Allain (1959). It is slightly less sensitive than the passive haemagglutination test but still provides a very adequate diagnostic procedure. In their comparison of serological tests, Kagan *et al* (1966) found it equal to the passive haemagglutination test in detecting hydatid infected individuals, but it was

slightly less specific, giving reactions in 8% of normal persons or those with other diseases. Cyst fluid from pig is recommended as the antigen of choice.

A latex flocculation test was introduced by Fischman (1960). It was later modified by Szyfres & Kagan (1963), who reported it to be equal in sensitivity to the passive haemagglutination and the bentonite tests with positive sera. Fischman (1965) found that cyst fluid from human hydatids was the most satisfactory antigen for the latex test, and best results were obtained when serum was used in the non-inactivated state. This test has much to recommend it since it is simple to perform, and Fischman (1965) recommends it as a full diagnostic test (as opposed to a screen test) possibly supported by a carefully standardized complement fixation test as a secondary technique for positive reactors. In comparative studies Kagan *et al* (1966) found the latex test equal to the bentonite technique in specificity and approximately equal in sensitivity.

Other serodiagnostic tests for echinococcosis which are being developed are a fluorescent antibody technique using protoscolices of fertile hydatid cysts (Sorice, Castagnari & Tolu 1966) and the 'scolexo-precipitation', which is an *in vitro* test utilizing living protoscolices (Schulz & Ismogilova 1962).

Intradermal test

Originally introduced by Casoni (1911), this test has had wide use in all parts of the world and is still in use as a method of diagnosis. It is possibly the most sensitive of the diagnostic tests, but its specificity may at times be alarmingly low. This is due to several factors, including the high level of skin reactivity in comparison to clinical evidence of infection, the persistence of reactivity following death or surgical removal of the cyst, but a major source of lack of specificity may be, in many cases, traced to the lack of any standardization of the antigen used for the test. Very frequently, crude hydatid fluid of unknown N content has been used, and the volumes injected have varied from 0.05 ml to 1.0 ml. The nitrogen content of hydatid cyst fluids may vary greatly, and for this reason Kagan *et al* (1966) recommend the use of a skin test antigen prepared from *E. multilocularis* which can be produced under laboratory conditions. These authors found that high concentrations of nitrogen in the skin test antigen led to many false positive reactions in control individuals, amounts over 100 γN/ml giving 30 to 40% positive reaction in normal patients. The specificity of the test increased as the antigen N was decreased, and Kagan *et al* (1966) found 12 to 15γ antigen N/ml to give the highest specificity.

As well as standardization of the antigen nitrogen, there is also a need to standardize the criteria for a positive reaction. The reaction is best measured with the stencil technique described by Pellegrino & Macedo (1956) from which the area of the wheal and erythema can be calculated.

If used with the above facts in mind, the intradermal test can serve as a useful epidemiological tool.

TREMATODES

Schistosomiasis (Bilharziasis)

This disease occurs in the tropics and the Far East. Infection is produced by a skin penetrating larval stage (cercaria), and the adult worms live in the venules draining the bowel or bladder.

The majority of diagnostic work has been concerned with the human schistosomes *S. japonicum*, *S. haematobium* and *S. mansoni*. A large number of immunodiagnostic tests have been used over the last 50 years, but until recently all have suffered from a lack of standardization of techniques and antigens. In the absence of such, opinions have differed about the value of the tests as diagnostic aids, particularly with an infection which may show many grades of severity. A critical review of the immunological methods for the diagnosis of schistosomiasis is given by Kagan & Pellegrino (1961).

Source of Antigen

Antigens prepared from the homologous parasite are the most satisfactory for immunological work, however, if this is not possible then other human strains may suffice. Cercariae from snails and adult worms from laboratory animals are usually used, and the modern techniques in practice in various parasitological laboratories now provide a readily available supply of material produced from homologous parasites.

Diagnostic Tests

Complement fixation test

This test has a high degree of sensitivity and specificity using either cercarial or adult worm antigen. The most satisfactory antigenic preparation appears to be that of Chaffee, Bauman & Shapilo (1954), modified by Anderson (1960). This is essentially a triethanolamine buffered saline extract of lipid-free cercariae or adult worms. One of the advantages of the complement fixation test is that it will detect infection before worms are mature and at the egg producing stage. The complement fixation test remains positive for long periods after active infection has ceased and in endemic areas the antibody titre of the serum may not decline for several years. It does not provide a satisfactory means of assessing therapeutic cure, but despite this the complement fixation test is regarded as a reliable test when performed under satisfactory conditions.

Flocculation tests

A slide flocculation test has recently been described for the diagnosis of *S. mansoni* infections by Anderson (1960). This is essentially a modification of the VDRL for syphilis and consists of cholesterol lecithin crystals coated with cercarial

antigen prepared in the Chaffee manner. It is a simple and sensitive test for the diagnosis of schistosome infections. Cross reactions occur between *S. mansoni* antigen and sera from patients with *S. japonicum* and *S. haematobium* infections, but this is not surprising and the findings are consistent with those obtained by other workers using soluble antigens. The main value of the slide flocculation test lies in its reliability, relative simplicity and economy. Its value as an epidemiological tool has been enhanced by the development of a 'plasma card test' (Sadun, Anderson & Schoenbechler 1963), which utilizes plasma from a finger-prick blood sample mixed on a plastic coated card surface with the antigen suspension and charcoal. The result of the test can be read in a few minutes after rotation. Evaluation of this technique in Africa (Sadun *et al* 1963) and Brazil (Pellegrino 1963) have shown it to be very useful as a screening test.

Fluorescent antibody tests

The fluorescent antibody technique using fluorescein labelled anti-human globulin has been developed for the serological diagnosis of bilharziasis in man by Sadun, Williams & Anderson (1960). It has the advantages that it is easily standardized and appears to be as sensitive and as specific as the complement fixation test and the slide flocculation test. Originally, its main difficulty was the need for fresh cercariae and fresh serum; however, Sadun, Anderson & Williams (1961) have modified the techniques for use with dried blood smears on filter paper. In this way minute drops of blood from finger pricks can be obtained in endemic areas, placed on filter paper and mailed to a central laboratory and there extracted for the fluorescent antibody technique. To obviate fresh cercariae, a method has been deveoped by Sadun *et al* (1961) whereby cercarial specimens can be stored for varying periods of time without demonstrable loss of activity. Such a technique, in which all reagents can be standardized, seems to be well suited for wide-scale investigations of human infections. By using dried blood smears on filter paper, it can be envisaged that a large number of specimens may be collected in endemic areas with ease by relatively untrained personnel.

A soluble antigen fluorescent antibody (SAFA) technique for the diagnosis of parasitic diseases has been developed by Toussaint & Anderson (1965). This employs the indirect fluorescent antibody procedure with a soluble antigen fixed to an artificial matrix such as cellulose acetate paper discs punched from 'millipore' filter paper. The results are read in a fluorometer. Advantages of the technique include the ability to select a soluble antigen for the test and the avoidance of highly subjective microscopic interpretation of fluorescence. Toussaint (1966) has improved this technique for the diagnosis of schistosomiasis using a soluble antigen prepared in the Chaffee manner from adult *S. mansoni*. Comparison of the test with the complement fixation procedure on sera from a schistosomiasis endemic area showed good correlation between the two tests

and specificity was excellent in that none of forty sera from healthy donors reacted.

Serological tests using living stages of schistosomes

The *circumoval precipitin test* was described by Oliver-Gonzalez (1954) and arose from the observation that when living schistosome eggs are placed in serum from infected persons or monkeys, precipitates occur around the eggs. The precipitates result from the reaction of specific antibodies with secretions which have diffused through the egg membrane and which are produced by the living miracidium within the egg. The antibodies responsible are specific for the egg and can be absorbed from serum only with living eggs. The circumoval reaction also shows some species specificity since no cross reaction occurs when the eggs of *S. haematobium* or *S. japonicum* are placed in *S. mansoni* serum and only a slight reaction occurs when *S. mansoni* eggs are incubated in serum from *S. haematobium* or *S. japonicum* patients. The reaction is more intense in the chronic egg-producing stage of infections than in acute or recent infections. Since the reaction decreases in intensity after treatment and may be negative 6 months after successful therapy, the test may be of use in evaluating treatment. When properly performed the circumoval precipitin test has high diagnostic value. However, it is too complicated and time consuming a test for routine use in mass diagnosis, but it may be of immense value in individual cases of infection.

The Cercarienhüllenreaktion (*CHR*) of Vogel & Minning (1949) has attracted attention as a diagnostic test. The reaction is characterized by the formation of a membrane or envelope around the body and tail of living cercariae when they are placed in immune serum. The reaction has been used in the diagnosis of bilharziasis by several workers, and the CHR reaction will detect early infection, the antibody causing the reaction appearing in the serum 40–47 days after experimental infection. Since the reaction becomes negative 5–7 months after successful therapeutic treatment, it is possible that it can be used as an index of the efficiency of therapy.

Other tests using living stages of schistosomes include the cercarial agglutination test and the miracidial immobilization test. However, the use of such tests is limited to laboratories equipped for research on schistosomiasis, and though they may provide useful adjuncts to other diagnostic tests, the necessity to handle living, and at times pathogenic cercariae, make them undesirable for use on a large scale.

Urine precipitin reaction

The principle of this test is that antigen or other substances in the urine of infected persons will react with antiserum against schistosomes prepared in a rabbit. The majority of this work has been carried out in *S. japonicum* infections in the Far East. Okabe & Tanaka (1958) reported that in all instances, urines

from infected persons were positive with this reaction, whereas normal individuals were negative. Cross reactions with urine from patients with other trematode infections were negative. The test appears to be of most value for *S. japonicum* infections since when used with *S. mansoni* it was not satisfactory. No information is available concerning the value of this test in *S. haematobium* infections. This test may detect early schistosomiasis, and in some acute cases a positive reaction was obtained within the month after infection. A fuller evaluation of this test is required.

Intradermal test

The intradermal test has been used extensively in the immunological diagnosis of bilharziasis, and practically every stage of the life cycle of the schistosome parasite has, at one time, been used as an antigen.

In infected individuals the intradermal reaction becomes positive about 4–8 weeks after infection and thereafter persists for many years even after satisfactory treatment. The sensitivity of the intradermal test is difficult to evaluate since a diversity of antigens and criteria of interpretation have been used throughout the years. It is, however, recognized that the intensity of reactions is greater in adults than in children, the reaction is more pronounced in men than in women, and similarly it is more intense in coloured persons than in white (Kagan, Pellegrino & Memoria 1961). The intradermal test in adults will usually reveal a larger number of positive cases than would be found by a stool examination alone; however, in children the intradermal test does not give such favourable results, and this is especially true when the tests are made on the forearm. In fact the site chosen for test in the child appears to be of particular importance, and a higher degree of sensitivity is found when the test is performed on the back than on the forearm. In spite of the sensitivity of the intradermal test, a positive reaction is not sufficient evidence to confirm a clinical diagnosis of bilharziasis since sensitivity of the skin can persist for a considerable time. A variety of antigens can be used, but preparations of adults or cercariae of *S. mansoni* are most commonly employed. No significant differences have been noted with antigens from various sources if the nitrogen content of the antigen was adjusted to the same level before injection (Kagan *et al* 1961).

As a diagnostic test the intradermal test can best be used for epidemiological surveys when large numbers of persons can be screened by a relatively simple technique.

Other Trematode Infections

Fascioliasis

Fasciola hepatica infection in man is usually due to the consumption of raw watercress (occasionally other raw vegetables) collected from vector snail infested land or streams. Pautrizel *et al* (1962) successfully used a skin test in the diagnosis

of human infections, the antigen being prepared from adult *F. hepatica*. An antigen nitrogen content of 0.03 mg/ml proved most sensitive and specific. Other French workers, Capron *et al* (1964), have developed an immunoelectrophoretic diagnostic test for the infection.

Clonorchiasis

Infection with *Clonorchis sinensis*, the Chinese liver fluke, is widespread in the Far East. Infection is acquired through the consumption of raw fish containing the metacercariae of the parasite. Immuno-diagnostic tests available include complement fixation and a skin test. Sadun *et al* (1959) and Sawada *et al* (1964) have produced a purified protein antigen which in 0.3γ amounts produces a marked immediate type skin reaction in *C. sinensis* infected persons. Cross reactions were minimal in persons infected with a number (12) of other helminth parasites.

Paragonimiasis

The lung fluke *Paragonimus westermani* is a major public health problem in the Far East. Infection is acquired through the consumption of fresh water crabs or crayfish infected with metacercarie. Immuno-diagnostic tests available include the complement fixation test (Yokogawa, Tsuji & Okura 1962), which usually shows a close relationship to the presence of parasites and becomes negative in a comparatively short time after a parasitological cure, and an intradermal test (Hunter *et al* 1958). The latter is sensitive and relatively specific, but a positive reaction will persist for several years after complete parasitological cure. Capron *et al* (1965) have attempted immunoelectrophoretic diagnosis of paragonimiasis but have found the method unsatisfactory when the level of serum precipitins is low.

REFERENCES

Abrabatzis G. & Papapanagiotou J. (1963) Laboratory Tests in Hydatid Disease: A comparison of the indirect hemagglutination, the complement fixation and the intradermal tests. *Bull. Wld. Hlth. Org.* **28,** 266.

Anderson R.I. (1960) Serologic diagnosis of *Schistosoma mansoni* infections. 1. Development of a cercarial antigen slide flocculation test. *Amer. J. trop. Med.* **9,** 299.

Anderson R.I., Sadun E.H. & Schoenbechler M.J. (1963) Cholesterol-lecithin slide (TsSF) and charcoal card (TsCC) flocculation tests using an acid soluble fraction of *Trichinella spiralis* larvae. *J. Parasit.* **49,** 642.

Beaver P.C. (1966) Zoonoses, with particular reference to parasites of veterinary importance. In *Biology of Parasites*, ed. Soulsby E.J.L. New York and London: Academic Press.

Biguet J., D'Haussy R., Aubry M. & Rose F. (1964) Étude des anticorps precipitants chez les sujets atteints d'onchocercose. *Bull. Soc. Path. exot.* **57,** 1098.

Bozicevich J. (1951) Immunological diagnosis of parasitic diseases. In *Parasitic Infections in Man*, ed. Most H. New York: Columbia University Press.

BOZICEVICH J., DONOVAN A., MAZZOTTI L., DIAZ F.A. & PADILLA E. (1947) Intradermal and complement fixation reactions elicited by various antigens in persons infected with *Onchocerca volvulus*. *Amer. J. trop. Med.* **27,** 51.

BOZICEVICH J., TOBIE J.E., THOMAS E.H., HOYEM H.M. & WARD S.B. (1951) A rapid flocculation test for the diagnosis of trichinosis. *Publ. Hlth. Rep.* (*Wash.*) **66,** 806.

CAPRON A., BIGUET J., TRAN VAN KY P. & ROSÉ G. (1964) Possibilités nouvelles dans le diagnostic immunologique de la distomatose humaine à *Fascioloa hepatica*. Mise en évidence d'anticorps sérique par immunoélectrophorese. *Presse Med.* **72,** 3101.

CAPRON A., YOKOGAWA M, BIGUET J., TSUJI M. & LUFFAU G. (1965) Diagnostic immunologique de la paragonimose humaine. Mise en evidence d'anticorps sériques specifiques par immunoélectrophorese. *Bull. Soc. Path. exot.* **58,** 474.

CASONI T. (1911) La diagnosi biologica dell'Echinococcosi Umana mediante l'Intradermoreazione. *Fol. Clin. Chem. Micro* **4,** 5.

CHAFFEE E.F., BAUMAN P.M. & SHAPILO J.J. (1954) Diagnosis of schistosomiasis by complement fixation. *Amer. J. trop. Med.* **3,** 905.

COWLING D.C. (1964) Hydatid Disease: a summary of laboratory and other findings at the Royal Melbourne Hospital. *Med. J. Australia* **1,** 146.

CRANDALL R.B., BELKIN L.M. & SAADALLAH S. (1966) Complement Staining with Fluorescent Antibody for Detection of Antibody in Experimental Trichinosis. *J. Parasit.* **52,** 1219.

DUGUID I.M. (1961) Features of ocular infestaton of Toxocara. *Brit. J. Ophthal.* **45,** 789.

FISCHMAN A. (1960) A rapid latex test for hydatid disease. *New Zealand Med. J.* **59,** 485.

FISCHMAN A. (1965) Reactivity of latex and complement fixation tests in hydatid disease. *J. Parasit.* **51,** 497.

FLECK L. (1946) Swoiste substancje antygenowe w moczu chorych na dur plamisty. *Pol. Med. Wkly.* (*Pol. Tyg. lek.*) **2,** 663.

GAASE A. (1949) Der immunobiologische Nachweis der Trichinose bei Mensch und Tier. *Z. Hyg. Infekt.-Kr.* **129,** 570.

GARABEDIAN G.A., MATOSSIAN R.M. & DJANIAN A.Y. (1957) An indirect hemagglutination test for hydatid disease. *J. Immunol.* **78,** 269.

GHEDINI G. (1906) Richerche sul siero di sangue di individuo affetto da cisto da echinococco e sul liquido in essa contenuto. *Gazz. Osp.* (*Milan*), **27,** 1616.

GOULD S.E. (1945) *Trichinosis*. Springfield, Ill.: Charles C. Thomas.

GRÄFE H.H. (1964) Kritischer Beitrag zur Serodiagnostik der Echinokokkose des Menschen. *Arch. Hyg. Bakt.* **148,** 367.

GREENE R.A. & BREAZEALE E.L. (1952) The incidence of *Trichinella spiralis* infections in an asymptomatic group as indicated by the Kline test. *Ariz. Med.* **8,** 36.

HOGARTH-SCOTT R.S. (1966) Visceral Larva Migrans—An Immunofluorescent Examination of Rabbit and Human Sera for Antibodies to the ES antigens of the Second Stage Larvae of *Toxocara canis*, *Toxocara cati* and *Toxascaris leonina* (Nematoda). *Immunology* **10,** 217.

HUNTER G.W.III, RITCHIE L.S., PAN C., LIN S., SUGIURA S., NAGANO K. & YOKOGAWA M. (1958) Immunological Studies—II. Intradermal tests and their application in the field for the detection of schistosomiasis japonica, paragonimiasis and clinorchiasis. *Milit. Med.* **122,** 85.

INNELLA R. & REDNER W.J. (1959) Latex-agglutination serologic test for trichinosis. *J. Amer. med. Ass.* **171,** 885.

KAGAN I.G. (1960) Trichinosis: a review of biologic, serologic and immunological aspects. *J. infect. Dis.* **107,** 65.

KAGAN I.G. (1963) Seminar on immunity to parasitic helminths. VI. Hydatid disease. *Exptl. Parasit.* **13,** 57.

KAGAN I.G., NORMAN LOIS & ALLAIN DOROTHY S. (1959) Studies on the serology of visceral larva migrans. I. Hemagglutination and flocculation tests with purified Ascaris antigens. *J. Immunol.* **83,** 297.

KAGAN I.G., NORMAN LOIS, ALLAIN DOROTHY S. & GOODCHILD C.G. (1960) Studies on Echinococcosis: non-specific serologic reactions of hydatid-fluid antigen with serum of patients ill with diseases other than echinococcosis. *J. Immunol.* **84,** 635.

KAGAN I.G., OSIMAN J.J., VARELA J.C. & ALLAIN DOROTHY S. (1966) Evaluation of intradermal and serologic tests for the diagnosis of hydatid disease. *Amer. J. trop. Med.* **15,** 172.

KAGAN I.G. & PELLEGRINO J. (1961) A critical review of immunological methods for the diagnosis of bilharziasis. *Bull. W.H.O.* **25,** 611.

KAGAN I.G., PELLEGRINO J. & MEMORIA J.M.P. (1961) Studies on the standardization of the intradermal test for the diagnosis of bilharziasis. *Amer. J. trop. Med.* **10,** 200.

KENT H.N. (1960) Isolation of specific antigens from *Ascaris lumbricoides* (var. *suum*). *Exptl. Parasit.* **10,** 313.

MACHNICKA-ROGUSKA B. (1963) The occurrence of antigen in the blood and urine of rabbits in the course of experimental infection with *Trichinella spiralis. Wiadom. Parazytol.* **9,** 465.

MELCHER L.R. (1943) An antigenic analysis of *Trichinella spiralis. J. infect. Dis.* **73,** 31.

NORMAN LOIS, DONALDSON A.W. & SADUN E.H. (1956) The flocculation test with a purified antigen in the diagnosis of trichinosis in humans. *J. infect. Dis.* **98,** 172.

NORMAN LOIS & KAGAN I.G. (1961) The maintenance of *Echinococcus multilocularis* in gerbils (*Meriones unguiculatus*) by intraperitoneal inoculation. *J. Parasit.* **47,** 870.

NORMAN LOIS & SADUN E.H. (1959) The use of metabolic antigens in the flocculation test for the diagnosis of trichinosis. *J. Parasit.* **41,** 476.

NORMAN LOIS, SADUN E.H. & ALLAIN DOROTHY S. (1959) A bentonite flocculation test for the diagnosis of hydatid disease in man and animals. *Amer. J. trop. Med.* **8,** 46.

NORMAN LOIS, SADUN E.H., REDDING R.W. & COOPERRIDER D.E. (1955) Flocculation-test in sera from hogs experimentally and naturally infected with *Trichinella spiralis. J. Parasit.* **41,** 162.

OKABE K. & TANAKA T. (1958) A new urine precipitin reaction for *Schistosomiasis japonica*, a preliminary report. *Kurume. med. J.* **5,** 45.

OLIVER-GONZALEZ J. (1954) Anti-egg precipitins in the serum of humans infected with *Schistosoma mansoni. J. infect. Dis.* **95,** 86.

OLSON L.J. (1960) Serology of visceral larva migrans: *in vitro* larval precipitate test. *Texas Rep. Biol. Med.* **18,** 473.

PAULUZZI S. (1964) La fissazione del complemento per l'idatidose. II. Standardizzazione biologica del potere antigene del liquido cistico e di altri materiali idatidei. *Boll. Ist. Sieroter. Milan.* **43,** 1.

PAUTRIZEL R. & BAILENGER J. (1961) Diagnostic immunologique de l'echinococcose. *Ann. Biol. Clin.* **19,** 243.

PAUTRIZEL R., BAILENGER J., DURET J. & TRIBONLEY J. (1962) Utilization d'un antigene distomien délipidé dans le diagnostic allergique de la distomatose a *Fasciola hepatica. Rev. Immunol.* **26,** 167.

PELLEGRINO J. (1963) Immunological methods for the diagnosis of schistosomiasis mansoni. *Proc. 7th Congr. Trop. Med. & Malar.* 1963, p. 21.

PELLEGRINO J. & MACEDO D.G. (1956) Novo criterio de lectura de reaçao intradermica na esquistossomose. *Rev. Brasil. Malariol.* **8,** 499.

RICHARDS B., OLSON L.J. & BOX D.T. (1962) Survey of Galveston children for antibodies to *Toxocara canis*. *J. Parasit.* **48,** 501.

ROTH H. (1946) Employment of serological and skin tests at outbreaks of Trichinosis in the Alingoås and Boras districts (Sweden). *Acta med. scand.* **126,** 17.

SADUN E.H., ANDERSON R.I. & SCHOENBECHLER M.J. (1963) A plasma card test for rapid serodiagnosis of schistosomiasis (SPC). *Proc. Soc. Exp. Biol. (N.Y.)* **112,** 280.

SADUN E.H., ANDERSON R.I. & WILLIAMS J.S. (1961) Fluorescent antibody test for the laboratory diagnosis of Schistosomiasis in humans by using dried blood smears on filter paper. *Exp. Parasit.* **11,** 117.

SADUN E.H., ANDERSON R.I. & WILLIAMS J.S. (1962) Fluorescent antibody test for the serological diagnosis of trichinosis. *Exptl. Parasit.* **12,** 423.

SADUN E.H., WALTON B.C., BUCK A.A. & LEE B.K. (1959) The use of purified antigens in the diagnosis of clonorchiasis sinensis by means of intradermal and complement fixation tests. *J. Parasit.* **45,** 129.

SADUN E.H., WILLIAMS J.S. & ANDERSON R.I. (1960) Fluorescent antibody technic for sero-diagnosis of Schistosomiasis in Humans. *Proc. Soc. exp. Biol. (N.Y.)* **105,** 289.

SAWADA T., KONO M., SATO S., YAMAMOTO T. & TAKEI K. (1962a) Intradermal and precipitin tests with *Dirofilaria immitis* antigen in canine and human filariasis. *Gunma J. Med. Sci.* **11,** 7.

SAWADA T., KONO M., SATO S., YAMAMOTO T. & TAKEI K. (1962b) Intradermal tests with purified antigen in canine and human filariasis. *Gunma J. Med. Sci.* **11,** 7.

SAWADA T., NAGATA Y., TAKEI K. & SATO S. (1964) Studies on the substance responsible for the skin tests on clonorchiasis. *Jap. J. Expt. Med.* **34,** 315.

SAWADA T., TAKEI K., KATAMINE D. & YOSHIMURA T. (1965) Immunological Studies on Filariasis. III. Isolation and Purification of antigen for intradermal skin test. *Jap. J. Exp. Med.* **35,** 125.

SCHOLTENS R.G., KAGAN I.G., QUIST K.D. & NORMAN LOIS (1966) An evaluation of tests for the diagnosis of trichinosis in swine and associated quantitative epidemiological observations. *Amer. J. Epidemiol.* **83,** 489.

SCHULZ R.S. & ISMOGILOVA R.G. (1962) (A new immunological reaction in hydatidosis). *Bestnik Selskokhozyaistvennoi Nauki Alma-Ata.* **6,** 45. (In Russian.)

SORICE R. CASTAGNARI L. & TOLU A. (1966). La diagnosi biologica dell'idatidosi amana *Giorn. Malatt. Infett. Parass.* **18,** 192.

SOULSBY E.J.L. (1957a) Precipitin and intradermal tests in *Ascaris lumbricoides* infection in pigs. *Brit. vet. J.* **113,** 492.

SOULSBY E.J.L. (1957b) Intradermal tests on pigs with antigens prepared from *Trichinella spiralis* and *Ascaris lumbricoides*. *Brit. vet. J.* **113,** 447.

SOUTHAM C.M., THOMASON A.E. & BURCHENAL J.H. (1949) False positive trichina precipitin reactions in neoplastic disease. *Proc. Soc. exp. Biol. (N.Y.)* **72,** 254.

SUESSENGUTH H. (1947) An improved antigen for the slide test for trichinosis. *Amer. J. med. Technol.* **13,** 213.

SUESSENGUTH H. & KLINE B.S. (1944) A simple rapid flocculation slide test for trichinosis in man and swine. *Amer. J. clin. Path.* **14,** 471.

SZYFRES B. & KAGAN I.G. (1963) A modified slide latex screening test for hydatid disease. *J. Parasit.* **49,** 69.

TADA I. & KAWASHIMA K. (1964) Studies on the skin reaction in human filariasis with a purified antigen from *Dirofilaria immitis*. *Jap. J. Parasit.* **13,** 472.

TANNER C.E. & GREGORY JUDITH (1961) Immunochemical study of the antigens of *Trichinella spiralis*. I. Identification and enumeration of antigens. *Canad. J. Microbiol.* **7,** 473.

TOUSSAINT A.J. (1966) Improvement of the soluble antigen fluorescent antibody procedure *Exptl. Parasit.* **19,** 71.

TOUSSAINT A.J. & ANDERSON R.I. (1965) Soluble antigen fluorescent antibody technique. *Appl. Microbiol.* **13,** 552.

VOGEL H. & MINNING W. (1949) Hüllenbildung bei Bilharzia-Cercarien im Serum Bilharziainfizierter Tiere und Menschen. *Zbl. Bakt.* **153, 91.**

WITEBSKY E., WELS P. & HEIDE A. (1942) Sero-diagnosis of trichinosis by means of complement fixation. *N.Y. St. J. Med.* **42,** 431.

WOODRUFF A.W., BISSERU B. & BOWE J.C. (1966) Infections with Animal Helminths as a factor in causing poliomyelitis and epilepsy. *Brit. Med. J.* **1,** 1576.

WOODRUFF A.W. & THACKER C.K. (1964) Infection with Animal helminths. *Brit. Med. J.* **1,** 1001.

YOKOGAWA M., TSUJI M. & OKURA T. (1962) Studies on the Complement fixation test for Paragnonimiasis as the method of Criteron of cure. *Jap. J. Parasit.* **11,** 117.

ZAPART WANDA (1961) Odczyn uroprecypitacji u rozpoznawaniu wlosnicy. *Pol. med. Wkly. (Pol. Tyg. lek.)* **16,** 661.

CHAPTER 7

SKIN TESTS IN DIAGNOSIS

J. PEPYS

INTRODUCTION

The introduction of skin testing in a scientific manner by von Pirquet marked a great advance, which has provided accurate, fundamental information on immunological phenomena in man and animals. The names of Jenner, Blackley, Koch, von Pirquet, Mantoux, Arthus, Prausnitz and Küstner, Bloch, Casoni,

Dick, Schick, Schultz-Charlton, Frei, Kveim and Mitsuda are associated with, and many survive as eponymous names for common skin tests. These tests may show decreased reactivity-immunity, or increased reactivity-hypersensitivity. The contribution of hypersensitivity to enhanced resistance is one of the most controversial yet important problems in immunology.

The immediate (Type I), Arthus (Type III), and delayed (Type IV) reactions are demonstrable by skin tests and may be present in the subject at the same time. Arthus reactions are the least well defined in man and difficulty arises in trying to distinguish them from delayed reactions.

Another type of reaction, about which knowledge is now beginning to accumulate, consists of granulomatous infiltration which develops slowly and is best seen after 2–4 weeks. This obscure reaction occurs in hypersensitivity to zirconium and beryllium and in some cases to tuberculin, and resembles the Kveim reaction in sarcoidosis and perhaps the lepromin (Mitsuda) reaction in leprosy.

An understanding of the nature of the various reactions, of the methods for eliciting them, and of the circumstances under which the tests are employed, is necessary for their scientific application. Wherever possible, skin tests should be observed for the appearance of reactions within minutes, hours, days and weeks. It is almost the rule to find that little attention is paid to later reactions where immediate reactions are expected, and vice versa. We have only a limited, though valuable, understanding of the significance of many reactions, and more careful observations of this type are needed to extend our knowledge.

IMMEDIATE TYPE I (ANAPHYLACTIC) REACTION

Skin tests for immediate type hypersensitivity are employed chiefly in the investigation in atopic subjects, of disorders such as asthma, rhinitis, urticaria migraine and gastro-intestinal allergy. Immediate reactions may be elicited by tests in non-atopic subjects as well.

Dermographism may complicate skin testing. Ebken, Bauschard & Levine (1966), using a controlled scratch test with a weight of 400 g, obtained wealing reactions larger than 3 mm in 2.7% of patients with allergic rhinitis, 9.4% with chronic urticaria, and in 4% of patients with mental disorders.

The immediate reaction is mediated by non-precipitating, heat-labile antibody, termed reagin, which is readily produced in atopic subjects after ordinary exposure to otherwise innocous substances in the environment, hence there is a group of commoner allergens to which most atopic subjects react. It is of interest that patients with hypogammaglobulinaemia or so-called agammaglobulinaemia (Gitlin, Janeway, Apt & Craig 1959) can give immediate skin test reactions, thus showing the presence of skin-sensitizing antibody.

The immediate reaction develops rapidly, is maximal in 10–20 min and resolves within 1–1½ hr. The urticarial weal and erythematous flare is frequently accompanied by itching, which can be helpful in deciding whether a small reaction is positive or negative. The weal results from increased arteriolar flow through permeable capillaries and lymphatics leading to the exudation of serum. The peripheral arterio-capillary unit is a focal point in the production of the tissue oedema, which is responsible for most of the clinical manifestations. Psychological stimuli, infective or endocrine disturbances, or irritants, exert a potent effect on the peripheral circulation and in consequence upon the amount of exudation from the permeable capillaries. This influence of non-immunological factors on the end result of a primarily immunological reaction deserves more attention. The mechanisms of the inhibitory effect of hypnosis on both Type I and Type IV skin test reactions are not known. Interference with peripheral vascular reactions may be responsible, since histological examination of inhibited Type IV reactions shows the characteristic cellular infiltration (Fry, Mason & Pearson 1964; Black, Humphrey & Niven 1963).

Eosinophil cells predominate early in positive reactions and can persist for many days. The eosinophil cell is characteristic of immediate, Type I, hypersensitivity, and though its role is obscure, immediate reactions are the most certain method of producing an eosinophilia, locally or in the peripheral blood.

Selection of Allergens for Testing

Of the commoner allergens, inhalants are more reliable than food allergens for skin testing. The speed and ease with which allergens are dissolved out of pollens, dusts, moulds and danders when they come into contact with the tissue fluids of the respiratory tract, corresponds with the efficacy of aqueous extracts of these substances. The usual food extracts are more likely to give positive skin test reactions in patients in whom the food produces a rapid clinical response; where the response is slow, skin tests cannot be relied upon.

There are wide differences of opinion as to whether all available allergens should be tested, or only a limited number, and in particular the commoner allergens and those suggested by the patient's history and environment. This problem is more pressing where the skin testing is separated from the clinical management and where treatment is based without discrimination upon skin test results. The author prefers to test a limited number of the commoner allergens, on the understanding that further and repeated tests will be performed if necessary.

Confirmation of skin tests results by the history or by other tests is desirable, though this may not be possible even where the allergen is subsequently proven to be of clinical importance. Extracts of the following materials provide a basis for testing: house dust; the moulds, *Alternaria, Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum, Tricothecium roseum*, yeasts and dry rot;

cat, dog and horse hair; feathers; grass pollens which can be tested as a group, and where possible, individual tree pollens. The commoner food allergens, such as milk, wheat, eggs, chocolate, fish, nuts, may also be used, or the clinician may prefer to test these by clinical trials, which are necessary in any case to confirm or check the skin test findings.

Allergen extracts are notoriously variable in potency, and are not as a rule standardized on a chemical basis, except for protein nitrogen estimations of pollen extracts. The variability of extracts makes precise comparisons of the results in patients tested with different preparations unsatisfactory. The potency of extracts is best assessed by skin tests on suitable patients.

The size of a reaction is a measure of the potency and concentration of the extract and the degree of hypersensitivity, but it does not necessarily correspond to the clinical importance of the allergen in the particular patient.

Methods of Testing

The chief methods are the prick, scratch and intracutaneous tests. Many workers start with prick or scratch tests and then proceed, if these are negative, to intracutaneous tests. There are differences of opinion regarding the relative value of these methods. Tabart (1961) found the prick superior to the scratch test, and whilst it was comparable with the intracutaneous test for pollen sensitivity, it gave fewer reactions with other inhalant allergens. Intracutaneous tests tend to give far more non-specific reactions than prick tests. Although rarely if ever required with prick testing, it is advisable to have adrenalin 1/1000 available for emergency treatment of immediate general reactions to intracutaneous tests, and antihistamine drugs can be used in addition.

The author employs the prick test almost exclusively, because of its precision and lack of non-specific trauma. Reliance is placed upon improved allergens to increase its sensitivity. Squire (1952) has calculated that the prick test introduces three-millionths of a millilitre into the skin.

The correlation of the prick test with the clinical history is seen in the reactions of a large series of asthmatic patients attending the Brompton Hospital. Positive reactions to prick tests with a house dust extract were obtained in 62%. Positive reactions were obtained in 73% of those with a history of sensitivity, and in 44% without such a history, this difference being statistically significant at the 5% level. Similarly, tests with grass pollen gave positive reactions in 83% of patients with a definite history and in 32% without such a history, the difference being significant at the 0.1% level.

A comparison of carbol saline extracts of animal hair and feathers with extracts of whole minced skin of these animals show how improved allergens enhance the efficacy of the prick test. Of thirty-six patients with a definite history of sensitivity to cats thirty reacted vigorously to the cat skin and six weakly to the cat hair. In twelve patients with a history of sensitivity to dogs

eleven gave vigorous reactions to dog skin and four to dog hair. Of eleven patients with a history of sensitivity to feathers nine gave positive reactions to hen skin and two gave weak reactions to hen feathers.

More attention will have to be paid to cross reactions because of their fundamental and possibly clinical significance. For example, the author has obtained positive skin test reactions to extracts of hen's egg in almost all patients suffering from asthma or bird breeder's lung due to budgerigar and pigeon and in some of these cases allergic reactions affecting different parts of the body have followed the ingestion of the hen egg on the one hand and inhalation of the avian antigen on the other. Positive reactions to nuts are also obtained frequently in pollen sensitive subjects, and here too allergic reactions to both may be present. Another striking example of cross-reactivity is the production in patients with aspergillus allergy of wealing reactions to prick tests with glycopeptide extracts of the *Aspergillus* genus, the dermatophytes, pneumococcal C-substance, nematode parasites such as *Ascaris*, *Fasciola hepatica*, etc. and extracts of vegetable dusts such as malt and palm kernels. All of these extracts have in common the capacity, like pneumococcal C-substance, to combine with C-reactive protein in the serum (see Chapters 3 and 37).

Routine prick tests with moulds gave positive reactions to *Alternaria* in 17%; *A. fumigatus* in 13%; *C. herbarum* in 16%; dry rot in 14%; *P. notatum* in 7%; *T. roseum* in 9% and to yeasts in 7%. Almost all reactors to moulds gave positive reactions to house dust, though not necessarily vice versa. In patients with broncho-pulmonary aspergillosis of the hypersensitive type, all the patients gave positive reactions to aspergillus extracts (Pepys *et al* 1959).

The relationship of skin and bronchial tests to the clinical history is reported by Ten Cate (1961). Bronchial tests did not produce reactions in patients with negative skin test reactions, whereas bronchial reactions were obtained in 22% of those with positive skin test reactions, in 46% with a doubtful history and in 75% with a definite history of sensitivity.

Significance of Positive Reactions

Positive reactions, where the technique and test materials are satisfactory, are specific in the immunological sense. Where they are not obviously related to the clinical condition they are regarded as evidence of latent or subclinical allergic sensitivity. Difficulties arise from positive reactions in so-called normal subjects, and where they cannot be clearly related to the disorder in atopic subjects. Positive reactions reported in normal subjects have been found to be due to inadequate screening of the subjects by Curran & Goldman (1961), who obtained positive reactions to scratch tests in 5% of a non-allergic group and together with intracutaneous tests in 9%. In non-allergic subjects without a family history of allergy, positive reactions were obtained in 4%, compared with 30% in those with a family history (Pearson 1937); similar results were

obtained by Curran & Goldman (1961). Furthermore, about half of a group of allergic subjects who gave positive reactions to pollen but were not suffering from pollinosis, developed clinical symptoms subsequently, the majority within 5 years (Chambers & Glaser 1958). Scratch test reactions were obtained by Hagy & Settipane (1966) in 63.8% of students with rhinitis or asthma; 27.1% with other allergic disorders and 17.4% of those with no allergic disorders.

Significance of Negative Reactions

The variability and arbitrary nature of many allergenic extracts must be kept in mind in evaluating negative reactions. The clinical history is more important than a negative skin test reaction, which cannot be taken as absolute evidence of a lack of sensitivity. It is also not uncommon, for example, to find children suffering from typical pollinosis in whom skin test sensitivity only appears 1–2 years afterwards. The author has observed that a number of patients with a definite history of house dust sensitivity, who failed to react to prick tests, gave unusually vigorous local and sometimes focal reactions to subcutaneous injections of house dust extract. There is no doubt that these patients are hypersensitive to the house dust extract, although the explanation of their unfavourable response is obscure.

Past treatment can decrease or even abolish skin test reactivity though this may have little relation to the clinical response. Antihistamine and related tranquillizing drugs depress or inhibit immediate reactions and it may take several days before the capacity to react is restored. Adrenalin, ephedrine and to a lesser extent theophylline derivatives, if administered shortly before testing, can decrease the reactivity of the skin, whereas corticosteroids have little if any effect.

Passive Transfer (Prausnitz-Küstner) Test

The usefulness of the passive transfer test for clinical purposes is limited by the danger of homologous serum jaundice. In practice, clinical trials can usually be made with suspected allergens, thus making the test even less necessary. The technique is described in detail by Walzer (1947).

Cooke (1947) used the passive transfer test for the demonstration of 'blocking' antibody produced by the injection of allergenic extracts. The mixture of the allergen with post-treatment serum gave weaker reactions than with the pre-treatment serum in tests on passively sensitized skin. Quantitative estimations of 'blocking' antibody have been made, but unfortunately do not correspond with the clinical status of the patient. Cooke (1947) also used the test for the comparison of allergens.

Bacterial Allergy in Atopic Disorders

The production of immediate and delayed reactions to bacterial products, in particular to ubiquitous and universal pathogens and commensal organisms

has not, so far, been related in a convincing manner to the pathogenesis of clinical allergic disorders. Distinction must be made between atopy, i.e. immediate type hypersensitivity commonly attributed to polysaccharide and delayed type hypersensitivity to protein from bacteria. The clinical and histological manifestations of immediate type hypersensitivity are the same whatever the nature of the allergen.

Immediate Reactions to Bacterial Products

The use of pure bacterial preparations like the type-specific pneumococcal polysaccharide of Heidelberger & Avery (1923) and polysaccharides from other organisms giving immediate reactions (Burger 1950), would enhance the value of skin tests. Tillett & Francis (1929) found that pneumococcal polysaccharide gave immediate reactions, regarded as a good prognostic sign, in patients convalescent from pneumonia, whereas the nucleo-protein gave delayed reactions. When anti-pneumococcal sera were used for treatment, the production of an immediate reaction by the polysaccharide was evidence of the introduction of sufficient antiserum into the patient.

Skin test reactions to bacterial extracts are more probably related to past infection than to the allergic disorder under investigation, and other diagnostic tests and purer antigens are needed to establish an aetiological relationship. The literature on this subject is reviewed by Baird (1957). Swineford & Holman (1948) performed tests with vaccines and with crude polysaccharide and nucleoprotein fractions of a wide variety of bacteria. They found that of thirty-six patients who gave strong reactions, twenty-six gave immediate and ten delayed reactions; thirty-one suffered from hay fever and asthma and three from rheumatoid arthritis. Whilst individual patients tended to give either immediate or delayed reactions there were numerous exceptions. Streptococcal polysaccharides and nucleoprotein gave both immediate and delayed reactions, probably because of the crudity of the preparations. There was doubt as to whether the reactions which were maximal at 24 hr, were delayed or Arthus in type.

ARTHUS TYPE REACTIONS (TYPE III)

Arthus reactions are dependent upon precipitating antibody and, like immediate Type I allergy, can be passively transferred with serum. The severe and not uncommon Arthus reactions to repeated injection of large doses of heterologous serum reviewed by Ratner (1943) became rare when chemotherapy replaced antipneumococcal serum. Little attention was given subsequently to the Arthus type skin test reaction until recently. Attention has now been drawn once again to this important reaction because of its appearance in patients with pulmonary diseases due to inhaled organic antigens (see Chapter 36) in whom precipitins are a feature. A characteristic dual skin test reaction is obtained in these patients,

consisting of an immediate, Type I, wealing reaction which may resolve completely or run into a second oedematous reaction often strikingly raised above the surrounding skin. It becomes obvious after 3–4 hr, is maximal at 7–8 hr, and is subsiding within 24 hr. This second, Arthus type, reaction is itchy, and the soft oedema usually present may be very extensive. There is very little, if any, central induration, and the borders of the reaction are not well defined. It may be difficult to evaluate small swellings appearing some hours after skin test. If, however, larger doses of antigen are administered the extent of the reaction in the appropriate subjects helps to distinguish it from swellings due to other reasons. It is not uncommon for the response to intracutaneous tests, in the forearm, with 0.01 ml of pigeon, budgerigar, bovine or porcine serum or of solutions of 1 to 10 mg of protein antigen from *A. fumigatus* to consist of oedema extending from wrist to elbow. The reaction causes surprisingly little discomfort. The absence of haemorrhagic reactions and necrosis, which are features of Arthus reactions in experimental animals, can be attributed to the relatively small test dosage in man. There can be no doubt, from the extent of the reactions often seen, that larger doses of the order described by Ratner (1943) would be likely to cause much tissue damage. The distinction between the two phases of the dual reaction is best seen with prick tests in very sensitive subjects in whom the immediate weal resolves completely at 1½–2 hr, to be followed by the second reaction after 3–4 hr. The duality of the reaction is also shown by the inhibitory effect of corticosteroids on the Arthus type component, whilst the immediate wealing component is not obviously affected. In some cases a triple reaction may occur in which, after the subsidence of the second reaction, an indurated, erythematous reaction of the delayed Type IV nature may appear at 48–72 hr.

The Type III skin test reaction appears to be dependent upon a preceding Type I reaction. As a rule dual reactions are not obtained in patients with only precipitins against *A. fumigatus* or other antigens, nor do they appear in patients with reagins only. It must be emphasized that the prick test is preferable for deciding that Type I allergy is present, since intracutaneous tests with heterologous sera and other potent antigens may give non-specific wealing. It has been exceptional in the author's experience for prick-test Type I reactions to be absent in those patients with precipitins who give dual reactions to intracutaneous tests. Bier, Passos and Siqueira (1968) report a similar dependence in guinea-pigs of the passive Arthus, Type III, reaction on a preceding Type I (P.C.A.) reaction.

Reactions to Heterologous Serum Proteins and Related Antigens

In addition to the Arthus reactions during treatment with rabbit antipneumococcal sera and horse and other antitoxic sera, Arthus type skin reactions can be elicited with the serum proteins or related antigens from many other species.

BIRD BREEDER'S LUNG (see Chapter 36)

Dual reactions with a vigorous Arthus type component are produced by prick and more often by intracutaneous tests (0.01 to 0.02 ml) with undiluted pigeon and budgerigar serum in patients with bird breeder's (fancier's) lung, or with extracts of the droppings which contain antigen derived from, or related to, serum proteins, as well as additional antigens. These latter extracts are prepared by defatting the droppings with ether followed by carbol-saline extraction for 5 days or more. The Seitz-filtered extracts are freeze-dried and put up in sterile, carbol-saline solution at 1 to 10 mg/ml for skin tests and at appropriate concentrations for inhalation and serological tests. Both serum and droppings extracts are heated at 56°C for 30 min to inactivate any ornithosis group virus. Suitable extracts containing serum protein-like antigens may also be prepared by extracts of skin and of pigeon and budgerigar egg, especially the yolk. Dual reactions have been obtained in almost all patients with alveolar disease due to the dust of the avian droppings and with precipitins against the avian extracts. In atopic patients with asthma due to avian antigens, Type I reactions only are elicited and precipitins are usually not present (Hargreave, Pepys, Longbottom & Wraith, 1966).

PITUITARY SNUFF-TAKER'S LUNG (see Chapter 36)

Dual skin test reactions to undiluted porcine and bovine serum may be obtained in those patients with diabetes insipidus who give Type I reactions and in whom precipitins have appeared following on prolonged nasal insufflation of porcine and bovine pituitary snuff.

Type I asthmatic reactions to pituitary snuff and urticarial and anaphylactic reactions to injected pitressin may also be produced. Some subjects are allergic to both the bovine or porcine serum proteins and pituitary snuff, and others to the pituitary snuff alone, that is, specifically to pituitary antigens (Pepys, Jenkins, Lachmann & Mahon 1966).

REACTIONS TO FUNGAL, BACTERIAL AND HELMINTH ANTIGENS

PULMONARY ASPERGILLOSIS (see Chapters 3 and 36)

In about 10% of patients with asthma and pulmonary eosinophilia and precipitins in their serum against *A. fumigatus*, dual reactions are obtained on prick tests with commercial extracts. The protein fraction of *A. fumigatus* extracts (see Chapter 3) gives dual prick test reactions in 52% of cases, whilst intracutaneous tests with this fraction at 1 and 10 mg/ml (not yet available commercially) gives dual reactions in almost all of these cases. By contrast dual reactions are not given by patients with reagins only, nor by patients with pulmonary aspergilloma who have abundant precipitins, except in some of the small group of the latter who give Type I reactions to prick tests.

As in the dual reactions to avian antigens, Type I reactivity as estimated by prick tests appears to be essential for the subsequent development of the Type III component. In patients with asthma and pulmonary eosinophilia, a negative prick-test reaction to *A. fumigatus* would argue strongly against the diagnosis of pulmonary aspergillosis (Longbottom & Pepys, 1964).*

Reactions to Candida Albicans

Intracutaneous tests (0.01–0.02 ml) with the cell-wall mannan of *C. albicans* Group A (10 mg/ml) described by Summers, Grollman & Hasenclever (1964) gave Type I followed by Type III reactions in seventeen out of twenty-two subjects with mannan A precipitins (see Chapter 3) as compared with four out of twenty-eight in those without precipitins. Repetition of the tests after 1–2 weeks resulted in stronger reactions or in the appearance of reactions in previously negative subjects, corresponding with increases in the precipitation reactions found in all subjects after a single skin test (Pepys *et al* 1967). Intracutaneous tests with culture filtrate extract of *C. albicans*, which contained protein as well as polysaccharide antigens, was responsible for Type IV reactions, which in some cases were preceded by less vigorous Type III reactions than were produced by the mannan A. Nevertheless, typical Type III reactions were also followed at 24–48 hr in one-half of the cases by small indurated reactions, which showed a strong tendency to recurrent flare-ups for several weeks, and which could not be distinguished macroscopically from Type IV reactions.

Reactions to Tuberculo-Polysaccharide

The coexistence of all types of hypersensitivity is well seen in tuberculin sensitive subjects. In addition to delayed reactions, immediate reactions have been reported in experimental animals by Miles (1955) and Pepys (1955). Furthermore, Cournand & Lester (1939) have reported that tuberculo-polysaccharide produced immediate reactions in man which lasted for 2 hr, followed by a second reaction appearing at 4 hr and lasting for 24 hr, at which time the reaction to tuberculoprotein was developing. The reaction to the polysaccharide was attributed by McCarter & Watson (1942) to the presence of traces of protein, but it is difficult to accept this explanation, since even strong doses of tuberculoprotein do not produce immediate and late reactions of the type described. Arthus-like reactions to fractions of *M. tuberculosis* H 37 Ra have been reported by Glenchur, Fossieck & Silverman (1965). These reactions developed after 2 hr and were marked at 4–8 hr. A similar reaction has been described to the higher doses of 10 to 250 TU by Duboczy (1965).

*Biopsies of dual reactions to *A. fumigatus* and to avian antigens in man have shown a perivascular infiltration of early and scanty polymorphonuclear neutrophil cells and a predominantly mononuclear cell infiltration. IgG, IgM, IgA and β_1c component of complement were present, both extra- and intra-cellularly. The latter was found in the vascular endothelium of some cases (Pepys, Turner-Warwick, Dawson and Hinson, 1967).

Reactions to Ascaris Extracts

Immediate reactions to ascaris extracts are well known, and increasing reactions to intracutaneous tests over a period of 24 hr are reported by Boucher, Roumagoux, Souquet & Bonnefoy (1960) in patients with Loeffler's syndrome. The possibility that the late reaction to the ascaris extract is of an Arthus type needs further study.

Reactions to Insect Antigens

Lunn & Hughes (1967) report on asthma in mill-workers due to the wheat weevil *Sitophilus granarius* and on a case of asthma and a farmer's lung type reaction in whom dual reactions were obtained on skin testing and who had precipitins in the serum.

Reactions to Tissue Extracts and Dextran

Thyroid Extracts

Reactions of the Arthus type to an extract of thyroid from thyrotoxic patients were elicited in patients with thyroid disease and precipitins. The skin test modified the precipitin reaction. The danger of transmission of jaundice with tissue extracts was emphasized (Buchanan, Anderson, Goudie & Gray 1958).

White Blood Cell and Calf Thymus Extracts and DNA

In patients with a diagnosis of systemic lupus erythematosus, positive skin test reactions were obtained to homogenates of leucocytes in eighteen out of thirty-one by Holman (1960), and to DNA preparations by Ores & Lange (1964) in nineteen out of nineteen, twenty-three other patients giving negative reactions. Fordal & Winkelmann (1965) obtained positive reactions to calf thymus DNA in 40 out of 115 patients with skin diseases, the most frequent reactions being obtained in systemic and discoid lupus erythematosus, and in necrotizing vasculitis, though the authors concluded that there was no definite evidence of DNA allergy in collagen diseases.

DNA prepared from the patient's own leucocytes and from calf thymus was found by Chandler & Nalbandian (1966) to give more vigorous skin test reactions in patients with a clinical picture like that of systemic lupus erythematosus but with negative LE tests. In the above reports the reactions, which were painful, tended to be maximal after several hours, suggesting that they may be of the Arthus type, though Fordal & Winkelmann (1965) did not find precipitins in fifteen cases, seven of whom had antinuclear factor, and more information is necessary before deciding whether they belong in this group.

Reactions to Dextran

Hypersensitivity of the immediate type, in particular to high molecular weight, long chain, branched dextrans was correlated with the presence of precipitins

(Kabat & Mayer 1961). The production of an Arthus reaction in himself by active sensitization with a high molecular weight, long-chain branched dextran is reported by Humphrey (personal communication). Reactions to skin tests came on after several hours without immediate reactions. These reactions were oedematous and showed capillary haemorrhage with infiltration of neutrophil and eosinophil cells.

DELAYED TUBERCULIN-TYPE REACTION (TYPE IV REACTION)

The production of delayed reactions is best seen in skin tests for the 'allergy of infection' and for contact sensitivity. The classical example, the tuberculin reaction, becomes evident, as a rule, at about 24 hr and is maximal at 48–72 hr, showing, except with weak reactions, a well-defined area of induration surrounded by erythema. If the reaction is vigorous there may be oedema, vesicles and bullae, and residual pigmentation is not uncommon.

The Tuberculin Skin Test Reaction

A vast amount of effort has been devoted to the tuberculin test, which has a reputation for precision and specificity. It is surprising, however, to see how many aspects are subject to rigorous questioning. Admirably comprehensive analyses of the tuberculin test in man are provided by Gernez-Rieux *et al* (1961) and Edwards & Edwards (1960).

The tuberculin test was first used qualitatively for the diagnosis of tuberculosis. Subsequent quantitative studies of the sizes of reactions to various doses have raised problems regarding the significance of high and low degrees of sensitivity. More recent quantitative comparisons of reactions to antigens from tubercle bacilli and from atypical mycobacteria promise to reveal qualitative differences of diagnostic value in the investigation of low degrees of sensitivity to tuberculin.

Test Antigens

The tuberculin preparations in general use at present are:

1. Old Tuberculin (OT) prepared by concentration of the autoclaved culture filtrate of *Mycobacterium tuberculosis*, and standardized to contain 100,000 tuberculin units (TU)/ml (International standard).

2. Purified protein derivatives (PPD) of tuberculin prepared by ammonium sulphate (PPD-S) (Seibert 1934), or trichloroacetic acid precipitation of culture filtrates. Concentrated PPD contains 2.0 mg/ml and is equivalent to 100,000 tuberculin units (TU) (International standard). The introduction of PPD appeared to provide a more precise antigen than crude Old Tuberculin, which contains lipids, polysaccharides and constituents of the medium, but it is now clear that OT has certain other advantages. Although OT and PPD are prepared from heated

material, the protein which seems to be of major importance in the tuberculin reaction remains very highly active. Gell & Benacerraf (1959) have found that whilst the delayed reaction is more sensitive than the immediate, it is also less specific and can be elicited by denatured proteins. PPD-S contains 92.9% protein, 1.2% nucleic acid and 5.9% polysaccharide, and is similar to that prepared at Weybridge. The French PPD, prepared by the Pasteur Institute (IP48) contains 30% polysaccharide.

Old Tuberculin and PPD cannot be standardized in terms of one another because of qualitative factors which lead to differences in reactions in subjects of varying degrees of sensitivity (Seibert & Dufour 1954; Paterson & Leech 1954). Furthermore, PPDs from the same and different sources may differ in their reactions in different populations or depending upon whether the sensitivity was due to natural infection or BCG vaccination in the same population (Meyer 1952; Guld 1957; Comstock 1960). W.H.O. have prepared a large batch of PPD, RT23 (Magnusson & Bentzon 1958), for standardized testing, since surveys and serial tests should be made with the same material throughout. An additional difficulty arises because of the rapid adsorption, particularly of low concentrations of PPD, to walls of containers. This may reduce its potency by 60% (Waaler, Guld & Magnus 1958). The addition of Tween 80 reduces adsorption of the PPD (Magnusson, Guld, Magnus & Waaler 1958), but Griep & Bleiker (1960) report that Tween 80 tends in itself to reduce reactions. This is probably due to irritation of the tissues by the Tween 80.

Methods of Testing

Von Pirquet used the scratch method in introducing the tuberculin test. Many other methods have been used, such as inunction of a tuberculin ointment into the skin (Moro test), patch tests with gauze impregnated with tuberculin (Vollmer test) and the jelly patch test. These methods are less satisfactory than the intracutaneous (Mantoux) test and the more recently introduced multiple puncture test (Heaf 1951; Heaf & Rusby 1959).

The Mantoux test is the most reliable for quantitative studies, since the dose can be graded, a known amount injected and the reaction measured. The multiple puncture test is performed with a spring gun which introduces six needles to a depth of 1 or 2 mm into the skin, through solutions of concentrated OT or PPD, to which adrenalin may be added. The test is equivalent to an intracutaneous test with 10 TU.

A linear relationship between the diameter or thickness of the skin reaction and the log-dose of tuberculin has been reported in animals (Wadley 1949; Long & Miles 1950). Bruce (1961) has reported a linear relationship in man between the area of induration and the log-dose of tuberculin injected in a constant volume, the area being influenced by the volume as well as the dose.

Effects of Tuberculin Tests on the Degree of Sensitivity—Serial Tests

Serial tests are important for the demonstration of the development of tuberculin sensitivity in previously non-sensitive subjects and are used for diagnostic and epidemiological purposes. Serial tests or repeated administration of tuberculin can, however, increase or decrease the sensitivity to subsequent tests. Canetti (1946) and O'Grady (1956, 1957) have reported that tests with increasing doses of tuberculin may enhance the sensitivity to lower doses for prolonged periods and on occasion may cause a reaction to appear at previously negative tests. There is also less waning of tuberculin sensitivity in BCG vaccinated subjects in whom tuberculin tests are repeated. Gernez-Rieux *et al* (1961) state that tests with IP48 do not have this enhancing effect. When tuberculin is injected in increasing doses at short intervals, i.e. tuberculin desensitization, the opposite effect is seen and even large doses will fail to excite reactions.

Repetition of Tests on the Same Site

This may lead to decreased reactions if the interval between tests is short because of the enhanced lymphatic absorption from the inflamed tissues of the previous reaction. Repetition of tests at the same site at intervals of 3 months results in reactions which are more rapid, more vigorous, and resolve sooner than tests on normal skin (W.H.O. 1955). The enhancement of the reactions is also seen on histological examination (Bachi 1959).

Simultaneous Tests with Varying Doses

Rosenthal & Libby (1960) report that a single test with 10 TU (OT) gave much larger reactions than were obtained with either dose when 10 and 100 TU were tested simultaneously. Similarly Pollock, Sutherland & Hart (1959) found that there were fewer positive reactions in subjects tested with 10, 30 and 100 TU, than in those tested only with 100 TU. On the other hand, in subjects with high degrees of sensitivity they found that simultaneous tests with 0.3, 1 and 3 TU led to more reactions to 0.3 TU than where 0.3 TU alone was tested.

Multiple Repeat Tests with Increasing Doses

Furcolow, Hewell, Nelson & Palmer (1941) tested infants with doses of PPD ranging from 0.000001 mg (1/2 TU) to 1.0 mg (50,000 TU). Almost all reacted by the time the final test dose was reached. The reactions to high doses, which appeared in infants without known contact with the disease, differed in character from those to low doses. The possibility that the latter were Arthus type reactions must be considered, since precipitins against concentrated culture filtrates of photo-, scoto- and non-chromogenic atypical mycobacteria have been found by micro-tests in agar gel in almost all subjects tested, unrelated to tuberculous infection (Pepys & Jenkins, unpublished).

Stability of Tuberculin Sensitivity

Reversion or loss of tuberculin sensitivity to conventional test doses occurs most frequently in subjects with low degrees of sensitivity. Dahlstrom (1940) reported that over a period of 5 years 1% of subjects reacting with 10 mm or more of induration to 1 TU, reverted, whereas in those with induration of less than 10 mm or who reacted only to 100 TU, 35% reverted. Daniels *et al* (1948) found that the maintenance of tuberculin sensitivity is influenced by the degree of exposure to the organism, more controls than contacts losing their sensitivity. Tuberculin sensitivity is depressed on the 5th day after vaccination with poliomyelitis or measles vaccines and on the 9th day after yellow-fever vaccination (Brody, Overfield & Hammes 1964).

Development of Tuberculin Sensitivity

The larger the infecting or sensitizing dose the earlier the appearance of tuberculin sensitivity, so that this may appear in experimental animals from the 8th day (Canetti 1946). Hypersensitivity to the tubercle bacillus may be shown in man, as early as the 3rd to 4th day after BCG vaccination, by an accelerated reaction to a BCG test (Saye 1953). Bruce (1961) has found a linear relationship between the dose of organisms, BCG in man, and the area of induration at tuberculin tests.

Low Degree or 'Non-Specific' Sensitivity—Contact and Low Degrees of Sensitivity

The claims by Palmer (1953) and Edwards & Palmer (1958) that low degrees of sensitivity are due to mycobacteria other than mammalian *M. tuberculosis* were made because no relationship was found between low degrees of sensitivity and contact with the disease and secondly because of the geographical distribution of low degrees of sensitivity.

Pollock, Sutherland & Hart (1959), however, found that a low dose of 0.2 TU was sufficient to cause reactions in most subjects who were closely or recently exposed, so that no correlation of contact with sensitivity to higher doses could be expected. They consider that atypical mycobacteria need not be postulated as the cause of low degrees of sensitivity in Britain. An increase of low degrees of sensitivity in 13–14-year-old children has been reported in Britain (Pollock *et al* 1959), probably due to decrease in frequency and intensity of exposure to infection.

Pollock *et al* (1959) obtained positive reactions in from 76.6–88.3% of subjects in the no-contact to close-contact groups tested with doses up to 1000 TU. This is close to the 92% of positive reactions obtained by multiple puncture tests with a depot PPD cream by Pepys, Bruce & James (1958), which shows that very low degrees of sensitivity are not uncommon in Britain, a finding supported

by the almost universal presence, mentioned above, of precipitins against antigens from atypical mycobacteria (Pepys & Jenkins, unpublished).

More satisfactory evidence for non-tuberculous causes of reactions to tuberculin is based on geographic and immunological studies by Edwards & Palmer (1958) and Edwards & Edwards (1960). Comparative tests with tuberculins from human *M. tuberculosis* and atypical mycobacteria showed marked differences in patients harbouring these organisms as well as in subjects from different parts of the United States, and Keay and Edmond (1966) found them useful in the diagnosis of atypical mycobacterial infections in children.

Low degrees of sensitivity have been found to be associated with resistance to infection (M.R.C. 1956), and more tuberculosis to be present in subjects with high degrees of sensitivity (Edwards & Palmer 1958).

Comparative Tests with Tuberculin and Tuberculo-Lipopolysaccharide

A lipopolysaccharide (PmKo) extracted from the tubercle bacillus has been compared with the French PPD, IP48 (Choucroun, Gresland & Kourilsky 1960a and b). They found that 51% of patients with acute, recent disease gave stronger reactions to IP48 than to PmKo, whilst in those with chronic, limited or cured disease the PmKo gave stronger reactions in from 63.8–79% of subjects. It is suggested that resistance is more closely related to hypersensitivity to the lipopolysaccharide than to the protein.

Factors which Modify Delayed, Tuberculin, Skin Test Reactions

The reaction to tuberculin introduced into the skin depends on its local persistence, the availability of reactive cells and the capacity of the tissues to respond to inflammatory stimuli. Factors capable of modifying these are important in tuberculin tests and in the understanding of the mechanism involved (Pepys 1955), and it is very likely that they will have similar effects in delayed reactions to other antigens.

Local Persistence of the Antigen

This depends upon lymphatic absorption from the skin which is influenced by the local circulation. Tuberculin reactions are decreased by the introduction into test sites, within 10–40 min, of histamine or other wealing agents and of hyaluronidase or irritants, which spread, dilute and enhance the absorption of the tuberculin. Enhanced lymphatic absorption occurs in pregnancy, in the premenstrual period, in febrile and infectious disorders, hunger cachexia, oedema of the skin, and after exposure to sunlight or other irritants. The decreased tuberculin reactions obtained under these conditions have been attributed to negative anergy of an immunological nature, but the physiological disturbances are sufficient to account for them.

This effect of wealing may be important in subjects in whom immediate type reactions are excited by an antigen, since all weals lead to the rapid absorption of large molecular substances, dyes, haemoglobin, and even of erythrocytes (Pepys 1951). Thus, not only may the immediate reaction be associated with breakdown of the antigen, but the weal would lead to its removal. Both factors would tend to mask the presence of delayed hypersensitivity.

Increased Local Persistence of the Tuberculin

Prolongation of the retention of the antigen, by slowly absorbed vehicles such as liquid paraffin and wool alcohols, or decreased lymphatic absorption due to decreased blood flow results in increased reactions.

Slowly absorbed vehicles

Incorporation of tuberculin in an emulsion of liquid paraffin and lanolin increases its potency and prolongs its persistence in the skin (Seeberg 1951; James & Pepys 1956). An improved mixture of PPD 5 mg/g in Eucerine (anhydrous wool alcohol) has been used for multiple puncture tests (Pepys, Bruce & James 1958). The greatly enhanced sensitivity of this depot PPD is comparable with that of the BCG test and with tests with tuberculo-lipopolysaccharide (PmKo, Choucroun), in producing reactions in patients who have been desensitized to tuberculin, and in patients with sarcoidosis or Hodgkin's disease who have extremely low degrees of sensitivity. The depot PPD also persists in the skin for long periods, so that BCG vaccination leads to the appearance of reactions at the previously negative sites when hypersensitivity develops.

Decreased lymphatic absorption

The injection of adrenalin into skin on which tuberculin patch tests are performed decreases blood flow and lymphatic absorption and results in increased reactions. The retention of sodium fluorescein for many hours in skin into which adrenalin has been injected can be observed under ultra-violet light through a Wood's filter. Adrenalin added to tuberculin used for scratch and multiple puncture tests has an enhancing effect. Increased reactions are also obtained in tests below a constrictor band or in paretic limbs with a reduced circulation (Gernez & Marchandise 1935).

Fixation of antigen in the skin

Modification of skin tests by the introduction of agents which alter the persistence locally of the tuberculin, show that fixation of the tuberculin starts very soon and is completed within 1–2 hr. Tuberculin may also persist for long periods in the skin of non-sensitive subjects so that positive reactions appear at the time of primary infection, even many months after the test, thus giving a 'revivescence' reaction.

Availability of reactive cells

The ingress into the test area of circulating, reactive, lymphoid cells can be decreased by the injection of adrenalin into intracutaneous tests. Reactions to tuberculin are decreased in experimental animals when lymphoid cells are reduced either specifically by the intravenous injection of tuberculin, or non-specifically by X-irradiation or by the injection of anti-lymphocytic sera. Evidence that the lymphoid cells are the reactive cells is shown by their capacity to transfer delayed hypersensitivity passively in animals (Chase 1945) and in man (Lawrence 1949, 1959). The depression of delayed hypersensitivity in sarcoidosis, or Hodgkin's disease, may be due to a diminution in reactive lymphoid cells due to the involvement of the reticulo-endothelial system (Chase 1966).

Inflammatory Response of the Skin to Tuberculin Tests

Corticosteroids have an inhibitory effect and antihistamine drugs little effect on delayed reactions; the reverse applies to immediate reactions. Nevertheless, an increase in histamine associated with the infiltration of lymphoid cells has been reported in tuberculin reactions by Inderbitzin (1957, 1959), and in contact dermatitis by Prochazka Fisher (1959).

SKIN TESTS IN BACTERIAL INFECTIONS

Skin tests are of diagnostic use in a number of infections and since they frequently excite a secondary response of circulating antibody or may even lead to its appearance, blood should be collected for serological studies before skin tests are performed. The application of the quantitative and other procedures employed in tuberculin testing is desirable for evaluating the reactions obtained for prognostic and epidemiological purposes.

Negative reactions show lack of present or past infection, except in the early stages of disease before hypersensitivity has appeared and in some cases in infants, the aged, and patients with overwhelming infection. This 'anergy' may be due to the same physiological factors which can modify tuberculin reactions. An important diagnostic sign, just as in tuberculosis, fungal and other infections is the production of positive reactions in subjects who previously gave negative reactions.

Skin Tests with Streptococcal Extracts

Skin tests with streptococcal extracts may be used to show the presence of antitoxin responsible for immunity to the erythrogenic toxin of β-haemolytic streptococcus, and the presence of specifically-reacting cells which mediate delayed type hypersensitive reactions. Although hypersensitive reactions are

more frequent in patients with rheumatic disorders, their diagnostic or prognostic significance is not known.

Skin tests with culture filtrate of β-haemolytic streptococcus (Dick test) produce reactions in 100% of subjects susceptible to scarlet fever. The positive reaction caused by the toxin appears within 6–12 hr, is maximal at 24 hr and then resolves. There is no official standard preparation in Britain, but Banks (1951) states that the test is a reliable index of immunity for experienced workers and should be used if active immunization is contemplated. After immunization 88–92% cease to react and 72% are still non-reactive 5 years later (Laurent 1951).

The size of reactions is proportional to susceptibility; subjects giving reactions larger than 25 mm contract scarlet fever readily, and those with reactions of less than 10 mm rarely. Control tests with heated toxin seldom cause pseudo-reactions, which appear after active immunization in from 3–4% of subjects. These reactions which appear later than the reactions to the toxin are maximal at about 40 hr, and are associated with immunity.

The injection of a streptococcal antitoxic serum into the skin of a patient with a rash due to scarlet fever leads to an area of blanching within 6–14 hr. This Schultz-Charlton test is of limited value, though it may help to distinguish the scarlatiniform rashes of rubella and of allergic origin from scarlet fever.

Skin tests with the M antigen of group (A) haemolytic streptococcus and other streptococcal antigens have been used for the study of passive transfer of delayed hypersensitivity in man by Lawrence (1952, 1955).

In all the patients with recurrent aphthous stomatitis tested by Graykowski *et al* (1966) positive delayed skin test reactions were given to a vaccine of killed pleomorphic transitional ρ-form-cultures of α-haemolytic streptococcus isolated from the lesions, immediate reactions being given by a few of the patients. Some patients with aphthous stomatitis also give reactions to herpes simplex antigen (Anderson & Kilbourne 1961).

Skin Tests with Extracts of *C. diphtheriae*

The reactions to skin tests with toxin and other materials from *C. diphtheriae* are of considerable interest since in addition to demonstrating the presence of antitoxin (Schick test), both delayed and immediate type reactions may appear. The positive reaction to the toxin develops after 24–36 hr, is maximal at 4 days, persists for 7 days and may be followed by pigmentation. In subjects with adequate amounts of antitoxin no reaction occurs.

Control tests with heated toxin are performed, since hypersensitivity reactions may confuse the interpretation of reactions to the toxin. The pseudo-reaction is of the delayed type and runs a shorter course than the reaction to the toxin. Where reactions occur to both the toxin and the control (combined reaction) this difference in duration can help to distinguish them. In tests with

highly purified toxoid Pappenheimer (1958) found that it was safe to give the full immunizing dose of toxoid after 24–48 hr to subjects giving negative reaction at the control.

Allergic reactions to diphtheria toxoid such as urticaria and asthma occur particularly in allergic subjects. Relyveld, Henocq & Raynaud (1961) tested 200 subjects, some of whom suffered from clinical allergic disorders. Crude toxoid gave immediate positive reactions in 65% which could be transferred passively, whereas the purified toxoid gave no reactions, indicating that the allergen is something other than the toxin. In 10% delayed reactions were given to both the crude and purified toxoids and 25% reacted to neither. The majority of the subjects giving immediate and delayed reactions were Schick negative, showing the presence of circulating antitoxin. The toxin is responsible for the delayed hypersensitivity.

Skin Tests in Leprosy

The lepromin test introduced by Mitsuda (1924) is performed with extracts of nodules from the skin of patients with lepromatous leprosy. The original, integral lepromin consists of boiled, ground-up nodules after removal of the large particles of tissue. The lepromins of Dharmendra and of Fernandez consist respectively of bacilli separated from the skin with or without chloroform and ether extraction.

The reaction of lepromin consists of a tuberculin-type reaction, the Fernandez reaction, which appears at 24–48 hr, and a later nodular reaction, the Mitsuda reaction, which may appear at about 7 days and which is read at its maximum at 3–4 weeks. Integral lepromin gives more Mitsuda than Fernandez reactions, whereas the Dharmendra and Fernandez lepromins do the opposite. Grinding of the bacilli decreases the Mitsuda, and increases the Fernandez reactions.

The reaction to lepromin is not diagnostic of leprosy, but is diagnostic of the type of leprosy and is of considerable prognostic importance. Positive Fernandez and Mitsuda reactions are obtained in patients with tuberculoid leprosy, whereas those with lepromatous leprosy do not give either reaction.

Depot Lepromin

The introduction by Kinnear-Brown & Stone (1961) of a depot lepromin, analogous to depot tuberculin, has provided a lepromin of enhanced potency. Multiple puncture tests with depot lepromin enable twenty-five times the number of subjects to be tested, an important economy of scarce lepromin. Depot lepromin persists in the skin for prolonged periods and Kinnear-Brown & Stone (1961) found that vaccination with BCG leads to the appearance of reactions at previously negative test sites after 4 weeks, thus indicating the presence of related antigens in BCG and *M. leprae*. The depot lepromin test, with its economy, ease of administration, production of good but not severe reactions,

and its capacity to provide essential immunological information will have an important part to play in the clinical and epidemiological management of leprosy.

SKIN TESTS IN VIRAL RICKETTSIAL AND FUNGAL DISEASES

Skin tests with viral antigens give reactions of the delayed type of diagnostic value. In lymphogranuloma inguinale the Frei test is useful, rarely giving negative reactions in infective subjects. Cross reactions may be obtained with psittacosis virus, though treatment of the antigens with acid is said to give more specific reactions in these two conditions (Barwell 1952). Mumps virus gives delayed reactions which are of epidemiological rather than diagnostic value. Fifty per cent of infected subjects react by the 4th to 5th day and 80% by the 9th to 10th day. The majority of subjects with a past history give a positive reaction though 25% of older children and adults without a history of the disaese gave positive reactions, perhaps due to subclinical infections (Enders *et al* 1946). Shone *et al* (1966) have found that positive delayed skin test reactions to mumps virus antigen are obtained in 90% of children under 2 years with endocardial fibroelastosis, whereas 90% of controls were negative. Out of ten children with congenital mitral insufficiency usually associated with endocardial fibroelastosis seven gave positive reactions. These reactions were not correlated with serum antibody titres. Antigens from cat scratch fever, virus hepatitis and Western equine encephalomyelitis have all been found to give delayed reactions in infected subjects. Positive delayed reactions were obtained by Anderson & Kilbourne (1961) in patients with herpes simplex and some with aphthous stomatitis to extracts of the cell culture fluid of herpes simplex.

Grond, Capponi & Dumas (1961) report on skin tests with rickettsial extracts, some of which gave specific reactions and others cross reactions. Negative reactions to *R. prowazeki* indicate absence of past typhus infection, positive reactions appearing in infected subjects in about 2 months.

The skin test reactions to fungal extracts serve, as does the tuberculin test, as models for the different types of hypersensitivity and for assessing their relevance to resistance (see Chapter 3). Barker *et al* (1962) have reported that the protein component of trichophytin is largely responsible for delayed, and the polysaccharide component for immediate, reactions. Further investigations with purified antigens like these are essential for the proper use and interpretation of fungal reactions in general, and may help to explain the striking differences in reaction in patients infected with *T. mentagrophytes* or *T. rubrum* (Lewis *et al* 1958). In patients infected with *T. mentagrophytes* immediate reactions only were obtained in 1.5%, delayed only in 72.9%, both immediate and delayed in 15%, and negative reactions in 10.6%. In patients infected with

T. rubrum 53% gave immediate reactions only, 10% delayed reactions only, 32% reacted to both and 5% were negative.

The prognosis for infection with *T. rubrum* is far worse than that for *T. mentagrophytes* in which delayed reactions predominate. Similarly, in patients infected with animal ringworm, vigorous delayed reactions are produced by skin tests and the prognosis is good. The differences in type of reaction must be due to a greater production of skin-sensitizing antibody in response to infection with *T. rubrum*. It is also probable that the immediate weal produced by the test may mask the presence of delayed hypersensitivity.

Trichophytin tests are not regarded highly for diagnostic purposes, since a positive reaction does not necessarily indicate present infection. A negative reaction is of value in excluding these fungi as the cause of an 'id' eruption. In rare instances clinical allergic manifestations such as asthma and rhinitis follow on the injection of trichophytin. It may be that with improved antigens detailed studies of a possible relationship between these fungal infections and clinical allergic disorders would be worth seeking.

Immediate and delayed reactions to monilial antigens may be elicited in subjects suffering from allergic disorders, though their clinical significance is obscure. Reactions to intracutaneous tests with 1/10,000 to 1/100,000 Candida extract were obtained in 62–93% of allergic subjects compared to 29–60% controls. With higher concentrations, 15% of controls fail to react (Buffe *et al* 1961).

In general both immediate and delayed reactions may be obtained in subjects infected with *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Nocardia brasiliensis*, *Actinomyces* and *Sporothrix schenckii*.

Cross reactions may occur between histoplasmin, coccicioidin and blastomycin, though the reactivity is strongest to the homologous antigen (Lewis *et al* 1958; Wilson 1957).

SKIN TESTS IN PARASITIC INFECTION

Antigen from hydatid cysts (*Echinococcus granulosus*, *Trichinella spiralis* and *Dirofilaria immitis*, produce positive reactions, more immediate than delayed, in a high proportion of infected subjects. Antigens from *Toxocara canis* and *T. cati* have given promising though equivocal results in visceral larva migrans (see Chapter 6). Cross reactions are common in helminth infections (Baer & Yanowitz 1950). The use of these tests in various protozoal and helminthic diseases is discussed in Chapters 5 and 6.

SARCOIDOSIS AND SARCOID-LIKE REACTIONS IN HUMAN SKIN

A wide variety of substances produce nodular reactions containing epithelioid cell tubercles on injection into the skin. These reactions are fully developed

after 4–6 weeks, and must be confirmed by histological examination. Shelley & Hurley (1961) discuss one group of granuloma-producing agents, which have an immunological effect, only traces being required in persons with specific sensitivity, such as zirconium, beryllium and PPD, to which may be added the Kveim antigen. Another group of agents do not act immunologically; large doses of colloidal suspensions of the appropriate particle size produce reactions in all subjects. Examples are silica, stearate and palmitate, and other substances which produce foreign body granulomas. They suggest that the local reticulo-endothelial reaction is excited by the particulate material. It is of interest that the Kveim antigen is particulate and that antigen-antibody aggregates have been found by Germuth (1961) to produce granulomatous reactions.

In addition to their diagnostic value, skin tests in sarcoidosis present many challenging problems.

KVEIM TEST (1941)— A SKIN TEST IN SARCOIDOSIS

The reaction to the intracutaneous injection of suspensions of lymphoid tissue from patients with sarcoidosis has been used to support the diagnosis of the disease where the clinical picture is suggestive and histological information is not available, and to distinguish between sarcoidosis and other diseases in which granulomatous lesions appear and where the clinical picture is not typical.

The essential feature of the positive Kveim test reaction is that typical 'naked' tubercles consisting of an epithelioid cell granulomatous reaction should be obtained in sarcoidosis but not in other diseases. Varying methods of preparation of the antigen have led to conflicting results. Purified test material (Chase 1961) has established the specificity of the test for the diagnosis of sarcoidosis. The mechanism of the slowly developing papule, maximal in 4–6 weeks, is obscure. Some light may be thrown on this by the similar reaction in subjects with hypersensitivity to zirconium, beryllium and to tuberculin in some persons.

PREPARATION OF ANTIGEN

Lymph node or spleen removed from patients suffering from sarcoidosis is chopped up finely and the larger particles, haemoglobin and soluble serum proteins, are removed by washing and differential centrifugation. The test material consists of a particulate suspension which passes through a bacteriological filter and can be easily dispersed. The intracutaneous test dose of 0.15–0.20 ml contains 450 μg of alcohol precipitable material, compared with 1300–2000 μg in the cruder extracts (Chase 1961). Siltzbach (1961) advises that a Huber needle with a lateral opening be used, in order to prevent a core of epidermis from being pushed into the skin. Care is required to prevent contamination with cotton wool or other fibres which can provoke false-positive reactions. The histological appearances of the tissue used for preparation of the antigen

do not help in selection and about half of the preparations tested by Siltzbach were inactive.

Interpretation of Reactions

Reliance is placed exclusively on the histological appearances by Siltzbach (1961). A dusky brown nodule develops after about 7–10 days, and reaches about 3–8 mm in diameter after 4–6 weeks. It resolves slowly and is inhibited by corticosteroids but may flare up with recrudescence of the disease, or in the post-partum period. Tissue suspensions of normal spleen or lymph node do not give reactions in patients with sarcoidosis nor in controls.

'A biopsy taken from a positive reaction at 28 days shows spherical nodules, composed mainly of epithelioid cells with occasional giant cells. A few lymphocytes and other inflammatory cells are found. The tuberculoid nodules may coalesce giving a picture of diffuse, densely packed epithelioid cell infiltration, with discrete tuberculoid structures at its periphery. Small areas of fibrinoid change are not uncommon, like those in the lesion of the disease' (Siltzbach 1961).

Negative reactions may contain foci of lymphocytes and histiocytes sometimes containing brown pigment. Foreign body giant cells are seen containing birefringent and fibrillar foreign material, the source of which is not known. If epithelioid cells are scattered in small numbers through the dermis, without a true tuberculoid pattern, the test is considered equivocal and should be repeated.

Results

Positive reactions were obtained in 83% of patients in whom the diagnosis was confirmed by histological examination, and in 50% of suspected cases, who had only the clinical features. False-positive reactions were obtained in less than 2% of patients with other diseases, and 5% of the tests were repeated because the histological findings were equivocal. The percentage of reactions decreased to 76% in patients with subacute active disease, 64% in chronic active disease and 33% in inactive disease. Decreased reactivity was associated with clinical improvement.

Israel & Sones (1961), who reported a high percentage of positive reactions in tuberculous patients, subsequently obtained no reactions with a different preparation thus confirming the claims of Siltzbach (1961), James (1955), and others. The reliability and consistency of the histological interpretation have also been tested and found to be satisfactory. In serial tests, gradual loss of reactivity occurs with time. Reactions were obtained in 86% retested in the first 6 months, in 78% retested 6–12 months later, in 68% 1–2 years later and in 42% retested 4–14 years later. Tests with this material in patients in Puerto Rico and in Britain (James 1961) gave similar results, indicating that there is a similarity in the disease in different parts of the world.

Shelley & Hurley (1961) investigated the possibility that the Kveim reaction is isomorphic, i.e. typical of the existing lesions but provoked by unrelated stimuli. They found that sarcoid subjects did not produce more granulomatous reactions to injections of sodium stearate, solutions of metals, or whole blood, than did patients with hypersensitivity to zirconium and beryllium.

Skin Tests with Other Antigens in Sarcoidosis

There is a general decrease in reactivity to delayed type antigens in this disorder, of which the decrease in tuberculin sensitivity is common and is regarded as supporting evidence for the diagnosis. In communities where tuberculosis has been largely eradicated other delayed type antigens are required to provide this evidence.

Israel & Sones (1961) compared the delayed reactions to the following antigens in patients with sarcoidosis and controls:

	Sarcoid patients (%)	*Controls* (%)
Mumps antigen	29.6	80.0
Oidiomycin	59.0	80.0
Pertussis agglutinogen	13.2	57.0
Trichophytin	18.5	26.7
Tuberculin	36.8	66.7

Citron (1958) has reported that 40% of patients with sarcoidosis react to oidiomycin compared with 90% of controls.

The depression of tuberculin sensitivity has been attributed to anticutins, leading to a so-called 'positive anergy'. Magnusson (1956) has found that the inhibitory effect of the test serum with which the tuberculin was mixed for the demonstration of anticutins was related to its wealing capacity whether or not it was obtained from patients with sarcoidosis. A very low degree of tuberculin sensitivity has been demonstrated in these patients with depot tuberculins (Seeberg 1951; James & Pepys 1956; and Pepys *et al* 1958) and with tuberculo-lipopolysaccharide (PmKo) (Choucroun *et al* 1960a and b). The failure to develop and maintain delayed sensitivity is shown by the observation that only a third of patients with sarcoidosis become transiently sensitive to tuberculin after BCG vaccination, and that all the controls gave positive skin test reactions after pertussis immunization as against 45% with transient hypersensitivity in the sarcoid patients (Israel, Sones, Stein & Aronson 1950).

Pyke & Scadding (1952) and Citron & Scadding (1957) have reported that systemic or local administration of cortisone enhances tuberculin sensitivity in sarcoidosis. Whilst corticosteroids delay absorption from the skin and may produce their effect by enhancing the persistence of the tuberculin, the full

explanation of this phenomenon is not clear. Attempts at production of contact sensitivity by Epstein & Maycock (1957) show that potent antigens sensitize both controls and sarcoid patients whereas less potent antigens sensitize fewer sarcoid than control patients. However, circulating antibody capable of mediating clinical allergic disorders and of giving immediate type reactions on skin testing is present.

Skin Tests in Zirconium Hypersensitivity

Shelley (1957) reported that prolonged application of a zirconium deodorant to the skin resulted in the appearance of papules which contained sarcoid-like epithelioid cell granulomata. The reaction, in sensitive subjects, to a skin test with very small doses of zirconium appeared after 10 days and contained sarcoid-like granulomata. There was no preceding reaction.

Skin Tests in Beryllium Hypsersensitivity

Hypersensitivity to beryllium appears after prolonged exposure and is manifested by widespread granulomatous lesions in the lungs which are followed by fibrosis. Granulomatous skin lesions also appear in exposed subjects. Corticosteroid treatment is very effective.

Patch tests are very reliable and give positive reactions (Denardi *et al* 1953; v. Ordstrand 1954; Hardy 1956; and Sneddon 1958). The reaction here, however, differs in one important respect from the intracutaneous test in zirconium hypersensitivity, in that an erythematous reaction is present at 24–48 hr. This persists for several weeks, unlike the usual reaction in contact sensitive subjects. Biopsy after several weeks shows the presence of sarcoid-like granulomatous infiltration.

Hardy (1956) states that serum protein changes like those in sarcoidosis are found, but she found no difference in the tuberculin sensitivity of affected subjects as compared with the general population. Very small numbers of patients have been found to be Mantoux negative (Sneddon 1958; Rogers 1957) and in one case a tuberculin-positive subject was seen to become tuberculin negative (Agate 1948). Kveim tests in a few patients were also negative (Sneddon 1958).

Skin Tests with Tuberculin Giving Granulomatous Reactions

Hurley & Shelley (1960) found that papular reactions were present after 4 weeks in five out of thirty-six negro patients, who gave positive reactions to tuberculin at 24–48 hr. These slowly developing reactions were unrelated to the severity of the tuberculin reaction and were reproducible on repeated testing. The reactions at 4 weeks were typical of sarcoidosis and were not found in tuberculin negative subjects.

Non-Immunological Granulomatous Reactions

Shelley & Hurley (1961) report that colloidal silica (25–30 mμ particles) in sufficient quantity produced epithelioid granulomas in all subjects, whereas very finely ground silicon dioxide did not produce this reaction. These authors believe that similar granulomas occurring at tattoo sites may be due, in the absence of evidence of specific hypersensitivity, to the breakdown of the pigments to a colloidal state which results in infiltration of reticulo-endothelial cells.

REFERENCES

Agate J.N. (1948) Delayed pneumonitis in a beryllium worker. *Lancet*, **ii**, 530.

Anderson W.A. & Kilbourne E.D. (1961) A herpes simplex skin test diagnostic antigen of low protein content from cell culture fluid. *J. Invest. Derm.* **37**, 25.

Bachi J. (1959) Studies on tuberculin reactions repeated in the same site (Report II). Histological study in human skin (II). *Kekkaku* **34**, 879.

Baer R.L. & Yanowitz M. (1950) Skin tests in various infectious and parasitic diseases. *Arch. Dermat. Syph.* **62**, 491.

Baird K.A. (1957) Allergy to bacterial products. A review of the literature. *Ann. Allergy* **15**, 174.

Banks H.S. (1951) Scarlet fever and haemolytic streptoccal sore throat. In *Modern Practice in Infectious Fevers*, p. 133. London: Butterworth.

Barker S.A., Cruickshank C.N.D., Morris J.H. & Wood S.R. (1962) The isolation of Trichophytin Glycopeptide and its Structure in Relation to Immediate and Delayed Reactions. *Immunology* **5**, 672.

Bier, O.G., Passos, H.C. and Siqueira, M. (1968) Haemorrhagic reactions elicited at sites of passive cutaneous anaphylaxis by the intravenous injection of aggregated γ-globulin *Immunology*, **14**, 291.

Barwell C.F. (1952) Some observations on psittacosis and lymphogranuloma venereum viruses; treatment of viruses by various reagents and specific activity of acid extracts. *Brit. J. exp. Path.* **33**, 268.

Black S., Humphrey J.H. & Niven J.S.F. (1963) Inhibition of Mantoux reaction by direct suggestion under hypnosis. *Brit. med. J.* **i**, 1649.

Boucher H., Roumagoux J., Souquet R. & Bonnefoy G. (1960) Loeffler's syndrome of ascaridian origin with incomplete verminous cycle. *Presse méd.* **68**, 57.

Brody J.A., Overfield T. & Hammes L.M. (1964) Depression of the tuberculin reaction by viral vaccines. *New Engl. J. Med.* **271**, 1294.

Bruce R.A. (1961) Observations on the measurement of skin sensitivity to tuberculin. *Tubercle (Lond.)* **42**, 199.

Buchanan W.W., Anderson J.R., Goudie R.B. & Gray K.G. (1958) A skin test in thyroid disease. *Lancet* **ii**, 928.

Buffe D., Gross E., Dominjon-Monnier F., Burtin P. & Kourilsky R. (1961) Étude comparative des seuils de réactions cutanees à la poussiere de maison et à candida albicans chez les allergiques et les non-allergiques. *Rev. franç. Allerg.* **1**, 48.

Burger M. (1950) *Bacterial Polysaccharides*. Springfield, Illinois: Charles C. Thomas.

Canetti G. (1946) *L'allergie tuberculeuse chez l'homme*. Collection de l'Institut Pasteur. Editions Medicales Flammarion.

CHAMBERS V.V. & GLASER J. (1958) The incidence of subsequent ragweed pollinosis in symptom-free persons having positive reactions to ragweed pollen extract. *J. Allergy* **29,** 249.

CHANDLER D. & NALBANDIAN R.M. (1966) DNA autosensitivity. *Amer. J. Med. Sci.* **251,** 145.

CHASE M. W. (1945) The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc. Soc. exp. Biol. (N.Y.)* **59,** 134.

CHASE M.W. (1961) The preparation and standardization of Kveim testing antigen. *Amer. Rev. resp. Dis.* **84** (part 2), 86.

CHASE M.W. (1966) Delayed type hypersensitivity and the immunology of Hodgkin's disease, with a parallel examination of sarcoidosis. *Cancer Research* **25** (part 1), 1097.

CHOUCROUN N., GRESLAND P. & KOURILSKY R. (1960a) Hypersensibilité de type 'Retardee' au lipopolysaccharide du bacille tuberculeux. *Rev. Tuberc. (Paris)* **24,** 589.

CHOUCROUN N., KOURILSKY R. & GRESLAND P. (1960b) Étude comparative des epreuves intradermiques a la tuberculine et au lipo-polysaccharide PmKo chez les sujets faiblement allergiques ou anergiques. *Rev. Tuberc. (Paris)* **24,** 980.

CITRON K.M. & SCADDING J.G. (1957) The effect of cortisone upon the reaction of the skin to tuberculin in tuberculosis and sarcoidosis. *Quart. J. Med.* **26,** 277.

CITRON K.M. (1958) The immunological aspect of sarcoidosis. *Brit. J. Derm.* **70,** 48.

COMSTOCK G.W. (1960) A comparison of purified tuberculins in South Eastern U.S.A. *Bull. Wld Hlth Org.* **23,** 683.

COOKE R.A. (1947) *Allergy in Theory and Practice.* Philadelphia and London: Saunders.

COURNAND A. & LESTER M.S. (1939) Skin reactions due to tubercle bacillus polysaccharides, p. 621. *Proc. 3rd Internat. Congr. Microbiol. N.Y.*

CURRAN W.S. & GOLDMAN G. (1961) The incidence of immediately reacting allergy skin tests in a 'normal' adult population. *Ann. intern. Med.* **55,** 777.

DAHLSTROM A.W. (1940) The instability of the tuberculin reaction: observations on dispensary patients with special reference to the existence of demonstrable tuberculous lesions and the degree of exposure to tubercle bacilli. *Amer. Rev. Tuberc.* **42,** 471.

DANIELS M., RIDEHALGH F., SPRINGETT W.H. & HALL I.M. (1948) *Tuberculosis in Young Adults. Report on the Prophit Tuberculosis Survey* 1935–44. London: H.K. Lewis.

DENARDI J.M., v. ORDSTRAND H.S., CURTIS G.H. & ZIEBINSKI J. (1953) Berylliosis: summary and survey of all clinical types observed in 12 year period. *Arch. industr. Hyg.* **8,** 1.

DUBOCZY B.O. (1965) Nonspecific early type of tuberculin reaction. *Amer. rev. Resp. Dis.* **92,** 55.

EBKEN R.K., BAUSCHARD E.D. & LEVINE M.I. (1966) Dermographism. Its definition, demonstration and prevalence. *J. Allergy* **37,** 104 (Abstract).

EDWARDS L.B. & PALMER C.E. (1958) Epidemiologic studies of tuberculin sensitivity I. Preliminary results with purified protein derivatives prepared from atypical acid-fast organisms. *Amer. J. Hyg.* **68,** 213.

EDWARDS P.Q. & EDWARDS L.B. (1960) Story of the tuberculin test, from an epidemiologic viewpoint. *Amer. Rev. resp. Dis.* **81,** 1.

ENDERS J.F., KANE L.W., MARIS E.P. & STOKES J. jr (1946) Immunity in mumps; correlation of the presence of dermal hypersensitivity and resistance to mumps. *J. exp. Med.* **84,** 341.

EPSTEIN W.L. & MAYCOCK R.L. (1957) Induction of allergic contact dermatitis in patients with sarcoidosis. *Proc. soc. exp. Biol. (N.Y.)* **96,** 786.

FORDAL R.W. & WINKELMANN R.K. (1965) Hypersensitivity to deoxyribonucleic acid in cutaneous disease. *Arch Derm.* **91,** 503.

FRY L., MASON A.A. & PEARSON R.S.B. (1964) Effect of hypnosis on allergic skin responses in asthma and hay fever. *Brit. med. J.* **i,** 1145.

FURCOLOW M.L., HEWELL B., NELSON W.E. & PALMER C.E. (1941) Quantitative studies of the tuberculin reaction: I. Titration of tuberculin sensitivity and its relation to tuberculous infection. *Publ. Hlth Rep. (Wash.)* **56,** 1082.

GELL P.G.H. & BENACERRAF B. (1959) Studies on hypersensitivity II. *Immunology* **2,** 64.

GERMUTH F.G. (1961) The biologic significance of experimentally induced allergic granulomas. *Amer. Rev. resp. Dis.* **84** (part 2), 84.

GERNEZ CH. & MARCHANDISE CH. (1935) La specificité des réactions cutanées tuberculiniques. *Rev. Immunol. (Paris)* **1,** 315.

GERNEZ-RIEUX CH. TACQUET A., GERVOIS M., VOISIN C. & MACQUET V. (1961) Valeur théorique et pratique des réactions cutanées tuberculiniques. *XIII^e Congres de la Tuberculose* 1961.

GITLIN D., JANEWAY C.A., APT L. & CRAIG J.M. (1959) 'Agammaglobulinaemia'. In *Cellular and Humoral Aspects of Hypersensitive States*, p. 375, ed. LAWRENCE H.A. New York: Hoeber Harper.

GLENCHUR H., FOSSIECK B.E.C.J. & SILVERMAN M. (1965) An immediate skin test for the diagnosis of active pulmonary tuberculosis. *Amer. rev. Resp. Dis.* **92,** 741.

GRAYKOWSKI E.A., BARILE M.F., LEE W.B. & STANLEY H.R. (1966) Recurrent Apthous Stomatitis. Clinical, Therapeutic, Histologic and Hypersensitivity Aspects. *J. Amer. med. Ass.* **196,** 637.

GRIEP W.A. & BLEIKER M.A. (1960) Comparison of a Dutch and Danish purified tuberculin. *Ned. T. Geneesk* **104,** 1025.

GROND P., CAPPONI M. & DUMAS N. (1961) Reactions allergiques et rickettsioses. *Rev. franç. Allerg.* **1,** 77.

GULD J. (1957) Interpretation of tuberculin reactions in populations with a high proportion of BCG vaccinated subjects. *Bull. Wld Hlth Org.* **17,** 225.

HAGY G.W. & SETTIPANE G.A. (1966) The frequency of allergens and positive skin tests among college students. *J. Allergy* **37,** 107 (Abstract).

HARDY H.L. (1956) Differential diagnosis between beryllium poisoning and sarcoidosis. *Amer. Rev. Tuberc.* **74,** 885.

HARGREAVE F.E., PEPYS J., LONGBOTTOM J.L. & WRAITH D.G. (1966) Bird breeder's (fancier's) lung. *Lancet* **i,** 445.

HEAF F. (1951) The multiple puncture tuberculin test. *Lancet* **ii,** 151.

HEAF F. & RUSBY N.L. (1959) *Recent Advances in Respiratory Tuberculosis*, p. 61. London. Churchill.

HEIDELBERGER M. & AVERY O.T. (1923) The soluble specific substance of pneumococcus. *J. exp. Med.* **38,** 73.

HOLMAN H. (1960) Systemic lupus erythematosus. A review of certain recent developments in the study of this disease. *J. Pediat.* **56,** 109.

HUMPHREY J.H. Personal communication, 1961.

HURLEY J.H. & SHELLEY W.B. (1960) Sarcoid granulomas after intradermal tuberculin in normal human skin. *Arch. Derm.* **82,** 65.

INDERBITZIN T. (1957) The effect of acute and delayed cutaneous allergic reactions on the amount of histamine in the skin. *Int. Arch. Allergy* **7,** 140.

INDERBITZIN T. (1959) Histamine in allergic responses of the skin. In *Mechanisms of Hypersensitivity*, p. 493, ed. SHAEFFER J.H., LA GRIPPO G.A. & CHASE M.W. Henry Ford Hospital International Symposium. London: J. & A. Churchill.

ISRAEL H.L., SONES M., STEIN S.C. & ARONSON J.D. (1950) BCG vaccination in sarcoidosis. *Amer. Rev. Tuberc.* **62,** 408.

ISRAEL H.L. & SONES M. (1961) *Sarcoidosis in Advances in Tuberculosis Research*, p. 211, vol. II. Basel and New York: Karger.

JAMES D.G. & THOMSON A.D. (1955) Kveim test in sarcoidosis. *Quart. J. Med.* **24,** 49.

JAMES D.G. & PEPYS J. (1956) Tuberculin in aqueous and oily solutions. Skin test reactions in normal subjects and in patients with sarcoidosis. *Lancet* **i,** 602.

JAMES D.G. (1961) Course and prognosis of sarcoidosis. *Amer. Rev. resp. Dis.* **84** (part 2), 66.

KABAT E.A. & MAYER M.M. (1961) *Experimental Immunochemistry*, p. 294. Springfield, Illinois: Charles C. Thomas.

KEAY, A. J. and EDMOND, E. (1966) Differential Mantoux testing in the testing of atypical mycobacterial infection in children.

KINNEAR-BROWN J.A. & STONE M.M. (1961) The multipuncture depot lepromin test. *Int. J. Leprosy* **29,** 1.

KVEIM A. (1941) En ny og spesifik-kutan reaksjon ved Boecks sarcoid. *Nord. Med.* **9,** 169.

LAWRENCE H.S. (1949) Cellular transfer of cutaneous hypersensitivity to tuberculin in man. *Proc. Soc. exp. Biol. (N.Y.)* **71,** 516.

LAWRENCE H.S. (1952) Cellular transfer in humans of delayed cutaneous reactivity to haemolytic streptococci. *J. Immunol.* **68,** 159.

LAWRENCE H.S. (1955) Transfer in humans of delayed skin sensitivity to streptococcal M. substance and to tuberculin with disrupted leucocytes. *J. clin. Invest.* **34,** 219.

LAWRENCE H.S. (1959) Delayed hypersensitivity and the behaviour of the cellular transfer system in animals and man. In *Mechanisms of Hypersensitivity*, p. 453, ed. SHAFFER J.H., LA GRIPPO G.A. & CHASE M.W. Henry Ford Hospital International Symposium. London: J. & A. Churchill.

LAURENT L.J.M. (1951) Immunization. Scarlet fever. In *Modern Practice in Infectious Fevers*, p. 76, ed. BANKS H.S. London: Butterworth.

LEWIS C.E., HOPPER M.E., WILSON J.W. & PLUNKETT O.A. (1958) *An Introduction to Mycology*, p. 229. Chicago: The Year Book Publishers Inc.

LONG D.A. & MILES A.A. (1950) Opposite actions of thyroid and adrenal hormones in allergic hypersensitivity. *Lancet* **i,** 492.

LONGBOTTOM J.L. & PEPYS J. (1964) Pulmonary aspergillosis. Diagnostic and immunological significance of antigens and C-substance in *Apergillus fumigatus*. *J. Path. Bact.* **88,** 141.

LUNN J.A. & HUGHES D.T.D. (1967) Pulmonary hypersensitivity to the grain weevil. *Brit. J. indust. Med.* **24,** 158.

MAGNUSSON B. (1956) The effect of sarcoidosis on the tuberculin response. *Acta derm.-venereol. (Stockh.)* **36,** Suppl. 35.

MAGNUSSON M. & BENTZON M.W. (1958) Preparation of purified tuberculin. RT.23. *Bull. Wld Hlth Org.* **19,** 829.

MAGNUSSON M., GULD J., MAGNUS K. & WAALER H. (1958) Diluents for stabilization of tuberculin. *Bull. Wld Hlth Org.* **19,** 799.

MCCARTER J.R. & WATSON D.W. (1942) The relationship of the antigenicity, physical-chemical properties and polysaccharide content of tuberculins to their intracutaneous activity. *J. Immunol.* **43,** 85.

MEDICAL RESEARCH COUNCIL REPORT (1956) BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescents. *Brit. med. J.* **i,** 413.

MEYER S. NISSEN (1952) A method for standardization of tuberculin preparations by intracutaneous reactions in humans. Comparison of two purified tuberculins. *Amer. rev. Tuberc.* **66,** 292.

MILES A.A. (1955) Early tissue reactions to tubercle bacilli and their products. In *Experimental Tuberculosis*, ed. WOLSTENHOLME G.E. & CAMERON M.P. Ciba Foundation Symposium. London: J. & A. Churchill.

MITSUDA K. (1924) Inoculation with an emulsion of leprous nodules. *Proc. 2nd Int. Leprosy Conference, Strasbourg.*

O'GRADY F. (1956) Mantoux reaction patterns in active and arrested tuberculosis. *Brit. J. Tuberc.* **50,** 159.

O'GRADY F. (1957) Tuberculin sensitization in man. *Brit. J. Tuberc.* **51,** 74.

ORES R.O. & LANGE K. (1964) Skin test for the diagnosis of systemic lupus erythematosus. *Amer. J. Med. Sc.* **248,** 562.

PALMER C.E. (1953) Tuberculin sensitivity and contact with tuberculosis. Further evidence of nonspecific sensitivity. *Amer. rev. Tuberc.* **68,** 678.

PAPPENHEIMER A.M. jr (1958) The Schick test (1913–1958) *Int. Arch. Allergy,* **12,** 35.

PATERSON A.B. & LEECH F.B. (1954) Factors affecting the intradermal tuberculin reaction on the guinea pig. *Amer. rev. Tuberc.* **69,** 806.

PEARSON R.S.B. (1937) Observations on skin sensitivity in asthmatic and control subjects. *Quart. J. Med.* **30,** 165.

PEPYS J. (1951) Effects of the anti-histaminic drugs on tissue reactions. *Acta allerg. (Kbh.)* **4,** 115.

PEPYS J. (1955) The relationship of nonspecific and specific factors in the tuberculin reaction. *Amer. rev. Tuberc.* **71,** 49.

PEPYS J., BRUCE R.A. & JAMES D.G. (1958) Multiple puncture depot tuberculin (PPD) cream tests in man. *Tubercle (Lond.)* **39,** 283.

PEPYS J., FAUX J.A., LONGBOTTOM J.L., MCCARTHY D.S. & HARGREAVE F.E. (1967) Allergic reactions to *Candida albicans* in respiratory disease. To be published.

PEPYS J., JENKINS P.A., LACHMANN P.J. & MAHON W.E. (1966) An iatrogenic autoantibody: immunological responses to 'pituitary snuff' in patients with diabetes insipidus. *Clin. exp. Immunol.* **1,** 377.

PEPYS J., RIDDELL R.W., CITRON K.M., CLAYTON Y.M. & SHORT E.I. (1959) Clinical and immunologic significance of *Aspergillus fumigatus* in the sputum. *Amer. Rev. resp. Dis.* **80,** 167.

PEPYS, J., TURNER-WARWICK, Margaret, DAWSON, Patricia L. and HINSON, K.F.W. (1967) Proceedings of the VIth International Congress of Allergology, Montreal. (To be published).

POLLOCK T.M., SUTHERLAND I. & HART P. D'ARCY (1959) The specificity of the tuberculin reaction in man in Great Britain. *Tubercle (Lond.)* **68,** 678.

PROCHAZKA FISHER J. (1959) In the discussion on histamine in allergic responses of the skin. In *Mechanisms of Hypersensitivity,* p. 503, ed. SHAFFER J.H., LA GRIPPO G.A. & CHASE M.W. Henry Ford Hospital, International Symposium, London: J. & A. Churchill.

PYKE D.A. & SCADDING J.G. (1952) Effect of cortisone upon skin sensitivity to tuberculin in sarcoidosis. *Brit. Med. J.* **ii,** 1126.

RATNER B. (1943) *Allergy, Anaphylaxis and Immunotherapy.* Baltimore: Williams & Wilkins.

RELYVELD E.H., HENOCQ E. & RAYNAUD M. (1961) La sensibilisation aux antigènes diphtèriques, Fourth International Congress of Allergy. Excerpta Medica International Congress Series, 1961.

ROGERS W.N. (1957) Chronic beryllium poisoning treated with corticotrophin. *Lancet* **ii,** 267.

ROSENTHAL S.R. & LIBBY J.E.P. (1960) Simultaneous multiple tuberculin testing. *Bull. Wld Hlth Org.* **23,** 689.

SAVE L. (1953) Contribution à l'étude de la réaction de réinfection et de la sensibilité à la tuberculine chez les enfants vaccines avec le BCG sec par voie digestive. *Presse Med.* **61,** 524.

SEEBERG G. (1951) The response to 'depot tuberculin' studied in patients with lupus vulgaris. *Acta derm.-venereol.* **31,** 364.

SEIBERT F.B. (1934) The isolation and properties of the purified protein derivative of tuberculins. *Amer. rev. Tuberc.* **30,** 713.

SEIBERT F.B. & DUFOUR E.H. (1954) Comparison between the international standard tuberculins, PPD-S and old tuberculin. *Amer. rev. Tuberc.* **69,** 585.

SHELLEY W.B. (1957) Some reflections on certain new granulomata. *Trans. St. John's Hosp. derm. Soc. (Lond.)* (no. 39).

SHELLEY W.B. & HURLEY H.J. (1961) Experimental sarcoid reactions in human skin. *Amer. Rev. resp. Dis.* **84** (part 2), 45.

SHONE J.D., MUNOZ ARMAZ S., MANNING J.A. & KEITH I.C. (1966) The mumps antigen skin test in endocardial fibroelastosis. *Pediatrics* **37,** 423.

SILTZBACH L.E. (1961) Current status on the Nickerson-Kveim reaction. *Amer. Rev. resp. resp. Dis.* **84** (part 2), 89.

SNEDDON I.B. (1958) Beryllium disease. *Postgrad. med. J.* **34,** 262.

SQUIRE J.R. (1952) Tissue reactions to protein sensitization. *Brit. med. J.* **i,** 1.

SUMMERS D.F., GROLLMAN A.P. & HASENCLEVER H.F. (1964) Polysaccharide antigens of *Candida* cell wall. *J. Immunol.* **92,** 491.

SWINEFORD O.J. & HOLMAN J. (1948) Studies in bacterial allergy. III. Results of 3860 cutaneous tests with 34 crude polysaccharide and nucleoprotein fractions of 14 different bacteria. *J. Allergy* **20,** 420.

TABART J. (1961) Valeur de la technique du prick test par rapport à d'autres methodes d'examen en allergologie. In *Test Procedures Used in the Examination of the Allergic Patient,* p. 49. Proceedings European Academy of Allergy. Leiden: H. E. Stenfert, Kroese, N.V.

TEN CATE H.J. (1961) Provocation tests with inhalants and exposure tests with foods. In *Test Procedures Used in the Examination of the Allergic Patient,* p. 101. Proceedings European Academy of Allergy. Leiden: H.E. Stenfert. Kroese, N.V.

TILLETT W.S. & FRANCIS T. (1929) Cutaneous reactions to the polysaccharides and proteins of pneumococcus in lobar pneumonia. *J. exp. Med.* **50,** 687.

v. ORDSTRAND H.S. (1954) Berylliosis; real but preventable industrial disease. *Arch. industr. Hyg.* **10,** 232.

WAALER H., GULD J. & MAGNUS K. (1958) Adsorption of tuberculin to glass. *Bull. Wld Hlth Org.* **19,** 783.

WADLEY F.M. (1949) The use of biometric methods in comparison of acid-fast allergens. *Amer. rev. Tuberc.* **60,** 131.

WALZER M. (1947) In *Allergy in Theory and Practice,* p. 494, ed. COOKE R.A. Philadelphia and London: W. B. Saunders.

W. H. O. Tuberculosis Research Office (1955) Repeated tuberculin tests in the same site. *Bull. Wld Hlth Org.* **12,** 197.

WILSON J.W. (1957) *Clinical and Immunologic Aspects of Fungous Diseases.* Springfield, Illinois: Charles C. Thomas.

CHAPTER 8

BLOOD TRANSFUSION SEROLOGY

K.L.G.Goldsmith

INTRODUCTION

Up to 1900, numbers of blood transfusions had been given but it had not been understood why many of the recipients had died. This was explained by Landsteiner, when in that year he discovered three of the four groups of the ABO system (the fourth, AB, being discovered in the following year). The increased use of transfusion therapy has enabled great strides to be made in our knowledge of red cell antigen-antibody systems. Moreover, geneticists, anthropologists, forensic pathologists and others have come to realize that they too can use the

reactions which form the basis of blood grouping in their own particular investigations.

In this chapter the general principles involved are first set out and then each blood group system is considered in turn. The application of this knowledge to practical problems is described and the chapter concludes with a note on leucocyte and platelet antigens.

GENERAL PRINCIPLES

Antigens and Antibodies

As in other branches of immunology, we are concerned with antigens and antibodies, which are mutually dependent, for until an antibody has been discovered the corresponding antigen cannot be identified. The antigens that concern blood group workers are usually attached to the red cells, but some, as will be shown later, are also present in the serum or other body fluids. Antibodies, on the other hand, are usually free in the globulin fraction of the serum.

In most cases, a given antibody appears to be entirely specific for a particular antigen, but from time to time cross-reaction with other antigens, sometimes of other species, is found to occur.

Reaction of Blood Group Antibodies

Blood group antibodies are classified both by the red cell antigens with which they react, and also by the manner in which the reaction is detected. The former differences are those which define the various antigen-antibody systems, and are considered in the appropriate sections, but the manner in which they react is briefly considered here.

Saline agglutinating antibodies

Agglutination, the commonest reaction of red cells in the presence of an appropriate antibody, often occurs when the red cells are suspended in a saline medium, and in this case the antibodies are called 'saline agglutinating', or more simply 'saline' antibodies.

Incomplete antibodies

With the discovery of the Rh blood group system, it was found that mothers could give birth to babies suffering from haemolytic disease of the newborn, apparently due to anti-Rh, without any demonstrable saline antibodies being present in the mother's serum. Such antibodies were called 'incomplete' and were later found to be demonstrable by various techniques, of which the following are the most important (see Chapter 1).

Blocking test. Diamond, Race and Wiener independently discovered that certain anti-Rh sera, while not capable of agglutinating a saline suspension of

Rh-positive red cells, were capable of being adsorbed on to the red cell surfaces in such a way as to inhibit their subsequent agglutination by known saline Rh antibodies.

Albumin agglutination technique. Subsequently Diamond, Wiener and others found that red cells suspended in a protein medium, such as albumin, were agglutinated by incomplete Rh antibodies. This test, which is easier to perform and is more sensitive than the blocking test, has largely replaced it.

The antiglobulin test. Some antibodies cause no visible reaction in either a saline or protein medium. In this case, the presence of the antibody adsorbed on to the red cell surface can almost without exception be demonstrated by means of an antiglobulin test. This test was described by Coombs, Mourant and Race, and depends on the fact that red cells coated with incomplete antibody agglutinate when exposed to the serum of an animal previously immunized to human globulin (or to human whole serum), as the antibody adsorbed on their surface is a globulin. This test has a wide application as it will detect many of the blood group antibodies and, indeed, is in some cases the only test capable of doing so.

The use of enzyme-treated cells. Red cell surfaces may be modified by certain proteolytic enzymes, particularly trypsin, papain, ficin and bromelin, so as to render them more easily agglutinated by certain blood group antibodies. Of particular interest is the fact that such enzyme-treated cells in saline suspension will be agglutinated by incomplete Rh antibodies.

Effect of temperature

Antigen-antibody reactions are very considerably influenced by the temperature at which the reaction is carried out. Some antibodies, of which most anti-A and anti-B are examples, react best between 4°C and 18°C, whereas others, notably Rhesus antibodies, give optimal reactions at 37°C.

Prozone phenomenon

Normally, in the course of agglutination, antibody molecules each attach themselves to two red cells, thus causing the red cells to adhere. In the presence of potent agglutinins, an excess of antibody molecules may each attach themselves so rapidly, one to each of all available red cell antigen sites, that no one molecule can then attach itself to a second red cell. Only by diluting the potent antibody-containing serum can red cell agglutination occur. The absence or weakness of agglutination at low dilutions followed by stronger agglutination at somewhat high dilutions followed by the usual falling off at still higher dilutions constitutes a prozone.

Globulin fractions in which antibodies occur

Most antibodies in human serum are γ-globulins with a molecular weight of approximately 160,000, and a sedimentation coefficient of 7S. In a terminology recently introduced by the Immunology Section of the World Health Organization these globulins are called IgG or γG. Other antibodies which have a molecular weight of 1,000,000 are called IgM or γM and they have a sedimentation coefficient of 19S. In addition, a third group of antibodies which has the same sedimentation coefficient as γG antibodies but a higher carbohydrate content has been labelled γA.

Saline-agglutinating antibodies are usually γM while those that react only in a protein medium are usually γG. The relationship of the γA antibody fraction to blood group antibodies is at present being investigated by a number of workers.

The Cells of the Blood

Red cell antigens have been fully investigated but recently evidence has accumulated to show that leucocytes and platelets also bear antigens and that antibodies to these sometimes occur. The study of leucocyte antigens in particular is proving important in the choice of tissue donors for transplantation. For this reason, an outline of recent discoveries in this field will be found later in this chapter.

Blood Group Systems

The most important of the blood group systems of red cells are listed below, together with the year in which they were first discovered:

ABO	1900	Lewis	1946
MN	1927	Duffy	1950
P	1927	Kidd	1951
Rh	1939	Yt	1956
Lutheran	1945	Xg	1962
Kell	1946		

The above list is not exhaustive but it does include all those systems which any but the most highly specialized worker is likely to encounter. There are also a number of antigens found only in a very few individuals and which are known as 'private' antigens (Levay, Wright and Batty being examples) and others, present on the red cells of all but a few exceptional individuals, described by Race and Sanger as 'public antigens' such as Sm and Vel.

In this chapter no attempt will be made to deal with the many complexities of certain blood group systems and for these the reader is referred to *Blood Groups in Man* by Race & Sanger (1962).

Blood Groups and Anthropology

Hirszfeld and Hirszfeld, working in Salonica in 1918 at the end of the First World War showed that the relative frequencies of the ABO groups differed in different races. Since then great strides have been made in the application of blood groups to anthropology. Those interested are referred to the works of Mourant (1954) and Mourant, Kopéc & Domaniewska-Sobczak (1958).

Notation

In reporting upon cases referred by clinicians, serological details should be described in as simple a manner as possible. Nevertheless, the scientific worker will require something much more comprehensive, and for this reason the reader will find proposals for modifications of the basic notation in many technical papers. Ford (1955) for instance, declared that the notation of the human blood groups was chaotic. He felt that not only was it out of accord with that adopted in other branches of genetics, but that the usage was inconsistent from one blood group system to another. He proposed various changes, some of which have been fairly generally accepted, although others have not so far been widely adopted. He emphasized that italics should be confined to genes and genotypes, this already being fairly common policy, and not used for phenotypes, antigens or antibodies. He also suggested that a gene without a suffix might be used for one whose hypothetical antigen had not been recognized by an antibody. For instance, the red cells of some Negroes react with neither anti-Fy^a nor anti-Fy^b and are given the phenotype Fy(a−b−), the gene responsible being given the symbol *Fy*.

Ford's suggestions, just mentioned, will be followed in the present chapter.

THE ABO BLOOD GROUP SYSTEM

Antigens

Landsteiner, once he had shown that the red cells of some of his colleagues were agglutinated by the sera of others, was quick to see that people could be divided into three groups which are now called A, B and O while in the following year von Decastello and Sturli discovered the group AB. These four groups were not only the first to be discovered but proved to be the most important of all from the clinical point of view, as the transfusion of ABO incompatible blood to an unfortunate recipient may be followed by a severe or even fatal transfusion reaction.

The antibodies used to determine the ABO groups are anti-A found in the serum of group B persons and anti-B from those who are group A.

In 1911 von Dungern and Hirszfeld described subgroups of A, using the serum known as anti-A_1 which agglutinated A_1 but not A_2 red cells. Nearly all anti-A

from group B donors contains the two antibodies anti-A and anti-A_1 (or α and α_1). Anti-A (α) agglutinates A_1, A_2, A_1B and A_2B red cells while anti-A_1 (α_1) agglutinates only A_1 or A_1B cells. The latter occurs naturally in the serum of some 2% of A_2 people and in 25% of A_2B people. Usually, however, this naturally occurring anti-A_1 is too weak to be used as a grouping serum and the reagent is prepared by absorbing the serum of group B donors with A_2 cells. The red cell genotypes and phenotypes that can be differentiated by the use of anti-A, anti-A_1 and anti-B, together with the naturally occurring agglutinins that may be found in the serum as shown in Table 8.1.

TABLE 8.1

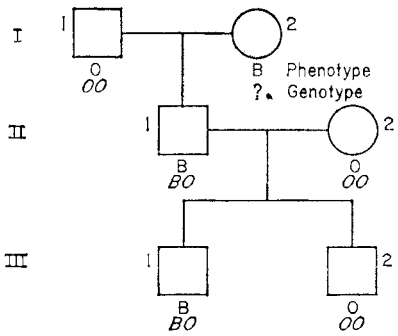
The demonstration of A_1, A_2, B and O groups as defined by anti-A, anti-B, anti-A_1 antisera, together with corresponding agglutinins

Genotype	Phenotype	Reaction of red cells with			Reaction of serum with	
		Anti-A	Anti-A_1	Anti-B	A_1 cells	B cells
A_1A_1						
A_1A_2	A_1	+	+	−	−	+
A_1O						
A_2A_2	A_2	+	−	−	+ or −	+
A_2O						
BB	B	−	−	+	+	−
BO						
A_1B	A_1B	+	+	+	−	−
A_2B	A_2B	+	−	+	+ or −	−

For most purposes these phenotypes give all the information required, but sometimes it may be helpful to know whether, for instance, an individual is *BB* or *BO*. Such a position would arise in the case of the father of a child found to be suffering from haemolytic disease of the newborn due to immune anti-B. It is likely that the mother would be group O, and the father B, and the clinician would want to know what were the chances of the parents having another similarly affected infant. If the father proved to be of the genotype *BB*, then all his children would carry the *B* gene and could be affected by haemolytic disease of the newborn, but if he were *BO* then half would lack the *B* gene and so would be unaffected. There are no antisera which can give this information by the examination of an individual blood specimen. However, under these circumstances a family study may sometimes give the required information. A pedigree illustrating the position is given in Fig. 8.1.

The father in question is shown as II-1. His phenotype is B, but by grouping

his own parents it is possible to show that his father must have transmitted an O gene to him, so that he must in fact have the genotype *BO*. Similarly, grouping of his children III-1 and III-2, would in this case show the same thing, for the very fact that he has had a group O child is evidence that he must have a group O gene to transmit to it.



Other A Subgroups

In 1936, Friedenreich described the rare phenotype A_3. Characteristically the appearance of the agglutination when A_3 blood is tested with anti-A, is of small clumps of cells surrounded by large numbers of free cells. Curiously, the presence of the *B* gene seems partially to suppress the agglutination of A_2 cells by anti-A serum, with the result that A_2B cells may react so weakly that they are erroneously classified as A_3B.

One other rare, but nevertheless clinically important weak A variant is A_x, also known as A_4 or A_0. The red cells are characterized by negative or weak reactions with anti-A serum, negative reactions with anti-B but positive reactions with the sera of some group O persons (i.e. anti-A + anti-B). The clinical importance of A_x lies in the fact that if such a person were a blood donor whose red cells had been tested only with anti-A and anti-B sera, he might erroneously be reported as Group O. Subsequent transfusion of this person's red cells into a group O recipient might well be followed by a haemolytic transfusion reaction. It is essential, therefore, that the red cells of all apparently group O donors are tested with a group O serum that is known to agglutinate A_x cells.

There are other weak forms of the A antigen but details of these will be found in Race & Sanger (1962). One, called A_g by van Loghem, Dorfmeier & van der Hart (1957) deserves mention as an interesting clinical problem. The individual concerned was group A but his red cells reacted extremely weakly with anti-A serum, and as he was suffering from myeloblastic leukaemia, the disease was thought to have modified the antigen. Other cases have been described, in some

of whom it has been possible to correlate the strength of the A antigen with progress and remission of the disease.

B Subgroups

B variants are rare, though a number, including B_3, B_k, B_v, B_w and B_x have been described, but more interesting was the demonstration by Marsh and Jenkins and by others of the presence in certain A_1 individuals of an acquired B-like antigen that makes the red cells react apparently as A_1B. Marsh (1960) showed that a particular bacterial filtrate was capable of modifying O and A cells to produce a weak B-like antigen. Unfortunately the organism from which the filtrate had been obtained was dead and could not be identified. Subsequently Andersen and later Springer showed that a B-like character could be produced on human erythrocytes by treatment with purified *E. coli* O_{86}.

The H Antigen

By the use of anti-H and anti-O considered below, red cells of all groups are shown with very rare exceptions to carry an antigen called H. This is more abundant in O cells than in those of other groups as may be demonstrated as follows:

$$O > A_2 > A_2B > B > A_1 > A_1B$$

There are now strong reasons for believing that H is a basic antigen from which the A and B antigens are developed by the action of appropriate genes, little being transformed in the production of group O cells, and most in the production of A_1B.

The 'Bombay' Blood Groups

A number of examples are known of blood samples which give no reactions with anti-A, anti-B, anti-A+anti-B, or anti-H. It has been shown that the peculiar phenotype is inherited and the condition is explained by assuming that such individuals carry a modifying *x* gene which when present in double dose suppresses the development of A, B and H antigens. It appears that *X*, the dominant allele of *x*, is necessary for the formation of the precursor antigen H from an even earlier precursor, immunologically related to the antigen of Pneumococcus Type XIV. The effect of the *Xx* genes on the Lewis system is further considered on page 241. The 'Bombay' phenotype is often given the symbol Oh and Levine has given it a superscript Oh^b, Oh^{A1}, Oh^{A2} when a family study has made it clear which antigen of the ABO system was suppressed.

Chimeras

It has been known for some time that in bovine twin embryos, vascular anastomoses can occur. In 1945, Owen showed that mixtures of blood groups in

non-identical cattle were due to the interchange of primitive blood cells, which results from the mixing of the two circulations.

Dunsford *et al* (1953) described a normal female donor whose red cells appeared to be a mixture of O and A_1. The mixture was separated by differential agglutination, and two different normal cell populations identified, one set being apparently derived from her twin brother, who died in infancy. A number of other similar human chimera pairs have now been investigated in which both individuals have survived.

Antibodies

So-called 'Natural' and 'Immune' anti-A

Mention has already been made of anti-A, anti-A_1 and anti-B. These antibodies are normally present in the serum (see Table 8.1), and show the characteristics of 'natural' antibodies. Blood group antibodies as a whole are commonly divided into 'natural' and 'acquired', the former being normally present and not attributable to any known immunization while the latter are thought to result from a definite immunizing stimulus. The 'natural' antibodies are not detectable in an infant until it is 3 to 6 months old, though at birth its serum may contain antibodies that have passed across the placenta from the mother.

The antibody titres of both anti-A and anti-B rise during early childhood to reach a maximum in adolescence, and then fall slowly during the remainder of life. These 'natural' antibodies react best in saline, optimally between 4°C and 18°C. Despite their name, it is not unlikely that these antibodies develop as a result of some as yet unidentified immune response, following ingestion or inhalation of antigens, which, though not blood group antigens themselves, resemble them in the essential part of their structure.

'Immune' anti-A and anti-B may develop following obvious stimuli such as heterospecific pregnancy or, happily rarely, incompatible blood transfusion. More commonly, however, they probably result from the injection of horse serum (anti-tetanus serum) or of vaccines which are prepared from organisms cultured in media containing horse or hog extracts. Schiff and Adelsberger showed that there was a connection between the Forsmann antigen and the human A antigen. Antigens of A-like specificity are by no means rare, and in the case of the pig, Winstanley, Konugres & Coombs (1957), Konugres & Coombs (1958) and Lockyer (1959) have demonstrated that immune anti-A, unlike natural anti-A reacts with pig group A red cells. As a result, the term anti-A^P is used for the human antibody which reacts with such pig cells.

'Immune' forms of anti-A and anti-B are albumin antibodies reacting best at 37°C, and, in the presence of complement, often behave as haemolysins. Witebsky devised a partial neutralization test in which he made use of the fact that while 'natural' anti-A or anti-B can readily be neutralized by the appropriate blood group substance, 'immune' antibodies cannot. By adding AB

substance to an immune anti-A serum he showed that the serum would then only weakly agglutinate A_1 cells in saline suspension and failed to agglutinate A_2 cells at all. By repeating the test with the red cells suspended in serum instead of saline, the titre was increased. For further details of the 'immune' characteristics of antibodies of the ABO system, reference should be made to Mollison (1967). Immune anti-A and anti-B antibodies are of great clinical importance, for not only is the transfusion of ABO-incompatible blood into a person possessing such antibodies likely to be followed by a serious transfusion reaction, but also the transfusion of plasma containing the antibodies into an A or B recipient may be equally dangerous. Recently, for instance, Keidan, Lohoar & Mainwaring (1966) reported acute anuria in a group A haemophiliac following transfusion of plasma containing immune anti-A.

*Anti-*H *and anti-*O

Mention has already been made of anti-H. There are in fact two antibodies which react with the H antigen, anti-H and anti-O. These antibodies were formerly thought to react with a specific product of the *O* gene but this is now not thought likely. Both were formerly indiscriminately called anti-O but Morgan & Watkins (1948) showed that some anti-O sera were neutralized by O secretor saliva and some were not; those which were inhibited they renamed anti-H. Sanger (1952) showed that most donors who had anti-H in their serum were Le(a+) and non-secretors of ABH substance while those with anti-O were Le(a−), and hence probably secretors, for as described in the section on Lewis groups, this system and secretion are related.

Anti-H antibodies occasionally occur as 'natural' agglutinins in the serum of individuals of group A_1B and A_1, who have the least H substance, but in its 'incomplete' form anti-H occurs widely as a cold agglutinin, sensitizing cells at 4°C. Those rare persons who have the Oh ('Bombay') group described above, always have anti-H in their serum. The antibody is also found in the sera of certain animals other than man, and even in the extracts of some plant seeds (see below).

*Anti-*A + *anti-*B (O *serum*)

In the discussion of A_x cells it was noted that anti-A + anti-B serum (from a group O donor) might agglutinate them, whereas anti-A (from group B donors would not. As long ago as 1907, Hektoen showed that A cells, when mixed with serum from group A persons, absorbed an antibody that would on elution agglutinate not only A cells, but also B cells.

There are two possible explanations for this phenomenon. Either there is an antibody molecule in the serum which has two combining sites, one for A and one for B cells, or there is a totally different antibody (sometimes called anti-C) which reacts with C antigens present on A and B cells but not on O cells.

(This C antigen must not be confused with that of the same name used in Fisher's Rh nomenclature.)

Lectins

Certain seeds, notably those of *Leguminosae*, yield extracts which behave like blood group antibodies. As there is no evidence that plants can elaborate antibodies to protect themselves from infective micro-organisms, the term 'lectin' rather than 'antibody' has been applied to them by Boyd & Shapleigh (1954). Readers interested may refer to the work of Mäkelä (1957) or of Bird (1959), who have investigated the subject very fully. It is not proposed here to give a detailed list of suitable plants, but mention must be made of extracts of the seeds of *Vicia cracca* and *Dolichos biflorus*, both of which agglutinate strongly A_1 cells and weakly A_2 cells, and those of *Ulex europaeus* which contain anti-H.

THE MN BLOOD GROUP SYSTEM

Unlike the ABO and Rh systems, the MN system is of relatively little clinical importance, yet it demonstrates many of the aspects of blood group serology. The antibodies which determined the M and N antigens were prepared in 1927 by Landsteiner and Levine, by the injection of human red cells into rabbits. By using the two available antisera, three phenotypes were established (as shown in Table 8.2). The three groups can be explained, as Table 8.2 shows, in terms of two allelomorphic genes, *M* and *N*, giving rise to three genotypes, *MM*, *MN* and *NN*, corresponding to the three phenotypes M, MN and N.

TABLE 8.2

The MN groups demonstrated by anti-M and anti-N

Phenotype	Genotype	Reactions with	
		Anti-M	Anti-N
M	*MM*	+	−
MN	*MN*	+	+
N	*NN*	−	+

Ten years after the discovery of the system, weak forms of M and N were discovered, and shown to be due to alleles called M_2 and N_2. This is by no means the end of the story for a number of other variants of M and N have now been described including M^e which react with some anti-M and some anti-N sera, M^g and M^v, each with a corresponding antibody, *MK* an apparently silent M

gene and M^a, an M antigen which reacts with all anti-M antibodies except for that contained in the patient's own serum.

The Ss Antigens

The most important addition to the MN system was the S antigen, discovered in 1947 by Walsh and Montgomery in conjunction with Race and Sanger. Unlike anti-M and anti-N, anti-S was found not in rabbits, but in man. It is of clinical importance, as it can be the cause both of haemolytic disease of the newborn and of transfusion reactions. In 1951, Levine and his colleagues discovered anti-s (and hence the antigen s, the product of the *s* gene, the allele of *S*) in the serum of a woman who had given birth to a baby suffering from haemolytic disease of the newborn. The genes *S* and *s* were shown to be closely linked to *M* and *N*

Table 8.3

The MNS groups demonstrated by Anti-M, Anti-N, Anti-S and Anti-s

Genotype	Reactions with			
	Anti-M	Anti-N	Anti-S	Anti-s
MS MS	+	−	+	−
MS Ms	+	−	+	+
Ms Ms	+	−	−	+
MS NS	+	+	+	−
Ms NS	+	+	+	+
MS Ns	+	+	+	+
Ms Ns	+	+	−	+
NS NS	−	+	+	−
NS Ns	−	+	+	+
Ns Ns	−	+	−	+

in a similar manner to that postulated by Fisher for the Rh genes. as described below. The groups that can now be identified using anti-M, anti-N, anti-S and anti-s antisera are shown in Table 8.3. Notice that except for *MsNS* and *MSNs*, the genotypes can be clearly distinguished if the four sera are used.

S^u

In 1953 Wiener and his co-workers discovered an antibody which they called anti-U which agglutinated the red cells of all Europeans but failed to react with the red cells of 12 out of 989 New York Negroes. Greenwalt showed in 1954 that the red cells which failed to react also failed to react with anti-S or anti-s so that anti-U appeared at this stage to be anti-S + anti-s though it was not possible to separate the two antibodies. Individuals whose red cells are not

agglutinated by anti-U are said to be S^u, though no antibody to this hypothetical antigen has so far been detected. A recent complication of the position has been the discovery by Francis & Hatcher (1966) of the phenotype S−s−U+ and it is suggested that anti-U is a distinct antibody of the MN system, not anti-Ss.

It has been shown that *MM* cells normally carry a certain amount of N antigen whereas *MMS*^{u} cells do not. This finding is of some practical importance since the M-positive cells used for the absorption of the anti-human species agglutinin from rabbit anti-N sera tend to remove some of the anti-N as well, thus reducing the potency of an otherwise useful reagent. This could be avoided by the use of *MMS*^{u} cells for absorption. A weak form of S antigen S_2 has recently been described.

The Hunter and Henshaw Antigens

Landsteiner and his colleagues prepared another antibody by injecting rabbits with the red cells of a Negro, Mr Hunter. The antigen thus identified was found to be present in 7% of American Negroes but in very few Europeans. That it was related to MN was shown by the fact that all persons who were Hunter-positive were N or MN. Ikin and Mourant produced another antibody, anti-Henshaw, by injecting rabbits with red cells bearing the Henshaw antigen also related to MN. It is interesting to note that anti-Hunter is the only blood group antibody to human red cells never so far detected in man.

The *Vw* and *Mi*^{a} Genes

Van der Hart and colleagues discovered an antigen with a frequency of only 1 in 2000 white people. It was called Vw and was found to be linked with the MNSs system. The family in which the antigen was first discovered was also shown to have the antigen Mi^a, discovered three years earlier by Levine and his colleagues, and the two were at first thought to be identical. Later, however, it was shown that only 50% of Mi^a positives are Vw-positive. The phenotype Mi(a−) Vw(+) has not so far been found, and it therefore appears that the *Vw* gene produces the antigen Mi(a+) Vw(+), whereas the *Mi*^{a} gene produces the phenotype Mi(a+) Vw(−).

Antibodies of the MNSs System

These antibodies fall into two main groups, those which occur mainly or only in rabbits (anti-M, -N, -Hu and -He) and those occurring in man, such as (anti-S, -s, -S, -Mi^a and -Vw). Anti-N is also found in extracts of the seeds of *Vicia graminea* and *Bauhinia purpurea*.

The treatment of red cells with proteolytic enzymes modifies the antigens of the MNSs system so that they will no longer react with their appropriate antibodies.

The antigens and antibodies of this system have been considered in some detail, to illustrate the amazing expansion that can occur in what at first seemed to be a simple blood group system.

THE P BLOOD GROUP SYSTEM

The P factor, as it was then called, was discovered by Landsteiner and Levine while attempting to produce antibodies to human red cells in rabbits. For many years only the one antibody was available, and people were classified as P+ and P− although it was recognized that P-positives could be divided into strong and weak reactors.

From what has already been said about other groups, it might well be expected that this system would become more complex and this indeed has happened. A very rare antibody, anti-Tj^a, discovered by Levine, was shown to be connected with the P system and the latter was expanded as is shown in Table 8.4.

TABLE 8.4

The P blood group system

Genotype	Phenotype	Reactions of red cells with	
		Anti-P_1 (Anti-P)	Anti-(P+P_1) (Anti-Tj^a)
P_1 P_1 P_1 P_2 P_1 p	P_1	+	+
P_2 P_2 P_2 p	P_2	−	+
pp	p	−	−

The nature of the relationship P_1, P_2 and p as shown in this table resembles that of A_1, A_2 and O. The phenotype P_1 is that which used to be called P-positive. P_2 used to be called P-negative and p used to be called Tj(a−). More recently Kortekangas and colleagues described a 'new' P antigen, P^k, the red cells being agglutinated by anti-P_1 and by a specific antibody, anti-P^k. For further details of this and other aspects of the P blood group system see Race & Sanger (1962).

ANTIBODIES OF THE P SYSTEM

Anti-P_1, first made in rabbits, and subsequently often obtained from the 'natural' sera of horses and pigs, is also frequently if not invariably found in the sera of P_2 (P_1-negative, formerly called P-negative) people. Unfortunately it is only rarely powerful enough to be used as a grouping serum. Its potency *in vivo*

may be enhanced by antigenic stimulation. It is known that living scolices of hydatid cysts will inhibit anti-P_1 and it might be expected that patients suffering from hydatid disease, if P_1-negative, would have potent anti-P_1 in their serum. Perhaps some of the more potent anti-P_1 sera have such an origin. The antibody has never been incriminated as a cause of haemolytic disease of the newborn though at least one transfusion reaction has been attributed to it. It may also cause reduction of *in vivo* red cell survival, even in the absence of obvious clinical effects.

Anti-$P+P_1$ (anti-Tj^a) occurs in the serum of pp (Tj^a-negative) individuals. Indeed, the fact that all those who had anti-Tj^a in their serum were P-negative was evidence of the connection of Tj^a and P antigens. The antibody is extremely potent, can be enhanced by antigenic stimulation and often shows haemolytic properties *in vitro*.

THE Rh BLOOD GROUP SYSTEM

The discovery of the Rh system started in 1939 with the finding of a 'new' antibody in the serum of a recently delivered woman who had had a reaction after a transfusion of her husband's (ABO compatible) blood and whose child was stillborn following what is now called haemolytic disease of the newborn. In 1940 Landsteiner and Wiener described the production of an antibody in rabbits immunized with the red cells of Rhesus Monkeys (Macacus rhesus), to thirty-nine out of forty-five human red cell samples. This antibody was found to give similar reactions to the 'new' human antibody. The Rhesus, or Rh factor, was soon found to be not a single entity, but, in fact, a very complex system. Here, only an outline of the present position will be given and for further details the reader is referred to Race & Sanger (1962).

Two Theories of the Rh System

In the course of parallel investigations by Wiener in America, and by Race and Taylor in England, it became clear that whereas in the other known blood group systems, a single gene never determined more than one antigen, in the Rh system a number of distinct antigens or blood factors were in some cases determined by a single genetical unit. There was seldom a dispute about the facts, but two distinct ways of interpreting them arose, one due to Wiener and the other due to Fisher in England. Wiener considered that there were eight 'Standard' allelic genes, any one of which might appear at a single locus, and which would govern the appearance of corresponding agglutinogens on the red cells. Each agglutinogen would in turn be made up of one or more blood factors. A specific antiserum could be produced for each of the several blood factors.

Fisher, on the other hand, postulated that there were three or more closely

linked genes on the chromosome, that each gene would be responsible for the production of a specific antigen, and that this, in turn, would be detected by a specific antibody. The genes would each have an allele, and these pairs were called *Dd*, *Cc* and *Ee*, a typical chromosome for instance being *DCe*, the genes being in that order on the chromosome, and not *CDe* as might be supposed. Any such combination could occur, but some are much more common than others. So far five of the six antibodies predicted by Fisher have been discovered, the exception being anti-d.

Rhesus Genotyping

Strictly speaking the results of testing a given specimen of blood represent a phenotype. However, from the frequencies of the phenotypes of a given population it is possible to calculate the frequencies of the Rh chromosomes or compound genes. Table 8.5 shows the frequency of the Rh chromosomes in the English population. Frequencies found in the various European populations differ somewhat from these, and those found in non-European populations are even more widely different. For further details see Mourant (1954).

Table 8.5

The frequency of the Rh Chromosomes in England
(Based on Race, Mourant, Lawler & Sanger (1948))

Fisher	Wiener 1949	Frequency
CDe	R^1	0.4076
cde	r	0.3886
cDE	R^2	0.1411
cDe	R^o	0.0257
C^wDe	R^{1w}	0.0129
cdE	r''	0.0119
Cde	r'	0.0098
CDE	R^z	0.0024
C^wde	r'^w	of
CdE	r^y	very
C^wDE		low
C^wdE		frequency

Using the five antisera anti-C, -D, -E, -c and -e the probable 'Rh-positive' genotypes in a population derived from European stock may be detected as in Table 8.6.

It is important to determine the genotype of the father of a child suffering from haemolytic disease of the newborn, so that the prognosis for future pregnancies may be determined. As most incompatible pregnancies are due to the D

antigen, the differentiation required will be most often between *DD* and *Dd*, although reference to Table 8.6 will show that in every case there is a possibility of error, which cannot be eliminated by any test made on the individual concerned.

TABLE 8.6

Probable positive genotypes of a white population (Based on Race *et al* (1948))

Reaction with anti					Most probable genotype	%	Other possible genotype	%	Error in 1st guess %
−C	−D	−E	−c	−e					
+	+	−	+	+	*CDe/cde*	32.7	*CDe/cDe*	2.2	7
+	+	−	−	+	*CDe/CDe*	17.7	*CDe/Cde*	0.8	5
+	+	+	+	+	*CDe/cDE*	12.0	*CDe/cdE* } *Cde/cDE* }	1.5	13
—	+	+	+	+	*cDE/cde*	11.0	*cDE/cDe*	0.7	6
—	+	+	+	−	*cDE/cDE*	1.9	*cDE/cdE*	0.3	16
—	+	−	+	+	*cDe/cde*	2.0	*cDe/cDe*	0.07	4

OTHER Rh ANTIGENS

THE D^u ANTIGEN

Some apparently D-positive red cells are agglutinated by some anti-D sera and not by others and are called D^u. Different grades of D^u exist, some cell samples agglutinating with almost all anti-D sera and some only with very few. All D^u samples, even the weakest, react by the indirect antiglobulin test, after sensitizing with a strong albumin anti-D serum. The D- antigen is usually inherited in a straightforward manner, but in some cases there is a partial suppression of an apparently normal *D* gene by a *Cde* combination inherited from the other parent. The injection of D^u cells into a D-negative person results in the development of an anti-D antibody, there being no specific anti-D^u antibody.

SUBDIVISIONS OF THE D ANTIGEN

Wiener has shown that Rh(D) positive individuals may have an anti-D antibody in their serum which fails to react with their own cells. He therefore divides the Rh_o, or D, antigen, into Rh_o, A, B, C and D. All normal D-positive individuals have all factors but anyone who lacks one or more may develop antibodies to the missing ones.

OTHER FACTORS IN THE RH SYSTEM

Among alleles of C and E may be mentioned C^w, C^x and E^w, each of which gives rise to a specific antibody and C^u and E^u which, like D^u, do not.

Over the last few years the Rh system has become far more complicated, and a number of people today think that Fisher's genetical theory, although fundamentally correct, will require considerable modification to fit the facts as they are now known and that some change will also be necessary in the nomenclature. Race, Sanger and colleagues explain almost all, but not quite all the findings of the hypothesis of interaction between genes at two or more of the CDE loci on a single chromosome.

One example of this interaction is the f factor, once thought to be a new gene but now regarded as a joint product of the *c* and *e* genes when these are present on a single chromosome. Another is the V factor which is regarded as the product of a normal *c* and a variant of *e* known as e^s on the same chromosome. Similarly, Rh_i is thought to be the product of C and e on the same chomosome. For further details of these and other Rh antigen complexes, reference should be made to Race & Sanger (1962).

Apparent RH Gene Deletions

Race, Sanger & Selwyn (1950), discovered a blood sample which gave no reactions with anti-C, -c, $-C^w$, or $-C^x$ and none with anti-E, -e, or $-E^w$, so that it appeared to be *D* — —/*D* — —. The D antigen appeared to be more powerful than was usual, as shown by the ability of a saline suspension of the cells to be agglutinated by incomplete anti-D sera. It has been suggested that in man all Rh antigens develop from a limited amount of precursor substance, and that if no antigens of the Cc or Ee classes are formed, more precursor is available to form D antigen. Other cases having this chromosome have now been described, and in addition other gene combinations, such as C^wD—/C^wDe and even blood samples giving rise to no Rh reactions at all, — — — —/— — — — (or Rh_{null}) have been found. Levine *et al* (1965) described an example of Rh_{null} together with a study of her family which suggested that the patient's cells might carry a modifying gene in double dose leading to prevention of the expression of the Rh genes. Such a picture resembles closely that of the 'Bombay' group of the ABO system.

Rh Antibodies

These may be classified both by the antigens (or 'factors' according to Wiener) with which they react and by the serological techniques which detect them. From a practical point of view it must be remembered that D is very antigenic, and for this reason the transfusion of a D-negative recipient with D-positive blood is very likely to be followed by the development of anti-D antibody. Should more D-positive blood be transfused into the same recipient at a later date, then a severe transfusion reaction may well occur. Equally serious is the position of the Rhesus-negative woman sensitized to the D antigen as a result of bearing an Rh-positive infant or by her having been given in error D-positive

blood. She is unlikely in future to bear Rh-positive children free of haemolytic disease of the newborn. Rhesus-negative females who have not reached the menopause must not, therefore, be transfused with Rh-positive blood and all other Rh-negative persons should be similarly treated unless it is absolutely unavoidable not to do so. The other Rh antigens may also cause trouble, but are less likely to do so. Nevertheless, a Rhesus negative *cde/cde* patient should never be transfused with D-negative, C- or E-positive blood lest anti-C or -E should develop. Donors classified as 'Rh-negative' must therefore be shown to be *cde/cde* by testing with appropriate antisera. Patients are normally divided into 'Rhesus positive' and 'Rhesus negative' simply by testing their red cells with anti-D serum.

When devising an appropriate compatibility test, or 'cross-match' test as it is often called, one must remember to employ techniques which will detect any Rh antibody, no matter how it may react. More will be said about this later in this chapter, but it should be remembered that such antibodies may be detected sometimes by a saline technique sometimes by an antiglobulin technique, or perhaps sometimes best with an albumin suspension of cells. Often Rh antibodies may best be detected by enzyme-treated red cells. A range of these techniques will also be employed when the sera of expectant mothers are screened for Rh antibodies. Reference should also be made to Mollison (1967) for details of the mode of action of Rh antibodies.

THE REMAINING BLOOD GROUP SYSTEMS

Most of the systems described below tend to conform to a pattern, starting with the discovery of an antibody demonstrating an antigen that is shown to be genetically determined and independent of all other systems. Subsequent work shows that there exists another antibody reacting with the antigenic product of an allelic gene. Sometimes further work has shown the existence of another gene, producing no antigen detectable with available reagents. In some cases further complexities have been discovered. All that one can hope to do here is to provide very brief details of individual systems and to suggest that those interested read more about the subject in relevant papers or textbooks.

The Lutheran Blood Group System

The system appears to consist of two allelic genes, Lu^2 and Lu^b, and antibodies to both the antigens have been found, though anti-Lu^b, especially, is extremely rare. Mollison has shown that anti-Lu^a can reduce the survival time of Lu^a-positive cells *in vivo* and haemolytic disease of the newborn due to anti-Lu^a has been described.

The Lutheran blood group system has been shown to conform to the pattern described above by the discovery of blood samples that react with no known

Lutheran antibody. Darnborough, Firth, Giles, Goldsmith & Crawford (1963), described the presence of anti-Lu^aLu^b antibody in the serum of a patient whose red cells lacked both Lu^a and Lu^b antigens. Sanger & Race (1958) showed that there is a linkage between the Lutheran and secretor genes.

The Kell Blood Group System

This system was discovered in the original process of investigating the potentialities of the antiglobulin test, and would hardly have been found by any previously available technique. Some 9% of random European blood samples carry the Kell (K) antigen which is probably next in importance after the ABO and Rh antigens as an immunizing antigen. The system now conforms to the usual pattern already described, the antigens to which antibodies exist being K and k, while a sample has been found that reacts to neither antibody and is thought to be homozygous for a gene, K^0.

Two other antibodies, anti-Kp^a and anti-Kp^b have been shown to be related to the Kell system. In addition, Stroup, MacIlroy, Walker & Aydelotte (1965), have shown that the Sutter antigens, Js^a and Js^b belong to the Kell system.

The Lewis Blood Group System

This system, while admittedly not as important from the clinical point of view as ABO or Rh deserves consideration not only because its antibodies are relatively common (present in 0.3% of a series of human sera examined by Kissmeyer-Nielsen, Bastrup-Maden & Stenderup (1955)), but also because the antigens show certain characteristics not seen in those of other human blood group systems.

Anti-Le^a was described by Mourant in 1946, though it had probably been encountered earlier by other workers. Two antibodies have been discovered, anti-Le^a and anti-Le^b while the red cell phenotypes Le(a+b−), Le(a−b+), Le(a−b−) and Le(a+b+) have been found, though the last occurs only in very young babies. The antigens of this system, like those of the J system in cattle, are now considered to occur primarily in serum and saliva, and only secondarily on red cells. Sneath & Sneath (1955) showed that red cells take up antigens if incubated with them, while Nicholas, Jenkins & Marsh (1957) found the same phenomenon to occur *in vivo* when they investigated chimerta twins. Red cells that would normally have been Le(a+b−) in one of the twins had become Le(a−b+) when in the circulation of the other twin, having acquired the phenotype of the latter, and vice versa.

Genetics of the Lewis Blood Groups (Table 8.7)

According to Ceppellini (1955), the Lewis antigens, both in the saliva and on the red cells, are controlled by two pairs of genes, the *Sese* genes, which also control ABH secretion and the *Ll* genes. Persons who are *LL* or *Ll* have some form of

Lewis antigen in the saliva and on the red cells, while those who are *ll* lack Lewis substances entirely. In the presence of the *Se* gene, the Lewis substance, both in the serum and on the red cells, usually appears entirely in the form of the Le^b antigen, though a small amount of Le^a is sometimes detectable. In the saliva both Le^a and Le^b substances are present. In persons who are *sese* the Lewis substances on red cells, in saliva and in serum are all in the form of Le^a.

As already mentioned, the Xx system which gives rise to the 'Bombay' blood group in *xx* individuals, also affects the expression of Lewis genes. If the L gene is present, the block to the production of the H antigen which occurs in *xx* individuals does not prevent the production of the Lewis antigen, which probably occurs like H from an earlier precursor. However, in *xx* individuals, the

TABLE 8.7

The Lewis blood group system

Lewis genotypes	Secretor genotype	Antigens in saliva		Antigens on red cells
		Le^a	Le^b	
LL	*Se Se*	+	+	Le(a−b+) except in newborn when they are Le(a+b+)
	Se se			
Ll	*Se Se*			
	Se se			
LL	*se se*	+	−	Le(a+b−)
Ll				
ll	*Se Se*	−	−	Le(a−b−)
	Se se			
	se se			

production of Le^b antigen is inhibited and hence the Lewis substance present in the saliva, serum, and red cells takes the form of Le^a, even if the individual possesses the *Se* gene. A person who was found by Giles, Mourant & Atabuddin (1963), to be of the 'Bombay' group, yet Le(a−b−) would have been of the genotype *xxll*.

ANTIBODIES OF THE LEWIS BLOOD GROUP SYSTEM

These consist of anti-Le^a and anti-Le^b. As stated above, these antibodies, especially anti-Le^a, are not extremely rare. They are often 'naturally occurring' may sometimes be detected in saline at 20°C or below, but the best method for their detection is usually by means of the indirect antiglobulin test, particularly if the two-stage complement-binding technique is used as described by Polley & Mollison (1961). Anti-Le^a may haemolyse Le^a-positive cells in the presence of complement and it may give rise to transfusion reactions. Anti-Le^b may occur

with anti-Le^a together with anti-H. An interesting example of the clinical importance of anti-Le^b is given by Mollison, Polley & Crome (1963), when they describe the problem of finding enough blood for a patient whose serum contained anti-Le^a and anti-Le^b and who was to undergo cardiac surgery. The anti-Le^b in the patient's serum was temporarily suppressed by the injection into him of Le^b-substance. Large amounts of Le(b+) blood were then transfused, followed by a marked immune response but not accompanied by a haemolytic transfusion reaction, apparently because the transfused Le(b+) cells rapidly lost Le^b substance and behaved as if they were Le(b−).

The Duffy Blood Group System

Two antibodies, anti-Fy^a and anti-Fy^b define two antigens Fy^a and Fy^b. The system conforms to the pattern described on page 239, the phenotypes being Fy(a+b−), Fy(a+b+) and Fy(a−b+). In addition, as in many other cases, specimens that react with neither antibody have been found. Indeed 68% of Negroes have the phenotype Fy(a−b−) and are thought to be homozygous for a gene *Fy*. Almost without exception the antibodies react by the indirect antiglobulin test. Anti-Fy^a can cause haemolytic transfusion reactions and, rarely, haemolytic disease of the newborn. Treatment of red cells with proteolytic enzymes destroys Duffy antigens so that they will no longer react with appropriate antibodies.

The Kidd Blood Group System

Two antibodies, anti-Jk^a and anti-Jk^b, define two antigens, Jk^a and Jk^b. The pattern is similar to that already described, with three phenotypes, Jk(a+b−), Jk(a+b+) and Jk(a−b+). The phenotype Jk(a−b−) has also been described.

The Yt Blood Group System

Anti-Yt^a was discovered eight years before anti-Yt^b and during that period the main interest in the Yt^a antigen was that it was carried by red cells of very nearly all persons tested. In other words the Yt^a antigen was shown to be what Race and Sanger call a 'public antigen'. The discovery of anti-Yt^b, defining the allelic antigen Yt^b, was made in 1964 by Giles and Metaxas thus making the Yt system genetically important.

The Xg Blood Group System

The great importance of this system is that the gene responsible must be carried on the X chromosome. One antibody, anti-Xg^a, has been found so far and it reacts only by the indirect antiglobulin technique. Race & Sanger (1962) give an exciting description of the discovery, inserted at galley proof stage of the fourth edition of their book, and to this the reader should refer.

Other Blood Group Systems

The Diego *antigen* is found exclusively in Mongoloid peoples. It has been responsible for a number of cases of haemolytic disease of the newborn.

The Ii *antigens.* In 1956 Wiener and his colleagues discovered an antibody in the serum of a patient suffering from a cold antibody type of haemolytic anaemia, that agglutinated nearly all human red cells. Positive reactions were said to be I-positive and negative reactors i. Marsh and Jenkins have since found anti-i and have shown that all red cells contain I and i, but foetal and cord samples contain very little I and much more i. In adult life the reverse is usually the case. Further information will be found in a paper by Marsh (1961). Recently, Booth, Jenkins & Marsh (1966), found a 'new' antibody, anti-I^T while Jenkins Koster, Marsh & Carter (1965) found anti-i in the sera of certain cases of infectious mononucleosis.

The T *antigen* is believed to occur on all normal human red cells, but is not able to react with its antibody unless the red cell surface is modified as may occur *in vitro* by the action of certain bacteria. Almost all human sera contain the antibody, anti-T, so that infected specimens of blood may show polyagglutination as a result of reactions between the T antigen and antibody.

PRACTICAL APPLICATIONS OF BLOOD GROUP SEROLOGY

Over the preceding pages of this chapter antigen-antibody reactions, some common, some rare, some of great clinical importance and others not, have been described. Out of this mass of information must now be extracted the points which are likely to be of use to the clinician or pathologist. Both will be concerned with:

I. The transfusion of blood
   (*a*) Grouping
   (*b*) Performance of compatibility (cross-matching) tests
   (*c*) Identification of antibodies, whether found in compatibility (cross-matching) tests, or after a transfusion reaction.

II. The diagnosis and treatment of haemolytic disease of the newborn.

III. The investigation of patients suffering from acquired haemolytic anaemia.

Sections II and III are dealt with elsewhere in this book so that Section I alone will be considered here. The methods of grouping red cells and of investigating antibodies are, however, the same, whether the case is one of transfusion reaction or of haemolytic disease of the newborn.

Red Cell Grouping

In the case of patients, the ABO and Rhesus (D) groups will be determined. Those who are D-negative will be classified as Rhesus-negative and will normally be of the genotype *cde/cde* as may be seen from Table 8.5. Donors who have been found to be D-negative should also be tested with anti-C and anti-E to exclude the presence of red cells carrying either C or E antigens which might give rise to the development of anti-C or -E in *cde/cde* recipients. It is particularly important, also, that apparently D-negative donors should be tested by a suitable technique to exclude the presence of the D^u antigen.

Techniques

Grouping sera are often issued with instructions and, if so, these should be strictly followed. Tube techniques are more reliable than those performed on slides. For agglutination tests, cells should be washed in saline and made up to a 2% suspension in the same medium or, if an incomplete anti-Rh serum is used, the 2% cell suspension may be made up in 20% bovine albumin instead of saline. Whatever the test, positive and negative controls in the form of red cells bearing and lacking the antigens under investigation must be included.

ABO grouping is performed using saline anti-A and anti-B sera, together with preferably anti-A + anti-B (group O) serum, since the latter differentiates true group O red cells from those which are A_x. At the same time, the serum of the specimen under test will be tested by means of known A_1 and B red cells for the presence of α and β agglutinins. Tests are carried out at room temperature. Rh grouping is performed at 37°C, tests being carried out by a saline or albumin technique according to the mode of reaction of the grouping serum used. For D^u testing an indirect antiglobulin technique is used, the red cells first being incubated with a potent anti-D serum and then tested with anti-human globulin reagent.

Performance of Compatibility (Cross-Matching) Tests

It is not sufficient to give blood which is simply known to be compatible with the patient's ABO and Rh group, for other antigen-antibody systems may cause incompatibility. It is not necessary to test specifically for the other antigens and antibodies, but the donor's red cells are tested against the patient's serum to ensure that there is no antibody in the latter which reacts with the cells. Sometimes, but not so frequently, the patient's red cells are tested in the presence of the donor's serum; this is less often done as it is considered that the donor's plasma, and hence any antibodies that it may contain, is considerably diluted when transfused into the patient. Nevertheless, it is essential for the serum of all donors to be initially screened to see that it does not contain any high titre

and potentially dangerous antibodies, especially immune anti-A+anti-B in group O donors.

The compatibility tests consist basically of testing a 2% suspension of the donor's cells against the patient's serum at 20°C for 'natural' saline antibodies and of testing the same cells and serum together at 37°C using albumin and indirect antiglobulin techniques for the detection of 'incomplete' antibodies. As a very useful control, the patient's own cells should be similarly matched in his own serum using the techniques mentioned above. It will be remembered that compatibility tests, detecting possible weak antigen-antibody reactions, call for more skill than do grouping tests in which high-titre reliable antibodies are used. Slide techniques are not reliable enough for cross-matching and only tube techniques should be used. For precise details of methods to be followed, see Mollison (1967).

Identification of Red-Cell Antibodies

Once an antibody has been detected, it must be identified and this is a task that may well have to be left to a specialist laboratory. A panel of red cell samples that have been very fully grouped for a wide range of red cell antigens will be required. Each of these cell samples will be tested against the serum using a comprehensive series of techniques which may be listed as follows:

(*a*) Saline agglutination tests at 4°C, 20°C and 37°C.
(*b*) Albumin-agglutination tests at 4°C, 20°C and 37°C.
(*c*) Indirect antiglobulin tests at 37°C.
(*d*) Indirect antiglobulin tests at 37°C using the complement-binding technique of Polley & Mollison (1961).
(*e*) Enzyme-treated red cells at 37°C.
(*f*) Tests for haemolysins.

The specificity of any one antibody may be recognized both by the groups of red cells with which it reacts and also by noting the techniques by which it is demonstrated. For instance, anti-Fy^a will agglutinate only Fy^a-positive cells, usually only by an antiglobulin technique, the reaction being prevented if the red cells are first enzyme treated. Anti-M or anti-N normally react with a saline suspension of cells, but again not if the cells have been enzyme treated. By making full use of such techniques, reactions due to mixtures of antibodies can be separated and the individual constituents identified. In addition use may be made of absorption and elution techniques to separate such antibody mixtures, but for further details of these methods, reference should be made to Mollison (1967).

One last point should be made with regard to antibody identification. Some antibodies are common and these are those which are likely to be detected by anyone starting to work in this field. One is sometimes tempted to jump to the

conclusion that what one has found is of the greatest rarity. Reference to Table 8.8, which is slightly modified from Stratton & Renton (1958) will serve as a guide as to what one is likely to find. An antibody believed to be of some rarity should certainly be checked by an experienced worker before the finding is accepted.

TABLE 8.8

Frequency of occurrence of red cell antibodies

Regular	Anti-A, B.
Common	Anti-A_1, D, C+D, O, H, P_1, Le^a, Le^b. Anti-Wr^a, Mi^b, Vw (but the antigens are rare).
Uncommon	Anti-c, c+E, E, D+E, M, S, K, Fy^a, Lu^a. Anti-C+e, e. (Usually in acquired haemolytic anaemia.)
Rare	All others

LEUCOCYTE AND PLATELET GROUPS AND THEIR CLINICAL SIGNIFICANCE

Forty years have elapsed since Doan described his 'leukotoxin' test with which he recognized what he called biologic differentiation in white cells. Today we still have a great deal to learn about leucocyte, and for that matter platelet, groups. Nevertheless it is quite clear that both leucocytes and platelets possess antigen-antibody systems of their own. Leucocyte antibodies, usually in the form of saline agglutinins, develop in those who have had multiple blood transfusions and may be the cause of non-haemolytic transfusion reactions. Platelet antibodies may also cause occasional transfusion reactions but are much more frequently associated with thrombocytopenia. Whether they are the cause or the result of the disease is a matter of dispute but at least they seem to be the cause of certain cases of neonatal thrombocytopenia. (See Jones, Goldsmith & Anderson 1961, and Pearson *et al* 1964). Recently, progress has been made, particularly with regard to the study of leucocyte antigens because of the increasing use of leucocyte typing as a means of choosing organ and tissue donors for transplantation. There is no doubt that leucocytes, unlike red cells, carry histocompatibility antigens, among the evidence for this being the fact that those who have received such grafts frequently develop leucocyte antibodies. In addition, if a volunteer is first immunized by being injected with leucocytes of a particular donor, and if the same volunteer is then grafted with tissue from the same donor, then the rate of rejection of that graft is accelerated.

Van Rood has been responsible for the discovery of a number of leucocyte antigens including 4^a, 4^b, 5^a, 5^b, 6^a, 6^b, 7^a, 7^b, 7^c, 8^a and 9^a. In addition Mac (which may be identical with Pl Gr Ly^{B1}), Pl Gr Ly^{C1}, X^a, X^b, LA1, LA2 and LA3 deserve mention. Specific platelet antigens include Zw^a, Zw^b, Ko^a, Ko^b and Duzo. For more details of this rapidly expanding subject see Dausset & Tangün (1965), Goldsmith (1965), Amos & van Rood (1965) and Russell, Amos & Winn (1965). The last two references are each to books containing papers by leading experts with especial reference to histocompatibility.

Techniques

Those employed for platelet or leucocyte antigen-antibody reactions tend to be unreliable, so doubtful positive findings should be ignored. For the demonstration of leucocyte agglutinins or platelet agglutinins saline suspension of the cells are added to the various sera being tested, with suitable controls, in much the same way that red cell saline antibodies are detected. Incomplete leucocyte or platelet antibodies are often demonstrated by the cumbersome antiglobulin consumption test described in Chapter 1 of this book. Complement fixation and cytotoxicity tests are also used for this purpose. Further details of some of these techniques will be found in papers by Dausset, Colombani & Okochi (1964), Dausset & Colombani (1964) and Engelfriet & Britten (1966).

Finally reference must be made to a very special group of diseases in which leucopenia or thrombocytopenia occur following drug ingestion. Often such a phenomenon results from a toxic depression of the marrow by the drug concerned, but in a few cases it has been possible to show that the patient has developed an antibody to a complex formed by the drug with either leucocytes or platelets. For further details on this subject see Ackroyd (1964) and Chapters 27 and 30.

REFERENCES

Ackroyd J.F. (1964) The diagnosis of disorders of the blood due to drug hypersensitivity caused by an immune mechanism. In *Immunological Methods*, p. 453. Oxford: Blackwell Scientific Publications.

Amos D.B. & Rood J.J. van (1965) *Histocompatibility Testing* 1965. Copenhagen: Munksgaard.

Bird G.W.G. (1959) Haemagglutinins in seeds. *Brit. med. Bull.* **15**, 165.

Booth P.B., Jenkins W.J. & Marsh W.L. (1966) Anti-I^T: a new antibody of the I blood-group system occurring in certain Melanesian sera. *Brit. J. Haemat.* **12**, 341.

Boyd W.C. & Shapleigh E. (1954) Specific precipitating activity of plant agglutinins (lectins). *Science* **119**, 419.

Ceppellini R. (1955) On the genetics of secretor and Lewis characters: a family study *Proc. Vth Congress of Blood Transfusion (Paris)* **207**, (1955).

Darnborough J., Firth R., Giles C.M., Goldsmith K.L.G. & Crawford M.M. (1963) A 'new' antibody anti-Lu^a Lu^b and two further examples of the genotype Lu (a−b−). *Nature (Lond.)* **198**, 796.

DAUSSET J. & COLOMBANI J. (1964) Detection of anti-leucocyte and anti-platelet antibodies by the antiglobulin consumption test. In *Immunological Methods*, p. 575. Oxford: Blackwell Scientific Publications.

DAUSSET J., COLOMBANI J. & OKOCHI K. (1964) Leucocyte and platelet agglutination. In *Immunological Methods*, p. 539. Oxford: Blackwell Scientific Publications.

DAUSSET J. & TANGÜN Y. (1965) Leucocyte and platelet groups and their practical significance. *Vox Sang.* **10**, 641.

DUNSFORD I., BOWLEY C.C., HUTCHISON A.M., THOMPSON J.S., SANGER R. & RACE R.R. (1953) A human blood-group chimera. *Brit. med. J.* **ii**, 81.

ENGELFRIET C.P. & BRITTEN A. (1966) Cytotoxic antibodies against leucocytes. *Vox Sang.* **11**, 334.

FORD E.B. (1955) A uniform notation for the human blood groups. *Heredity* **9**, 135.

FRANCIS B.J. & HATCHER D.E. (1966) MN blood types. The S− s− U+ and the M_1 phenotypes. *Vox Sang.* **11**, 213.

GILES C.M., MOURANT A.E. & ATABUDDIN A-H. (1963) A Lewis-negative 'Bombay' blood. *Vox Sang.* **8**, 269.

GOLDSMITH K.L.G. (1965) Donor selection and compatibility typing. *Brit. med. Bull.* **21**, 162

JENKINS W.J., KOSTER H.G., MARSH W.L. & CARTER R.L. (1965) Infectious mononucleosis: an unsuspected source of anti-i. *Brit. J. Haemat.* **11**, 480.

JONES T.G., GOLDSMITH K.L.G. & ANDERSON I.M. (1961) Maternal and neonatal platelet antibodies in a case of congenital thrombocytopenia. *Lancet* **ii**, 1008.

KEIDAN S.E., LOHOAR E. & MAINWARING D. (1966) Acute anuria in a haemophiliac due to transfusion of incompatible plasma. *Lancet* **i**, 179.

KISSMEYER-NIELSEN F., BASTRUP-MADEN K. & STENDERUP A. (1955) Irregular blood-group antibodies. Incidence and clinical significance. *Dan. med. Bull.* **2**, 202.

KONUGRES A. & COOMBS R.R.A. (1958) Studies on human anti-A sera with special reference to so-called 'immune' anti-A. II. Identification of the antibody detected by Witebsky's 'partial neutralization' test as anti-A^p and the occurrence of the A^p antigen on human and animal red cells. *Brit. J. Haemat.* **4**, 261.

LEVINE P., CELANO M.J., FALKOWSKI F., WHITE CHAMBERS J., HUNTER O.B. Jr & ENGLISH C.T. (1965) A second example of − − −/− − − or Rh_{null} blood. *Transfusion* (*Philad.*) **5**, 492.

LOCKYER J.W. (1959) Observations on the A^p antigen. *J. med. Lab. Technol.* **16**, 42.

LOGHEM J.J. VAN, DORFMEIER H. & HART M. VAN DER (1957) Two A antigens with abnormal serologic properties. *Vox Sang.* (*Basel*) **2**, 16.

MÄKELÄ O. (1957) Studies in haemagglutinins of Leguminosae seeds. (Thesis.) Supplement No. 11. *Ann. Med. exp. Fenn.* **35**, 1.

MARSH W.L. (1960) The Pseudo B Antigen. A study of its development. *Vox Sang.* (*Basel*) **5**, 387.

MARSH W.L. (1961) Anti-i: a cold antibody defining the Ii relationship in human red cells. *Brit. J. Haemat.* **7**, 200.

MOLLISON P.L. (1967) *Blood Transfusion in Clinical Medicine*, 4th edn. Oxford: Blackwell Scientific Publications.

MOLLISON P.L., POLLEY M.J. & CROME P. (1963) Temporary suppression of Lewis blood-group antibodies to permit incompatible transfusion. *Lancet* **i**, 909.

MORGAN W.T.J. & WATKINS W.M. (1948) The detection of a product of the blood group O gene and the relationship of the so-called O substance to the agglutinogens A and B. *Brit. J. exp. Path.* **29**, 159.

MOURANT A.E. (1954) *The Distribution of the Human Blood Groups*. Oxford: Blackwell Scientific Publications.

MOURANT A.E., KOPÉC A.C. & DOMANIEWSKA-SOBCZAK K. (1958) *The ABO Blood Groups: Comprehensive Tables and Maps of World Distribution*. Oxford: Blackwell Scientific Publications.

NICHOLAS J.W., JENKINS W.J. & MARSH W.L. (1957) Human blood chimeras: a study of surviving twins. *Brit. med. J.* **i,** 1458.

PEARSON H.A., SHULMAN N.R., MARDER V.J. & CONE T.E. (Jr.) (1964) Isoimmune neonatal thrombocytopenic purpura. Clinical and therapeutic considerations. *Blood* **23,** 154.

POLLEY M. & MOLLISON P.L. (1961) The role of complement in the detection of blood group antibodies: special reference to the antiglobulin test. *Transfusion (Philad.)* **1,** 9.

RACE R.R., MOURANT A.E., LAWLER S.D. & SANGER R. (1948) The Rh chromosome frequencies in England. *Blood* **3,** 689.

RACE R.R. & SANGER R. (1968) *Blood Groups in Man*, 5th edn. Oxford: Blackwell Scientific Publications.

RACE R.R., SANGER R. & SELWYN J.G. (1950) A probable deletion in a human Rh chromosome. *Nature (Lond.)* **166,** 520.

RUSSELL P.S., AMOS D.B. & WINN H.J. (1965) *Histocompatibility Testing*. Washington D.C.: National Academy of Sciences—National Research Council. Publication 1229.

SANGER R. (1952) A relationship between the secretion of the blood group antigens and the presence of anti-O or anti-H in human serum. *Nature (Lond.)* **170,** 78.

SANGER R. & RACE R.R. (1958) The Lutheran-Secretor linkage in Man: support for Mohr's findings. *Heredity* **12,** 513.

SNEATH J.S. & SNEATH P.H.A. (1955) Transformation of the Lewis groups of human red cells. *Nature (Lond.)* **176,** 172.

STRATTON F. & RENTON P.H. (1958). In *Practical Blood Grouping*, p. 312. Oxford: Blackwell Scientific Publications.

STROUP M., MACILROY M., WALKER R. & AYDELOTTE J.V. (1965) Evidence that Sutter belongs to the Kell blood group system. *Transfusion (Philad.)* **5,** 309.

WINSTANLEY D.P., KONUGRES A. & COOMBS R.R.A. (1957) Studies on human anti-A sera with special reference to so-called 'immune' anti-A. I. The A^p antigen and the specificity of the haemolysin in anti-A sera. *Brit. J. Haemat.* **3,** 341.

CHAPTER 9

IMMUNOLOGICAL ASPECTS OF FORENSIC SCIENCE

BARBARA DODD

INTRODUCTION

Most science subjects have a forensic flavour from time to time for almost all have potential usefulness in the solution of legal problems. Indeed forensic science can hardly stand as a speciality *per se*, being for the most part scientific knowledge applied in a particular, one might say peculiar, way.

However, some scientists are reluctant sleuths and unenthusiastic about adapting their field of study towards problems facing the legal profession. There are a number of reasons for this. As the law now stands every worker who has carried out a set of tests of value in a particular case usually has to appear as an expert witness to give account of his findings. This may entail hours in Court largely spent impatiently wishing to be tackling fascinating problems in his own laboratory.

There is also a certain understandable distaste for the methods of cross examination. 'So you repeated your tests on the following day, did you? Then

you couldn't have been very sure of them on the first occasion, could you?', a barrister once rapped out in Court. In these circumstances it takes experience to remain unruffled, realizing that such questions must be expected as part of the game. A cross-examining counsel may seem fierce indeed until one has seen him with arm thrown genially about the shoulders of his opponent at the lunch interval.

The reluctant approach to forensic science problems may be related to a lack of interest in what may be termed man-created problems as opposed to problems of nature. Shakespearean mixtures containing 'eye of newt and toe of frog' and including 'finger of birth-strangled babe' repel rather than allure. Fortunately more and more specialists are willing to undertake these tasks and forensic science expands almost daily. It is therefore not surprising to find immunology playing an important role in a field where problems of identity so frequently occur.

The wide variety and high degree of individuality of animal proteins and polysaccharides, together with new developments in techniques, are leading us towards a state of affairs where more and more precise information can be gained by immunological methods concerning the origin of biological material found in a variety of situations.

A tiny stain may not only be identified as blood, but its species of origin may be ascertained and some of the blood groups to which it belongs identified as well.

Immunological variations, however, are still outrivalled by the unique nature of fingerprints. Whereas it may be true to assert that every individual is immunologically different from every other, it is not possible at the moment to show this and it is doubtful whether it ever will become possible to arrange a set of immunological tests which will, as rapidly and efficiently, pinpoint an individual as do fingerprints.

Nevertheless, immunology is a tool frequently used in forensic science laboratories to assist in the identification of both individuals, fragments and fluids from all individuals.

Identification of Individuals

The specialized branch of immunology known as blood grouping is of assistance here. As will be apparent below, the blood group factors or antigens are inherited according to the ordinary laws of Mendelian inheritance. Therefore, a knowledge of the blood groups of the individuals concerned can help in cases of doubtful paternity. It can be proved, in certain circumstances, that a man cannot possibly be the father of a particular child. Similarly confusion of children at birth has been known to occur in hospitals or nursing homes and a mother may have a child given to her which is not her own. Blood grouping is of assistance here if it can be shown that of two women, one cannot possibly be the mother of one of the children but can be the mother of the other.

Blood grouping may also be of value in solving the parentage of children abandoned, dead or alive, on doorsteps or railway waiting-rooms.

In all these cases, however, it must be realized that a positive assertion can only be made about negative findings. It is possible, in certain circumstances to show that two individuals are unrelated. The only alternative to such a conclusion being that they may be related—never that they positively are—although the probability of their relationship may occasionally be statistically significant.

Identification of Fragments

Animal fragments include bones, pieces of skin, dander, hairs, etc. If any of these contain proteins there is a chance, through precipitin and other tests, of assigning these to their species of origin and, if human, of obtaining their blood groups. It is frequently desirable in accident cases to be able to identify fragments and in criminal cases to obtain information about hairs, dander and skin.

Identification of Animal Fluids and Stains

Animal fluids include blood, saliva, sweat, seminal fluids and milk, etc. Very often these have to be identified from stains. Immunological tests are not the only ones used on such occasions. Any investigation is usually a combination of chemical, histological as well as immunological methods.

In order to give readers an insight into the procedure for identification of stains a step-by-step description will be given later in this chapter. Problems in connection with stain detection will also be touched upon and outlines of several new and rather exciting approaches to the work described. Problems, of course, include ageing of stains and the variety of conditions in which they may be found. Frequently the investigator is also faced with an inadequate amount of material for all the tests that he would like to carry out.

Immunological differences between the milk of different species of animal can be seized upon and used to determine whether or not, for example, milk alleged to be pure cow's milk is mixed with that of a goat.

BLOOD GROUPING APPLIED TO CASES OF DOUBTFUL PATERNITY

The blood group antigens are situated on the membrane of the red cells. They are also situated in other cells and fluids of the body as will be appreciated below, but the normal procedure of blood grouping is to test the red cells and when appropriate the serum for blood group antibodies. Their presence is revealed by clumping or agglutination of the red cells when these are mixed with suitable antisera.

Genetic Principles

The usefulness of blood groups in cases of doubtful paternity is due to the fact that they are inherited characteristics and are passed on from parent to child according to the normal Mendelian laws which govern the inheritance of most human characteristics, e.g. eye colour, hair colour, etc. Any individual has two genes contributing to a character, one from the father and one from the mother. If these genes are the same, the individual is said to be homozygous for the character, and if the two genes are different, heterozygous. All blood group genes (with exceedingly few doubtful exceptions) are Mendelian dominants; the chief differences in inheritance being in the number of allelomorphs controlling a particular blood group system and the way in which they are designated.

It should, of course, be realized that in each body cell in the human species there are forty-six chromosomes in twenty-three pairs, and the different genes controlling the various blood group systems are not all situated on the same pair of chromosomes. In fact, most of them have been shown conclusively to be independent of each other and, therefore, to occur on different chromosome pairs.

Two rules of inheritance as applied to blood groups can be formulated:

1. A blood group factor cannot appear in a child unless present in one or other (or both) parents.

2. If one or other parent is homozygous for a particular blood group factor, this must appear in the blood of the child.

Of these rules, the first can be most reliably applied. Provided the individuals are tested on the same occasion with the same antisera an agglutination of the child's red cells by a particular antiserum not agglutinating the red cells of either mother or putative father, demonstrates the presence of a blood group factor in the child which the adults both lack. This is contrary to the first rule of inheritance cited above and so excludes the putative father from paternity of the particular child. Emphasis must be laid on the use of the same set of reagents for all samples related to a given case, otherwise there is always the possibility that an antiserum may have been used for the child which is capable of showing, and does in fact demonstrate in the child the product of a rare gene; this rare antigen might remain undetected in the blood of one or other parent because they had been tested on another occasion with antisera not able to detect it. In this case not only would exclusion of paternity be made on a false basis, but if, in fact, the puta ve father and child each possessed the rare antigen, the very opposite conclusion should have been reached, namely that there was a high probability that he was actually the father.

It is essential, therefore, that clinicians and others involved in arrangements for paternity tests, should recognize the importance of the serologist's request

that the individuals concerned, even though far apart, should have their blood tested by the same expert on the same occasion.

The second rule of inheritance is not so reliably applied, as homozygosity for a particular blood group factor cannot be so accurately determined.

Although homozygosity may be apparent because only one of two alternative alleles is present in the blood, the presence of a third rare allele, which has been passed to the child and goes undetected in the testing, must be considered. For instance, a rare allele Y_1 may occur in a hypothetical blood group system YZ and the corresponding antibody anti-Y_1 may be but rarely present in anti-Y testing sera. Then an individual who is Y_1Z would almost always be indistinguishable in the tests from a ZZ individual, which happening could readily give rise to false conclusions in doubtful paternity cases.

One possible cause of error in the interpretation of results which is sometimes made much of in legal argument is mutation. If the child has an antigen not possessed by the mother or putative father, it is argued that a mutation has occurred, i.e. a change in the character of the gene between one generation and the next. The weight of such an argument is extremely small. It is difficult to obtain an accurate idea of mutation rates in man, but rough estimates with several human genes give a figure of the order of 1 in 50,000. If blood group genes alone are considered the rate must be less than this and if the child should happen to have two antigens, lacked by both adults, then the possibility of mutation having occurred can be ruled out of Court!

Blood Group Systems Used

At first, in cases of doubtful paternity, tests were confined to the ABO, MN and Rh systems (giving about a 50% chance of exclusion where the man is not the father) but now a number of other systems, such as Kell, Duffy, Lutheran, Gm, Ss, haptoglobins and Gc groups are included. Not every antigen known to belong to a system is tested routinely owing to the rarity of the reagents needed. For example, whereas anti-Fy^a of the Duffy groups would not be omitted, anti-Fy^b would be reserved for circumstances specially requiring its use.

Naturally the greater the number of blood group systems used in the work, the greater the chance of excluding paternity. One of the most recent systems to be added to the list has been the haptoglobins. These are serum proteins which exhibit characteristic bands revealed by starch gel electrophoresis. Family studies have shown quite conclusively that these are inherited characteristics. Individuals can belong to one of three types designated Hp 1-1, Hp 2-1 or Hp 2-2. The approximate frequencies among Europeans are 16%, 47% and 37% respectively. Rare alleles exist which have to be taken into account when interpreting results based on these groups.

In Scandinavia haptoglobins have been used in cases of doubtful paternity for many years but in England, although tests have been made for some time, the

first judgment based on these tests, unsupported by evidence gained from other blood group findings was made early in 1966 in the case of Stocker v. Stocker. A certain amount of misleading publicity was afforded to this situation in the national press and haptoglobins were described as providing a 'near infallible' test for paternity. In fact, they merely add another 'string to the bow' and increase the chance of showing *non*-paternity from 62% to 67% approximately. Haptoglobins will be mentioned again in relation to the identification of blood factors in blood stains.

The criteria for the suitability of a system for this type of investigation is (*a*) that sufficient family studies have been carried out to show that its inheritance is according to Mendelian laws and can be reliably predicted, and (*b*) that the required antisera are available in sufficient strength for use as typing reagents.

TABLE 9.1

ABO matings, showing possible and impossible phenotypes of children

Mating	Possible phenotypes of children	Impossible phenotypes of children
A × A	A and O	B and AB
A × B	A, B, O, and AB	None
A × AB	A, B, and AB	O
A × O	A and O	B and AB
B × B	B and O	A and AB
B × AB	A, B, and AB	O
B × O	B and O	A and AB
AB × AB	A, B, and AB	O
AB × O	A and B	AB and O
O × O	O	A, B, and AB

There is little doubt that in the near future genetically inherited enzymes will be included in the list of characters used for the solution of the riddles of paternity. Enzymes such as the red cell acid phosphatases and phospho-glucomutases (PGM) can be investigated by means of starch gel electrophoresis but here we are crossing the boundary between immunology and biochemistry.

Commonly used blood group systems are presented in Tables 9.1 and 9.2. For more detailed description of some of the blood group systems used in cases of doubtful paternity, the reader is referred to Chapter 8, particularly to the section on the Rh system, and Tables 8.5 and 8.6.

In Tables 9.1 and 9.2 the commonly used blood group systems are presented because clinicians may very well find themselves in receipt of grouping

reports from serologists, which without some knowledge of the groups concerned, might well have originated from rune stones!

Tables 9.1 and 9.2 are self explanatory, Table 8.5 lists the eight main Rh gene complexes. Every individual has two of these gene combinations making up his genotype, one inherited from each parent.

Table 8.6 shows the common Rh subtypes and gives three nomenclatures for them:

1. The most commonly used in this country for referring orally to a subtype.
2. Wiener's nomenclature which is widely used.
3. Fisher's nomenclature in which the Rh subtypes are referred to in terms of the antigens they contain.

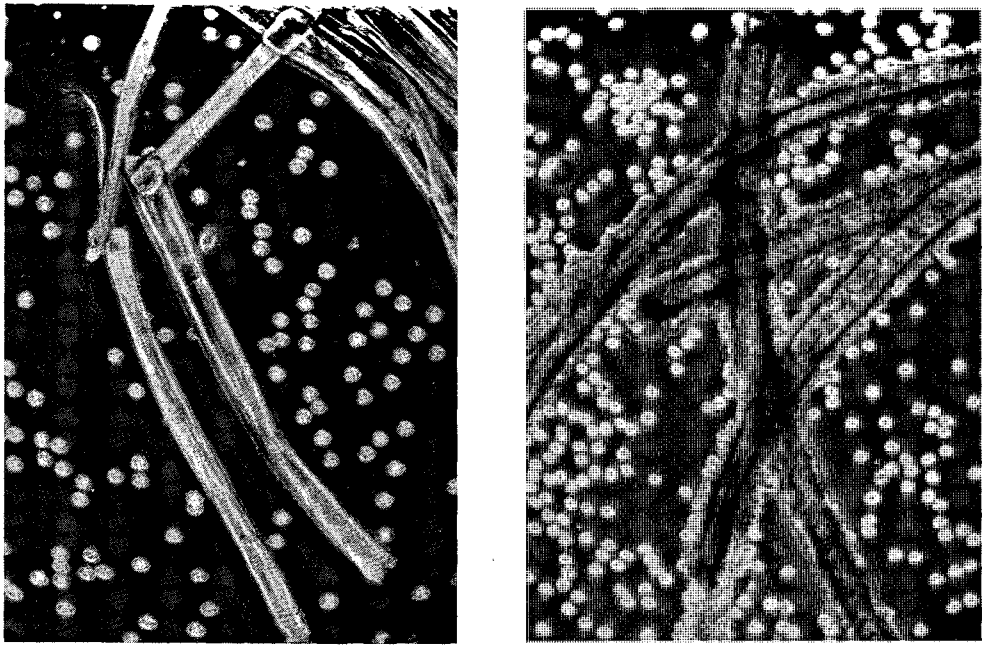
The subtype usually comprises several genotypes, each of which gives the same pattern of reaction with the standard Rh antisera, the name of the most common genotype being given to the sub-group as a whole. For instance, the subtype CDe/cde (R_1r) is composed of three genotypes, namely CDe/cde (93.7 %), CDe/cDe (6.2%) and Cde/cDe (0.1%).

TABLE 9.2

Matings within the MN system

MN group of parents	Possible MN groups of children			Impossible MN groups of children		
M × M	M				N	MN
M × N			MN	M	N	
M × MN	M		MN		N	
N × N		N		M		MN
N × MN		N	MN	M		
MN × MN	M	N	MN			

The Rh system is admittedly complicated, nevertheless, from the point of view of exclusion of paternity, it is perhaps a relief to know that the four main antisera and the corresponding antigens detected by them, may be considered absolutely independently of each other. For example, tests with anti-E may reveal the presence of E in the child and its absence in both mother and alleged father, thus permitting the conclusion that the alleged father is not the father of the child; which conclusion can be arrived at without having to place any of the individuals into their correct Rh phenotypes, or having to make any assessment of probable genotypes. One way of expressing a given person's Rh type is to list his antigens without any attempt at arrangement into actual genotype. For instance, persons of genotypes Cde/cde, cDe/Cde, CDe/cDe, whose red cells all give the same pattern of reaction with the anti-sera D, C, E



(*a*) (*b*)

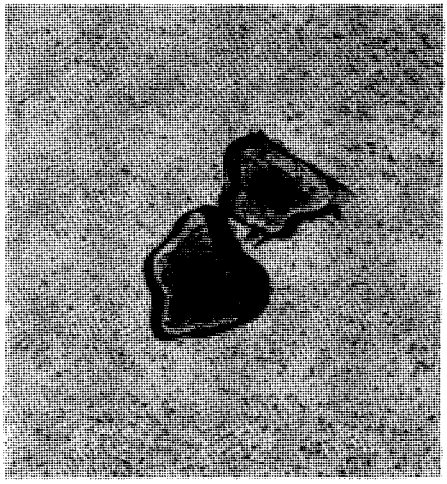
PLATE 9.1. Mixed agglutination reaction for the detection of blood group antigens on blood-stained cloth.

(*a*) Microscopical appearance of negative mixed agglutination reaction on blood-stained cloth fibrils. Phase microscopy × 10 eyepiece × 20 objective.

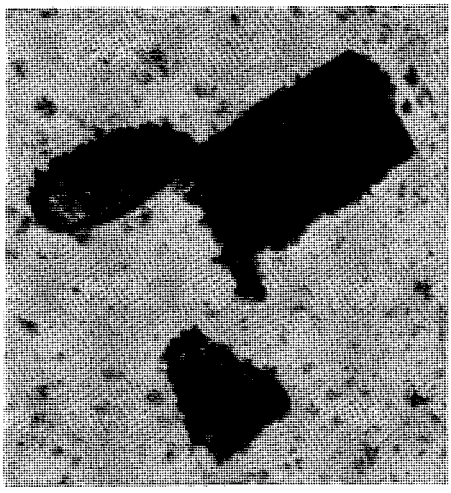
(*b*) Microscopical appearance of positive mixed agglutination reaction on blood-stained cloth fibrils. Phase microscopy × 10 eyepiece × 20 objective.

Taken, with permission, from Coombs & Dodd (1961).

facing p. 256



(a)



(b)

PLATE 9.2. Mixed agglutination reaction for A, B and O grouping of male beard hairs.

(*a*) Negative reaction: male beard of group A individual with anti-B and indicator B red cells.

(*b*) Positive reaction: male beard of group A individual with anti-A and indicator A red cells.

facing p. 257

and c (see above) may have their Rh type expressed thus: DCcec. This is an accurate statement based on the reactions obtained and bears no reference to gene arrangements. It is a convenient way of expressing results of paternity tests.

The Possibility of Proof of Paternity from Blood Groups

Proof of paternity (i.e. determining that only one particular man can be the father of a particular child) must, at least at the present time, be based on mathematical probability. If only common blood group combinations occur in the family tested, then the probability of paternity would not be statistically significant. However, if the child has to inherit less common or rare blood group genes from its father, then the chance of picking a man (or, more accurately, a sperm!) from the random population possessing these genes might range from one in twenty (the lowest level of statistical significance) to one in many hundreds. In such cases, if the putative father has the relevant genes and he is named as being associated with the case, then the probability of his paternity is significant.

What is the likelihood of very uncommon characters occurring in the families being examined? Some antigens are of such low incidence that they are called 'private'. At first sight these might not appear to be sufficiently common to be useful but it has been shown by Cleghorn (1964) that, if tests were made for all the known 'private' antigens, about 1 in 220 British individuals would be found to possess one or other of them.

An Unusual Case Investigated (Camps & Dodd 1966)

A newborn baby was found drowned. Murder was suspected. Blood samples from two putative fathers, the mother and the baby were grouped. One of the two men was described as being the father of the baby's mother. Thus incest became a factor to be considered, in addition to the possibility of murder. The C-c alleles of the Rh system proved most illuminating, as shown below in Table 9.3.

Mr Smith (the mother's father) was excluded from paternity since his genotype was CC and that of the baby C^wc. Mr Jones was likely to be the father since he and the child each possessed the rather infrequent gene C^w.

The third quite unexpected finding was that Mr Smith was, after all, not the father of Miss Joan Smith since she was Rh negative (cc)!

The Present Position of Paternity Testing in England

In this country there is still, in spite of valiant attempts over the last ten years by various individuals and bodies, no legislature covering blood groups in

relation to doubtful paternity. As seems not uncommon, we drag our feet compared with much of Europe. In England more than 7000 affiliation cases are heard annually and it is doubtful whether more than five or six hundred of these are blood typed.

Because of the lack of legislation on this matter, standardization of procedure is woefully lacking. Moreover, solicitors and doctors, who may wish to make arrangements for blood grouping tests, are often ignorant of available facilities which are not at the moment extensive and are almost exclusively confined to a few University Departments of Forensic Medicine and Blood Transfusion Centres. Fees vary greatly and may range from two to thirty guineas. There are other drawbacks. Any of the parties may refuse to have blood samples taken, without penalty, and for this as well as other reasons a blood grouping test is by no means a routine procedure in affiliation cases. In addition there is no standard certificate for the presentation of results to the Court and solicitors and barristers often lack knowledge of how to cross examine the serologist.

TABLE 9.3

Individuals tested	Rh antisera used					Phenotype
	D	C	E	c	C^w	
Mr Smith (putative father)	+	+	−	−	−	D C C e e
Mr Jones (putative father)	+	weak	−	+	+	D C^w c e e
Miss Smith	−	−	−	+	−	d d c c e e
Baby Smith	+	weak	−	+	+	D C^w c e e

The legal measures which have been proposed to remedy this state of affairs, have had an unfortunate history. A Blood Tests Bill introduced by Lord Merthyr in 1938 was on the brink of becoming law when war commenced. A second measure entitled 'The Affiliation Proceedings (Blood Tests) Bill' was prepared by Lord Merthyr in 1957 with the help of an informal committee (Grant 1961). Then in 1958 Lord Merthyr was elected Deputy Speaker and Chairman of Committees of the House of Lords and was unable to proceed further with the Bill. Later, Lord Amulree decided to sponsor the measure and introduced the Bill in 1961. Certain objections were raised and the Bill went to Committee for amendment. The main objection was that the Bill allowed a magistrate to order blood grouping tests at his discretion even though one or both parties might not acquiesce. It was considered that this would create too much of a precedent!

An amended Bill had a second reading on 21st March, 1961, but did not become law in that Parliamentary Session owing to lack of time. It now seems unlikely that the matter will ever be re-introduced as a Private Member's Bill but there is a chance that the impending reform of law relating to the family will include this aspect.*

The Position of Paternity Testing Elsewhere

In the rest of Europe blood grouping tests in cases of doubtful paternity vary from being practically non-existent (e.g. Russia), to being carried out routinely every time a man denies paternity of a child; for example, in Denmark where about 2000 tests are made each year in the Institute of Forensic Medicine in Copenhagen.

However, in general, other European countries show a commendable concern for the welfare of the illegitimate child which Britain would do well to emulate.

STAINS

Blood Stains

A blood stain cannot be properly identified as such unless it can be shown to have haemoglobin. Nor can it be assigned to a particular species of animal, from man downwards, until a reaction has been obtained to proteins or other antigens specific for that species. Only after satisfactory results have been obtained for these tests can attention go to finer distinctions, such as the blood group of the stain if it is human. Of course, the amount of blood stain available affects the ease with which all the tests can be carried out. A large blob of material, which to even the layman announces itself as blood, presents a far easier task than one which is practically invisible. Yet many garments and other objects have to be examined for blood because they are suspected to have been splashed by it, when there is no naked-eye evidence for this fact at all.

The usual procedure in such cases is to rub each of the suspected areas with damp filter paper, after which a drop of freshly prepared benzidine solution is added to each paper. The rapid development of a blue colour indicates the area upon which to concentrate. The production of a blue colour with benzidine is not necessarily caused by blood since other biological materials and some chemicals containing oxidizing agents will produce a blue colour. The area chosen as being hopeful is then examined minutely under a good dissecting

*At the present time (November, 1967), change is in the air! A Law Commission Working Paper on Proof of Paternity in Civil Proceedings is being circulated for comment. This deals with such matters as the power of the Courts to order blood tests and the extension of blood grouping to include a larger number of Divorce Cases. Moreover, the Law Society has already regularised fees for legally aided clients.

microscope. The appearance of small clots, or red cell debris, may obviate the necessity for a test for haemoglobin, thus precious material is saved.

Haemoglobin is usually sought for spectroscopically or by crystal test, the haemochromogen crystal test being a satisfactory test requiring only one well-stained thread for its performance. Interest is now being revived in immunological tests for haemoglobin. In the past haemoglobin has been found to be a weak antigen, but modern work on adjuvants (see Chapter 1) has facilitated the production of anti-haemoglobin precipitating sera. In this field it is possible to make distinction between adult and foetal haemoglobin and also between the haemoglobins of certain species of animal.

Having established the presence of haemoglobin in a stain, the next step is to assign it to its species of origin. This, at first sight, may seem an immense task since the animal kingdom is a large one! However, there is very often a strong indication from the history of the case that the blood is human so an anti-human reagent is tried first and in many cases is positive. On occasion it may be necessary to distinguish between the blood of man and domestic animals, cat, dog, rabbit or between various animals, such as cow, goat, sheep, deer and fox. Species identity is somewhat bedevilled by the problem of cross-reactions particularly between closely related species; for example anti-human globulin made in a rabbit reacts not only with human globulin but with the globulin of anthropoid apes as well. This difficulty can be overcome, but not easily. One either has to carry out a series of delicately adjusted absorption experiments or prepare another antiglobulin serum by injecting the appropriate anthropoid with human globulin. In both cases it is a matter of catching one's monkey! Fortunately in Britain distinction between the blood of apes and man is a very rare problem.

The classical species identification is by the 'ring' test carried out in precipitin tubes or capillaries (see Chapter 1).

A more modern method is the antiglobulin consumption test. The principle of this is the absorption of a standard antiglobulin reagent by the globulin contained in a portion or extract of stained material. Any reduction in strength (titre) of the antiglobulin caused by its absorption by globulin occurring in the stain is measured by means of indicator red cells coated with globulin. For the detection of human globulin, red cells sensitized by incomplete anti-D are used. These should normally become agglutinated by the anti-human globulin used in the test, but remain unagglutinated or less strongly agglutinated if the anti-human globulin is already taken up by the stain (see also Chapter 1).

The anti-globulin consumption test is highly sensitive and will detect globulin in dilutions of the order of 1 in 50,000. However, it is not so simple to perform for the detection of globulin of other species than human since such tests require indicator cells coated with globulin from a variety of different animal species. In this situation, the gel double diffusion principle scores (see Chapter 1), although it is less sensitive.

A piece of stained material or extract therefrom may be placed in a central well cut in agar gel while each of a set of (usually six) wells surrounding this contains a drop of antiserum against the globulins of a variety of species. In this way the extract in the central well may be tested against a number of antisera simultaneously. The sensitivity of this form of gel diffusion test has been cunningly increased by Culliford (1964) by combining the gel diffusion method with electrophoresis. This involves placing extracts of stains in small wells in an agar plate opposite to other wells containing appropriate antisera (anti-human, anti-cow, anti-chicken, etc.) and subjecting them to electrophoresis in such a way that the albumin and non-gamma globulins of the stain move towards and meet with the gamma globulins of the antisera which are being electrophorized in the opposite direction, whereupon a line of precipitation may form between the two potential reactants. This method also gives excellent results with stains that are contaminated with fat or dirt since this is left behind in the gel.

The species of origin of a blood-stain can also be determined by testing the antigenic specificity of γ-globulin adsorbed on cloth fibrils by an adaptation of the mixed antiglobulin reaction (see Styles, Dodd & Coombs 1963). As more becomes known about the allotypic determinants on γ-globulin, intra-species differences should also be able to be determined. In this connection, in a model study on stains of rabbit blood where allotypic forms of γ-globulin are well established, Hill, Kelus & Coombs (1968) found it possible to differentiate stains from different rabbits by means of either the mixed antiglobulin reaction performed directly on fibrils or double diffusion agar gel tests on extracts of the stains. Stains from rabbits of γ-globulin allotype A1 were differentiated from those of allotype A4. Eight-week-old stains could be differentiated.

Finally we come to the grouping of stains by means of red cell components. Valuable evidence can be obtained by establishing a difference in blood group between stain and individual. For example, a given blood stain of group A cannot have originated from a given person of group O; on the other hand a given stain of group O could originate from a given person of group O, but by no means positively does so since group O is a common blood group to which about 47% of the English population belong. Increasing the number of blood groups tested for naturally increases the chances of making distinctions, and, moreover, increases the chance of obtaining a statistically significant probability that the stain does originate from one particular individual.

There are problems attached to the grouping of stains which do not exist in the ordinary grouping of blood. Underlying blood grouping is the principle of agglutination or clumping of red cells by specific antisera. For this agglutination phenomenon to be successfully achieved, it is essential that the red cells remain intact. In the drying process which takes place in a blood stain, the red cells are usually damaged to such an extent that they are no longer able to agglutinate; fortunately, however, the cells are still capable of specifically absorbing the corresponding antibody and this forms the basis of the classic inhibition or

absorption technique. The antigens of the ABO blood group system display considerable toughness, withstand heating almost to boiling point, the passage of time and other vicissitudes and still remain active enough to detect. Other blood group antigens seem more delicate; of these M and N seem the most vigorous, but display some cross-activity (i.e. M absorbing anti-N). In a measure it is possible to get over this.

After a rather slow start research on the grouping of stains is going ahead more vigorously. Other well-known techniques in addition to the inhibition technique are being adopted for the detection of antigens in stains.

These are direct tests which detect the antibody actually taken up by the stain, instead of the antibody left behind, as in the inhibition technique. The first of these by Kind (1960) makes use of the principle that bound antibody can be eluted by raising the temperature to 56°C. The method is confined to the ABO groups. The stain is fixed by dipping in boiling water for a few seconds before treating with antiserum. After washing well, the antibody is eluted from the stain or crust in the presence of a dilute suspension of the appropriate red cells. On cooling, the red cells become agglutinated if the corresponding antibody has been taken up and eluted from the stain.

Coombs & Dodd (1961) have found that the 'mixed agglutination method' (described in Chapter 1), is adaptable to the detection of antigens on blood-stained cloth. The stained cloth fibres are cut into short lengths, 1 mm approximately, and then teased out under a dissecting microscope into the finest possible fibrils. A mere five or six of such fibrils are enough for ABO grouping. The fibrils are treated with appropriate antibodies, which are taken up specifically if the corresponding antigen is present in the stain. The sensitization of the fibrils is revealed when fresh indicator red cells containing the same antigen are added, these then coat the fibrils. Photographs of positive and negative fibrils are shown in Plate 9.1, facing p. 256. The technique is an elegant one and has been used successfully for the antigens of the ABO system as well as for MN and 'species' antigens.

Nickolls & Pereira (1962) followed up this work by producing an exceedingly successful adaptation of the method of Kind. This they termed absorption elution. Very recently Pereira and Bargagna have succeeded in demonstrating the Rh antigens D, C, E and c but larger amounts of stain are required than for the demonstration of A and B antigens. This fits in with the fact that, at least for the D antigen, it has been shown that there are several hundred fewer antigenic sites per red cell than for A or B (Lee & Feldman 1964).

Culliford (1967) has demonstrated haptoglobins by an immunoelectrophoretic method in blood stains and these are being used in case work. Beyond the realm of immunology, red cell enzyme allotypes such as phospho-glucomutases (PGM) are useful markers and are proving to be readily identifiable in stains (Culliford 1967).

The extent of the usefulness of being able to type stains further than the basic

ABO groups can be appreciated if the main situation in which such knowledge is required is now described. In a case of murder or serious injury caused by assault, very often three samples are available for comparison. A whole blood sample from the suspect; a similar sample from the victim who may have been killed or seriously injured; a blood stain or stains found on the suspect's clothing, weapon, etc.

The objective in such circumstances is to attempt to discover whether the blood stain could originate from the victim or whether it might merely be blood from the assailant himself. The investigator therefore has to find such blood group antigens as will make a distinction between the blood of assailant and victim and, in addition, be detectable, if present, in the stain. Naturally, the greater the number of systems tested for, the greater the chance of making the desired distinction.

An example will elucidate the point.

ABO system	Suspect and victim and stain all of group A. No distinction made.
MN system	Suspect type MN, victim type N, stain type N. Stain therefore could originate from victim but not from suspect.
Haptoglobin system	Suspect type Hp 2-1, victim and stain both 2-2. Further evidence in support of conclusion drawn from results of MN system.

The really striking fact emerging from the above example it that although approximately 42% of the English population are of group A, 37% are of Hp 2-2 and 22% of type N, a combination of these types occurs only in 3% of the population.

Thus if the stain on the suspected assailant's clothing is *not* from the given victim but from another source, the chance of it belonging to this combination of blood groups is 3 in 100.

We are now in a position to realize how important the blood typing of stains becomes immediately it is possible to type a stain for several blood group systems. A recent murder case in which the crime took place in a Berkshire village emphasizes the point still further. This case had the fortunate feature that the perpetrator of the crime left a blood stain, almost certainly from himself, in the vicinity. From the evidence, it became relevant to treat about 200 male members of the village as suspects. Let us suppose that this stain is of the common group combination given above and there are 200 male members of the community among which the murderer may lurk. On the basis of the above calculation, the suspects are likely to be whittled down to six approximately. The stain has but to have one or two less common (but by no means rare) blood group antigens for the expected number of men among 200 having this combination to be reduced to the order of one!

The grouping of blood stains is becoming a powerful weapon in the hands of the crime investigator.

An important question is that of the ageing of stains. How old can they be and yet retain their immunological activity? Experience varies and this, no doubt, is due to the conditions to which the stains are exposed. Using the 'mixed agglutination' technique, ABO antigens have been found active in a stain on a handkerchief which was 5 years old. By inhibition technique the A antigen has been found in a blood crust on floor boards 14 years after the blood was shed. On the other hand when stains have been exposed to adverse circumstances, then some blood group antigens may disappear in a few days or even hours. On the whole, stains which have the opportunity to become dry quickly retain their immunological properties for longer periods than those that remain wet and encourage the growth of bacteria. Accumulation of dust on stains may falsify results. Extracts of house dust usually show strong A and less strong B activity.

All these points call for caution in the interpretation of results and make carefully planned control tests essential. In spite of drawbacks, however, much valuable immunological evidence may be obtained.

Seminal Stains

The finding of spermatozoa in a stain identifies it as seminal. There is, as yet, no other completely diagnostic test in routine use although some early experimental results (Coombs, Richards & Dodd 1962) suggest that double diffusion agar precipitin tests using antisera specific for seminal proteins could, with advantage, be adapted for this purpose.

The ABO blood group antigens appear in seminal stains from secretor individuals (about 80% of the population) and even to a certain extent in stains from non-secretors. This last fact is emerging from work done with the new sensitive fibril techniques (see above). The remarks already made about the value and limitation of grouping blood stains apply to the grouping of seminal stains.

Saliva

Saliva can often be identified by means of its ability to convert starch into sugar. The presence of squamal cells consistent in appearance with buccal squames is confirmatory evidence for the presence of saliva. Secretor saliva is often rich in blood group substances and presents little difficulty in testing. Cigarette ends of even dry smokers show considerable blood group activity.

Both saliva and seminal stains can be successfully grouped by mixed agglutination and absorption elution. These methods are also sensitive enough to detect trace amounts of blood group substances in non-secretors (Dodd & Hunter 1963, Pereira 1963).

Mixtures of Stains

In practice, the forensic science laboratory often finds its cases far from clear cut. Blood, seminal, vaginal or saliva stains may be found contaminating each other. In such cases it is helpful to have available fresh samples of blood and saliva from persons known to be involved. Often it is not possible to interpret the results without some knowledge of the facts of the case; in such circumstances the immunologist has to guard against bias.

Two actual examples may serve to illustrate the problems.

1. In a case of rape, stains on a schoolgirl's tunic contained both spermatozoa and squamal cells and in addition blood group substance A.

The finding of spermatozoa and many squamal cells together in the same stained area suggested that the stain was made by both seminal and vaginal fluids. Then the question arose whether the blood group substance A originated from the seminal fluid or the vaginal fluid or from both.

A meticulous and laborious testing of many small areas of the stain revealed some which possessed squamal cells, A substance and no spermatozoa while others contained spermatozoa but no A.

These findings suggested that the girl was contributing an A activity which the man did not have.

The testing of blood and saliva samples from the accused man and the girl revealed that he was a non-secretor of group O while she was a group A secretor. If the investigator had not been alive to the possibility of mixed stains the accused man might have got off on the grounds that the tunic was stained with seminal fluid of group A, which could not have originated from him, he being of group O.

2. In a case of rape and murder, a blood-stained pillow was tested. Some of the stains behaved as group O, others as group B. A few areas without blood stain showed squamal cells and group B activity. It was considered highly possible that these areas were stained with saliva from a group B secretor. It was also considered possible that the blood-stained areas behaving as group B might consist of a mixture of group O blood and group B saliva which would be a legitimate alternative explanation to their being stained with group B blood alone.

Tests on blood and saliva from suspect and victim showed the suspect to be a group B secretor while the murdered girl was group O, secretor status unknown.

IDENTIFICATION OF SKIN

It is not difficult to see the value of being able to determine accurately the blood group of skin cells, particularly dandruff, in certain criminal cases. Work by Swinburne (1962) is establishing the value of the 'mixed agglutination' technique in this direction also.

She prepared epithelial squames from dandruff and skin scrapings of both live persons and cadavers. After special treatment with potassium hydroxide and ethanol the resulting suspensions of cells are used in the mixed agglutination technique already briefly described in this chapter in connection with blood stains. She successfully identified A and B on the material by this method. Later Poon & Dodd (1964) identified H.

Grouping of Hairs

Encouraging results are now being obtained by using mixed agglutination for the detection of A, B or H in young facial hairs (Lincoln & Dodd 1968). It is possible that the main blood group activity lies in the serum coating the hair. Successful prior treatment of the hairs requires that extraneous material should be removed from the sebaceous layer without dissolving away the layer itself. A photograph of the appearance of mixed agglutination on male beard is shown in Plate 9.2, facing p. 257.

So far, the grouping of head hairs has attained but limited success. False positives occur, particularly with anti-A and female hair that has been bleached or lacquered is not always satisfactory.

MILK AND MEAT TESTING

Precipitin and complement fixation reactions have been used for years in Germany, principally to distinguish between horse meat and the meat of other species. The techniques are included in German legislation.

In the United Kingdom precipitin tests are used occasionally by public analysts and others. Work on the differentiation of meats by precipitin tests has been done by Ginsberg (1948) and also by Pinto (1961) working in Ceylon on the problem of distinguishing ox, buffalo, goat and deer.

In all this work the problem of cross-reaction again has to be considered and adjustments made to allow for this.

Salt meat can be handled if water is used as a test diluent instead of saline. Cooked meat cannot be tested owing to denaturation of proteins by heat. There is the chance of a satisfactory result if the centre portion of a thick piece of cooked meat is tried.

There would seem to be a good opportunity of substitution in the case of canned meats. Research into suitable immunological methods for this kind of material might be rewarding.

The immunological aspects of milk testing seem to be little explored. Substituting or, at least, resort to immunological methods to detect it, does not seem to be a practical problem. Pinto, working in Ceylon, has been faced with the problem of distinguishing the contamination of cow with buffalo milk. For the solution of this problem he uses essentially the same techniques as those employed for the recognition of various types of meat.

IMMUNOLOGICAL CAUSES OF DEATH

It will be apparent from reading other chapters of this book that immunological tests may establish a cause of death. For example, the evidence for the occurrence of a fatal transfusion accident may rest almost entirely on the finding of an antibody in the patient's serum, incompatible with the donor's blood. There are also rare cases of fatal anaphylaxis in adults and infants.

Of particular interest is the recent work which postulates that the cause of death of some infants who die without apparent reason while sleeping peacefully in their prams, is a hypersensitivity reaction to cow's milk. It is known that many infants have antibodies to cow's milk in their serum and it is postulated that a modified anaphylactic reaction is initiated by the sleeping child regurgitating stomach contents containing as yet undigested cow's milk and aspirating some of this material into the lungs (Parish *et al* 1964).

There is no doubt that immunological methods have become essential aids to the forensic scientist which fact, it is hoped, has become clear from the above account. What is perhaps not so apparent, but nevertheless true, is that working on forensic problems deepens the immunologist's understanding of his own subject. This is not always appreciated by inhabitants of ivory towers!

REFERENCES

BARGAGNA M. & PEREIRA M. (1967) A study of absorption elution as a method of identification of Rhesus antigens in dried blood stains. *J. forens. Sci. Soc.* **7,** in press.

CAMPS F.E. & DODD, BARBARA E. (1966) Blood Grouping in a Case of Infanticide. *Medicine, Sci. Law.* **6,** 122.

CLEGHORN T.E. (1964) Personal communication.

COOMBS R.R.A. & DODD, BARBARA E. (1961) Possible application of the principle of mixed agglutination in the identification of blood stains. *Medicine, Sci. Law.* **1,** 359.

COOMBS R.R.A., RICHARDS C. & DODD, BARBARA E. (1963) Serological identification of seminal stains. *Medicine, Sci. Law.* **3,** 65.

CULLIFORD B.J. (1964) Precipitin reactions in forensic problems. *Nature (Lond.)* **211,** 872.

CULLIFORD B.J. (1966) Haptoglobin types in dried blood stains. *Nature (Lond.)* **211,** 870.

CULLIFORD B.J. (1967) The determination of phospho-gluco-mutase types in blood stains. *J. forens. Sci. Soc.* **7,** in press.

DODD, BARBARA E. & HUNTER D. (1963) A Comparison between the Inhibition and Mixed Agglutination Techniques for the Detection of A, B, and H′, Proceedings of the Third International Meeting in Forensic Immunology, Medicine, Pathology and Toxicology.

GINSBERG A. (1948) The Differentiation of meats by the precipitin test. *Vet. Rec.* **60,** 683.

GRANT A. (1961) The affiliation proceedings (Blood Tests) Bill (H.L.). *J. Forens Med.* **8,** 80.

HILL E.W, KELUS A.S. & COOMBS R.R.A. (1968) The recognition of allotypic forms of γ globulin in blood stains of rabbits: A model for future work on human stains. *Medicine, Sci. Law.* In press.

KIND S.S. (1960) Absorption-elution grouping of dried blood smears. *Nature (Lond.)* **185,** 397.

LEE R.E. & FELDMAN J.D. (1964) Visualization of antigenic sites of human erythrocytes with ferritin-antibody conjugates. *J. Cell. Biol.* **23,** 396.

LINCOLN P.J. & DODD, BARBARA E. (1968) *Medicine, Sci. Law*, in press.

NICKOLLS L.C. & PEREIRA M. (1962) A study of modern methods of grouping dried blood stains. *Medicine. Sci. Law.* **2,** 172.

PARISH W.E., RICHARDS C.B., FRANCE N.E. & COOMBS R.R.A. (1964) Further investigations on the hypothesis that some cases of cot-death are due to a modified anaphylactic reaction to cow's milk. *Int. Arch. Allergy* **24,** 215.

PEREIRA, MARGARET (1963). Observations on the grouping of dried stains of body fluids. Proceedings of the Third International Meeting in Forensic Immunology, Medicine, Pathology and Toxicology, London.

POON, WAI LUN & DODD, BARBARA E. (1964) The subdivision of blood stains into A_1 and A_2 and the detection of H on human epidermal cells. *Medicine, Sci. Law.* **4,** 258.

PINTO F.C. (1961) Serological identification of ox, buffalo, goat and deer flesh. *Brit. vet. J.*, **117,** 540.

STYLES W.M., DODD, BARBARA E. & COOMBS R.R.A. (1963) Identification of human blood-stains by means of the mixed antiglobulin reaction on separated cloth fibrils. *Medicine, Sci. Law.* **3,** 275.

SWINBURNE, LAYINKA M. (1962) The identification of skin. *Medicine, Sci. Law.* **3,** 3.

See also:

Bibliography on Immunology in connection with body fluids and tissues (1962) *Medicine, Sci. Law.* **2,** 497.

BOORMAN, KATHLEEN E. & DODD, BARBARA E. (1966) *An Introduction to Blood Group Serology* 3rd ed. London: J. & A. Churchill.

RACE R.R. & SANGER R. (1968) *Blood Groups in Man.* 5th ed. Oxford: Blackwell Scientific Publications.

CHAPTER 10

IMMUNOASSAY OF HORMONES AND BIOLOGICALLY ACTIVE SUBSTANCES

C.N.HALES

INTRODUCTION

That the second edition of this volume should contain a separate chapter devoted mainly to the immunoassay of hormones is entirely due to the work of Berson and Yalow who not only conceived the basic method but also realized its potential in the assay of many peptide hormones.

It was realized many years ago that the injection of peptide hormones gave rise to the production of antibodies. In 1941 Thompson in a review of the subject wrote: 'Conceivably the endocrinologist may utilize the action of specific antihormones in the study of the interrelations of the endocrine glands'. This prediction has certainly been fulfilled. No chemical technique available can specifically estimate a peptide hormone in plasma when the latter represents by weight approximately one part in fifty million of the plasma protein. However, the immunological system of many organisms is capable of recognizing and eliminating specific macromolecules when present at very low concentration. Immunoassays may indeed be regarded as the macromolecular equivalent of enzyme assays of metabolic intermediates. In the latter advantage is taken of enzyme specificity in the recognition of metabolites.

Early attempts to use antibodies in the detection and estimation of hormones in plasma were unsuccessful because the methods available lacked adequate sensitivity. For example, in one of the earliest attempts Wasserman & Mirsky (1942) observed complement fixation when an insulin antiserum was reacted with serum of patients suffering from chronic hypoglycaemia. In these subjects it might be expected that plasma insulin concentrations would be considerably elevated. However, no complement fixation was observed when serum from normal individuals was tested. The first major attempt to employ insulin antibodies in a sensitive assay for insulin was that of Stavitsky & Arquilla who in 1953 used a haemagglutination inhibition method. This method is, however, too subject to non-specific interfering factors for its use in the assay of insulin in plasma (Wolstenholme & Cameron 1962).

It was not until Yalow & Berson (1959) combined the use of antibodies and radioactively labelled hormones that methods with adequate sensitivity and accuracy became available. The use of radioactively labelled hormones began with their study of insulin binding by the plasma of normal and diabetic individuals. It was shown that insulin-treated diabetics possessed in their plasma a protein with the properties of a specific antibody and which was capable of combining with radioactively labelled insulin (Berson *et al* 1956). It was then demonstrated that unlabelled insulin was capable of preventing competitively this combination and thus the basis of an assay for unlabelled hormone was established.

The advent of radio-immunoassays for peptide hormones has had such an enormous impact on the study of the normal and abnormal regulation of metabolism by peptide hormones that most of this chapter is concerned with a description of these methods. So rapid has been the development of new radio-immunoassays, however, that it is not possible to give a detailed account of the full range of methods and their application. The technical details of the individual assays may be obtained from the original accounts of the methods which have been the subject of two excellent recent reviews by Greenwood (1967) and Potts *et al* (1967).

The present account considers radio-immunoassays in relation to other immunoassays of biologically active substances. It contains brief references to some of the more important applications of the different methods for the immunoassay of biologically active substances and a more detailed description of the fundamental aspects of radio-immunological techniques.

GENERAL PRINCIPLES

$$\underbrace{\text{antigen} + \text{antibody}}_{\text{'free' components}} \longrightarrow \underbrace{\text{antigen} - \text{antibody complex}}_{\text{'bound' components}}$$

All immunoassay procedures depend upon two fundamental properties of immunological systems:

1. The combination of antigen with antibody yields an antigen-antibody complex in which certain properties of each components are altered (Table 10.1).
2. For a fixed amount of antibody there is an upper limit to the amount of antigen which can be bound.

Most immunoassays involve the use of a fixed amount of antibody which is allowed to react with varying amounts of antigen. As increasing amounts of antigen are added three changes occur:

1. The amount of free antibody decreases.
2. The amount of complex increases.
3. Finally free antigen begins to appear and increase in concentration.

TABLE 10.1

Differences which may be found in the properties of antigen–antibody complexes when compared with free antigen

1. Solubility in buffers, salt solutions, water-alcohol mixtures and presence of anti-γ globulin antisera.
2. Complement fixation.
3. Molecular weight.
4. Adsorption on to cellulose, ion exchange resins and activated charcoal.
5. Isoelectric point.
6. Loss of biological activity.

The extent of any one of these changes, in the presence of a fixed amount of antibody, is a function of the amount of antigen added and therefore any one of these changes can be made the basis of a quantitative assay of antigen. The details of the assay procedure will be determined by the basic change which is measured. The presence of uncomplexed ('free') antigen or antibody may be detected because the properties of antigen or antibody are often altered when they are bound in the complex.When the antigen is in excess this is indicated by the presence of free antigen: when the antibody is in excess by the presence of free antibody. The point at which there are equivalent amounts of antigen and antibody may be defined as lying between either:

1. In a system in which free antigen is detected the largest amount of antigen which does not produce detectable free antigen and the smallest amount of antigen which does, or
2. In a system in which free antibody is detected the smallest amount of antigen which prevents the detection of free antibody and the largest amount of antigen which allows the detection of free antibody.

The amount of antigen-antibody complex formed may be measured in terms of one of the properties peculiar to the complex.

Thus immunoassay procedures depend upon the estimation or detection of one of the following:

1. Free antigen.
2. Free antibody.
3. Antigen-antibody complex;

and will be considered under these headings.

Measurement or Detection of Free Antigen in the Presence of the Antigen-Antibody Complex

The estimation of the amount of free antigen present when the antigen is a biologically active substance and the antibody one which neutralizes this activity is relatively simple. The procedure employed will depend upon the biological activity of the antigen:

Toxins

These substances, produced by bacteria, are usually assayed by injection into susceptible animals. The effect on the animal varies with the system but most commonly is death, tetanospasm or a skin reaction (Pope 1963).

Enzymes

A number of toxins are enzymes and may therefore be assayed by estimation of enzymatic activity rather than *in vivo* toxicity. An example is the *Clostridium perfringens* α toxin which is a phospholipase of the C type and hydrolyses lecithin to diglyceride and phosphorylcholine. This activity is neutralized by an antiserum to the toxin.

Production of antibodies to enzymes is not limited to those enzymes produced by bacteria and which have toxic properties. A wide range of enzymes has now been used as antigens (Cinader 1963). Antibodies to enzymes have been used to study isoenzymes, enzyme synthesis and localization in cells. Antibodies to enzymes do not necessarily inhibit enzyme activity and this aspect of the antigen-antibody reaction may change during the course of immunization.

When antibodies are of the inhibiting type this inhibition may be prevented by the presence of the substrate particularly if the substrate is macromolecular. However, the fact that an antibody inhibits enzyme activity does not necessarily indicate that it combines at the active site of the enzyme.

Hormones

Antibodies have been produced to many peptide hormones. They are frequently capable of neutralizing the biological activity of the hormone and are a cause of acquired resistance to treatment with peptide hormones (see Chapter 25).

The *in vivo* injection of neutralizing antibodies to hormones may produce an acute hormone deficiency state. An example of this is the induction of diabetes in rats by the injection of an antiserum to ox insulin (Moloney & Coval 1955). Anti-hormones therefore provide a convenient and specific tool with which to study the biological action of hormones. The biological activity of free hormone may be measured using an *in vivo* or *in vitro* bioassay. An assay for insulin using insulin antibodies in conjunction with an *in vitro* bioassay has been devised (Wardlow & Moloney 1961).

Haptens

Small molecules which are biologically active but not antigenic may be rendered antigenic by chemical coupling to a macromolecular substance, usually a protein or polysaccharide. The antibody produced may be capable of binding and neutralizing the biological activity of the uncombined small molecule. In this way an immunoassay may be devised for a low molecular weight compound. A sensitive radio-immunoassay for digitalis has been developed along these lines (Oliver *et al* 1966).

Detection of Free Antibody in the Presence of the Antigen-Antibody Complex

The reaction of antigen with antibody may be observed relatively easily if the antigen has been coupled to red cells prior to reaction with an antiserum (see Chapter 1). In this situation the antigen-antibody reaction may lead in the absence of complement to agglutination of the antigen-coated red cells or in the presence of complement to haemolysis of the cells. In the absence of complement the agglutinated red cells may be recognized by observing the pattern which is formed on settling at the bottom of a test tube or by microscopical examination. In the presence of complement the degree of haemolysis is estimated most conveniently by spectroscopic estimation of the haemoglobin which is liberated. Following the reaction of antibody with antigen which is not conjugated to red cells any remaining free antibody may be detected by subsequent haemagglutination or immune haemolysis of antigen-coated red cells. If the antigen is in fact present in amounts equivalent to or in excess of antibody the ability of the latter to agglutinate or haemolyse red cells coated in antigen is abolished. These procedures therefore are known respectively as haemagglutination or haemolysis inhibition.

Measurement of Antigen-Antibody Complex

Estimation of Amount Precipitated

The amount of the antigen-antibody complex which precipitates depends, among other things, upon the particular system involved, some antigen-antibody complexes remaining soluble under most conditions. In precipitating

systems the amount of precipitate formed depends on the ratio of antigen to antibody in the reaction and therefore the amount of precipitate formed does not necessarily represent the total amount of antigen-antibody complex present.

Provided that other conditions such as temperature, ionic strength, pH, etc. (Kabat & Mayer 1961) are kept constant the estimation of the amount of precipitate formed can provide a measure of the extent of the reaction, and is related to the amount of antigen added. Due to the complex nature of the curve relating amount of precipitate formed to increasing antigen concentration in the presence of a fixed amount of antibody, it is necessary to test the effect of a number of dilutions of the unknown antigen before the concentration can be calculated.

Complement Fixation

Complement which is in fact a group of serum factors (see Chapter 14) is bound by many antigen-antibody complexes. The amount of complement bound is a function of the amount of antigen-antibody complex formed and therefore the estimation of complement fixation (see Chapter 1) provides a measure of the amount of antigen-antibody complex formed.

Complement fixation has been used for the assay of hormones including human chorionic gonadotrophic hormone (Brody 1966) and parathormone (Tashjian, Levine & Munson 1964). The sensitivities of these assays were 0.5 I.U/ml and 10 mμg/ml respectively which may be compared with 0.0025 I.U/ml and 0.5 mμg/ml in the corresponding radio-immunoassays.

Use of Labelled Antigen

When antigen binds to antibody there is a change in its physical and chemical properties (Table 10.1). In many instances the antigen is considerably smaller than the antibody and the resultant antigen-antibody complex retains most of the properties of the free antibody but few of the properties of the free antigen. If the antigen is readily identifiable by means of a label such as a fluorescent group or radioactive isotope (provided the label does not prevent the antigen-antibody reaction) the antigen bound to antibody can be estimated. It represents that fraction of the total label added which possesses the characteristics of the antigen-antibody complex.

This system may be used as the basis of an assay of unlabelled hormone. The system most commonly employed involves antigens labelled to high specific activity with radioactive isotopes of iodine. The addition of increasing amounts of unlabelled antigen to the labelled antigen plus antibody system decreases competitively the amount of labelled antigen-antibody complex formed. The use of high specific activity labelled antigens means that very low concentrations of antigen and antibody can be used and therefore the sensitivity of the

systems is very great. In addition the only estimation required, that of radioactivity, can be made simply, automatically and accurately.

RADIO-IMMUNOASSAY OF HORMONES

Production of Antisera

The production of antisera specific for the assay of human peptide hormones has presented certain problems:

1. Many of the peptides are of low molecular weight and are poorly antigenic.
2. There is a scarcity of pure human hormones and the corresponding hormones from ox or pig may show structural differences.
3. The biological activity of the hormone in the inoculated animal may make the production of antisera difficult. For example insulin may produce fatal hypoglycaemia and ACTH hyperadrenocorticalism.

Antigenicity may be enhanced:

1. By careful selection of species. For example the guinea-pig produces good antibodies to ox, pig and human insulin whereas the rabbit does not. This difference may be related to the degree of 'foreignness' of the injected insulin since rabbit insulin differs little from ox, pig and human insulin whereas guinea-pig insulin differs very considerably (Smith 1966).
2. In some cases (e.g. parathormone) it has been found that impure preparations are more potent antigens than the highly purified preparations (Yalow 1966).
3. The antigen may be coupled to other substances such as proteins, polylysine and cellulose (Olovnikov & Gurvich 1965).
4. Injection in adjuvant (usually complete Freund's adjuvant).

The length of immunization may vary from a few weeks to several months. The danger of producing antisera to impurities is not important provided that very pure hormone is used for the production of labelled antigen. The most important requirement of the antiserum is that it should have a high affinity for the hormone to be assayed. The extremely low plasma concentration of most peptide hormones (in the region of 10^{-10}M) means that only antisera with a high affinity for antigen will have an adequate sensitivity. It is at the moment impossible to predict which immunization procedures will produce the best antisera and animal variation even in a single species is very great. The amount of antiserum required is very low since most procedures employ 0.1 ml of an antiserum diluted to 1/50,000 or more. Therefore 1 ml of a good antiserum is enough for half a million assays. This means that it is advantageous to immunize a number of animals and select the best antiserum. Once this has been defined it can be used for many years.

Preparation of High Specific Activity Labelled Hormone

It is essential that the hormone preparation used for labelling should be of the highest possible purity since all subsequent determinations will involve the estimation of this preparation.

The ideal labelled preparation would be a hormone in which some of the normal atomic constituents had been replaced by a radioactive isotope. Unfortunately there are at the moment no methods available for the production of an adequate specific activity (50–1,000 mC/mg depending on the sensitivity required) by this means. The most frequently used procedure has been to introduce an isotope of iodine (I^{131} or I^{125}) into the tyrosine residues of the peptides. This inevitably implies a chemical modification of the hormone. Fortunately, this procedure appears to produce very little effect on the immunological activity of most hormones provided that the degree of substitution is low. The usual level employed is to substitute an average of one or less atoms of iodine/molecule of hormone. An additional theoretical difficulty in studying the reactivity of the labelled hormone is that it is inevitably heterogeneous since even at low levels of iodination some molecules of hormone will contain more than the average amount of iodine. These molecules will of course also carry a disproportionate amount of radioactivity.

The method of iodination which has been most widely employed is that introduced by Hunter & Greenwood (1962). In this method the oxidizing agent chloramine-T is used in conjunction with 2–10 mC of I^{131} or I^{125} and 5 μg of protein. Some variability of incorporation rate with different samples of isotope has been noted but the method is capable of producing very high specific activities. Following iodination it may be necessary to remove damaged components as well as unincorporated iodide and a variety of procedures has been employed for this purpose. Lower specific activity preparations with less damage may be prepared using iodine monochloride as the iodinating reagent (Hales & Randle 1963). This reagent suffers from this disadvantage that it introduces carrier I^{127} and therefore high specific activities can only be achieved by the incorporation of large amounts of iodine. One of the difficulties associated with reproducible iodination to give a high specific activity labelled hormone is the uncertainty as to the specific activity of the I^{131} starting material. Methods for the investigation of the specific activity of commercial samples of I^{131} have been described recently (Berson & Yalow 1966; Glover & Shepherd 1966).

Iodination with I^{125} is becoming more common due to its longer half life and lower energy radiations with consequent increased ease of handling. The only disadvantage of this isotope is that the longer half life means that seven to eight times as many atoms of I^{125} than I^{131} must be incorporated to achieve the same specific activity.

Separation of Free from Antibody-bound Hormone

The original procedure introduced by Yalow and Berson involved chromatoelectrophoresis of the mixture of free and bound hormone. The mixture was applied to Whatman 3MM paper and the heat produced by the electric current led to evaporation of water from the paper strip. This in turn led to a flow of water along the paper. Many free peptide hormones are bound firmly by the paper employed (although batches of paper vary in this respect) whereas the bound hormone migrates with the γ-globulin and away from the origin. Following the rapid separation of free and bound hormones in this manner the radioactivity of each was measured by scanning the paper strip and measuring the area under each curve of radioactivity planimetrically. For each concentration of standard insulin the ratio of bound:free radioactivity was calculated and plotted against the concentration of unlabelled insulin to provide a standard curve (Fig. 10.1).

Modifications of the original immunoassay technique have been almost entirely concerned with attempts to make this separation procedure more convenient. Most frequently these employ procedures to precipitate the antigen-antibody complex and leave the free antigen in solution. One of the earliest methods involved salt precipitation but this has not been widely applied. Precipitation with an anti-γ-globulin serum has been very extensively used in what have come to be known as 'double antibody' methods. In the latter a second antibody is prepared against the γ-globulin or whole serum of the animal in which the first (anti-hormone) antiserum was raised. It is not necessary to use the serum or γ-globulin of immunized animals as antigen for the production of the second antiserum. If the precipitation reaction is carried out at the dilution of the first antiserum required for hormone assay the precipitate may form very slowly and remain very fine. Precipitation may be speeded by the addition of carrier normal serum and fine precipitates may be conveniently separated by filtration through micro-porous filter membranes. Separation by centrifugation is possible but requires much higher concentrations of the second antibody and large amounts of carrier normal serum.

The second antibody system provides additional problems:

1. It requires time.
2. There may be interference by γ-globulins which cross react with the second antibody. An example of this is the weak cross reaction with occurs between human γ-globulin (present in the plasma under assay) and a rabbit anti-(guinea-pig γ-globulin)serum and which is sufficient to interfere with complete precipitation.
3. Complement may interfere with the reaction.

These difficulties may be avoided if the antihormone antibody is precipitated

before being reacted with the hormone. This precipitation does not interfere with the subsequent hormone-antihormone reaction. Once precipitation has been carried out the mixture may be freeze-dried and stored at 4°C under which conditions the antibody remains stable for many months.

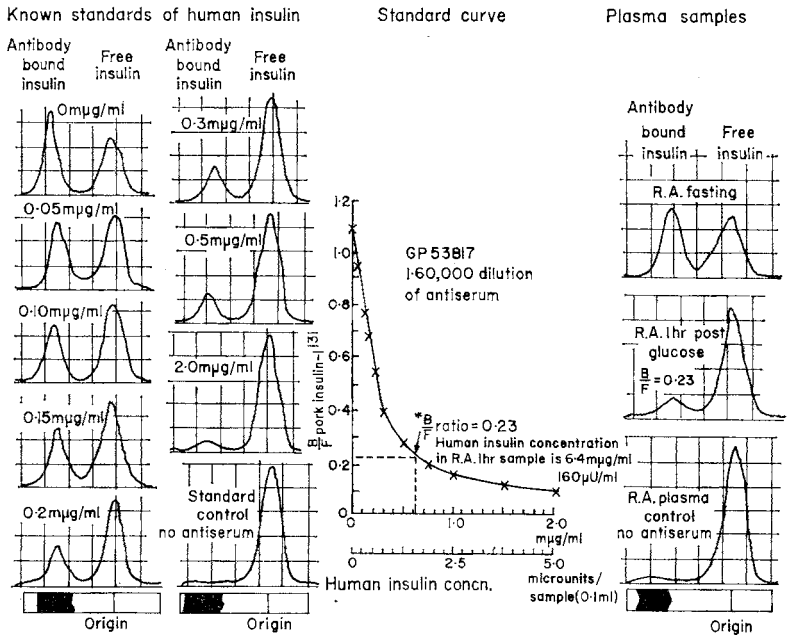


FIG. 10.1. Radio-immunoassay of human plasma insulin concentration using chromatoelectrophoretic separation of free and antibody bound labelled insulin. Insulin -I^{131}-antiserum mixtures containing known concentrations of human insulin are shown on the left and those containing unknown plasma on the right. At the bottom of each set is a control mixture to which no antiserum had been added; migrating activity in control tubes represents damaged fractions. The standard curve (middle) is obtained from measurement of areas under each of the two peaks in the complete series of chromatograms of which eight are shown. The insulin concentration in the 1 hour post-glucose specimen of patient R.A. is calculated as shown.

(Reproduced from Yalow R.S. & Berson S.A. *Immunoassay of Plasma Insulin.* In *Methods of Biochemical Analysis*, ed. D. GLICK **12**; 69–96. Interscience Publishers, New York, with permission.)

The advantages of using an assay in which the radioactivity of an immune precipitate is counted are:

1. The radioactivity assayed is specifically that bound to the precipitate and the assay can therefore be carried out in the presence of other labelled compounds which are not precipitated.

2. The assay may behave according to the theory of isotope dilution so that a linear function of the standard insulin concentration may be plotted (Hales & Randle 1963). This will be true if the antiserum binds a fixed amount of insulin at all the concentrations used in the assay and if the labelled and unlabelled insulin have the same affinity for the antiserum. Under these conditions the antiserum may be regarded as taking a fixed sample of the mixture of labelled and unlabelled insulin and the radioactivity of this sample will be proportional to the specific activity of the mixture. Then (Hales & Randle 1963):

$$\frac{C_o}{C_i} = i \frac{I}{i_o} + I$$

where i is the concentration of unlabelled insulin; i_o is the concentration of labelled insulin; C_o and C_i are the radioactivities of the insulin-antibody precipi-

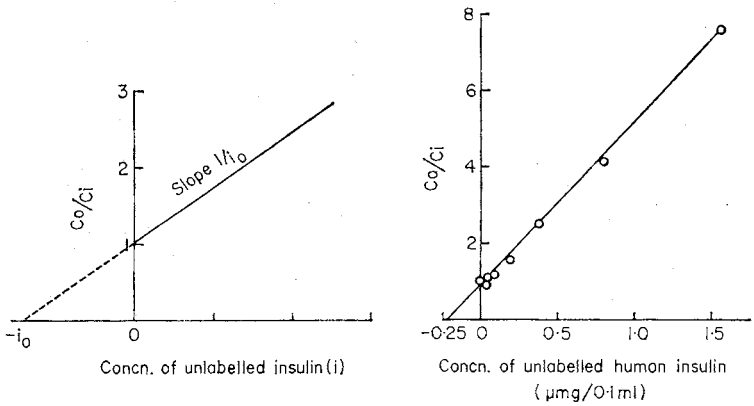


FIG. 10.2. Radio-immunoassay of human insulin using an anti-(γ-globulin) antiserum to separate free and antibody bound labelled insulin. The left hand graph depicts the relationship predicted from the theory of isotope dilution and the right hand graph results obtained experimentally (for the definition of terms see text.)

tate in the absence and presence of unlabelled insulin respectively. C_o/C_i will therefore be linearly related to i; C_o/C_i will be unity when i is zero; $i_o = -i$ when C_o/C_i is zero and the slope of the line is $1/i_0$ (Fig. 10.2).

There is a close correspondence between the theoretical predictions and results obtained in the assay of human insulin (Fig. 10.2).

More recently precipitation with alcohol has been used as an additional method of separation. Another way in which the antiserum may be rendered insoluble prior to reaction with hormone is by conjugation to an insoluble polymer such as sephadex (Wide & Porath 1966). Provided the antiserum is firmly bound and not eluted by subsequent exposure to high protein concen-

trations this may prove to be a most satisfactory way of avoiding the variables associated with precipitation techniques.

Other chromatographic and adsorption procedures have been developed for separation of free and bound antigen including ion exchange chromatography and adsorption on to activated charcoal or powdered cellulose. The higher molecular weight of the antigen-antibody complex also allows the separation of free and bound antigen by gel filtration. Variations of electrophoretic separation have included the use of cellulose acetate and polyacrylamide gel as supporting media.

Some Problems

Sensitivity

In spite of the very great sensitivity of radio-immunoassay (10^{-12}–10^{-10}M) there remain some hormones for which an increased sensitivity is desirable (growth hormone) or essential (ACTH and parathormone). Prior incubation of unlabelled hormone with the antiserum followed by addition of labelled hormone has been used to increase the sensitivity of some assays. In addition sensitivity is increased as the amount of antiserum and labelled antigen are decreased. It is only possible to work at very low concentrations when the antiserum has a high affinity for antigen and the antigen a very high specific activity. The specific activity which may be achieved by iodination is limited by the effects of radiation damage and excess iodine on immunological activity. As the concentration of the reactants is reduced the speed of reaction is reduced so that prolonged incubation periods, sometimes in excess of a week, may be necessary.

Incubation Damage

During the period in which antigen and antibody are reacting some of the antigen may be broken down. This is only a serious problem when plasma is present but in this situation a considerable amount of degradation of the labelled antigen may occur. If the damaged antigen has a reduced immunological reactivity there is a reduction in the amount of labelled antigen bound to antiserum. As a result, depending on the method of separation employed spuriously high, or low, concentrations of unlabelled antigen are calculated from the results of standard incubations in which there has been little or no incubation damage. A possible way of avoiding this would be to make the standard solutions up in control plasma but since the amount of damage varies from one sample of plasma to another this procedure is not satisfactory. The other alternative is to correct for damage. This is theoretically unsatisfactory since one is correcting for damage to the labelled hormone and ignoring possible damage to the unlabelled hormone. It is not possible to equate damage of labelled hormone with

damage to unlabelled hormone since the two preparations may easily have a different susceptibility to damage. Damage to the labelled hormone has the most serious implications since a 10% reduction in the label bound to antiserum may be equivalent to a doubling of unlabelled insulin concentration when read from the standard curve. In the Berson and Yalow system, correction for damage to labelled hormone is made by setting up a control incubation of plasma with labelled hormone in the absence of antiserum. Damaged components often migrate from the origin of the chromatoelectrophoretic strip so that the amount of damage can be estimated by calculating the proportion of the radioactivity which migrates. There are objections to the validity of this correction first because the criterion of damage is non-specific, i.e. it does not measure the immunological activity of the components which migrate and those which do not. In some assays damaged components remain at the origin (Potts *et al* 1967). Secondly, in the actual assay a considerable proportion of the labelled hormone is bound to antiserum and it is known that this protects it against plasma damage. Therefore the actual damage produced when antiserum is present may be significantly reduced.

It is possible, using a double antibody method, to provide a better estimation of incubation damage but even this is not entirely satisfactory. In order to assess the amount of immunologically active labelled hormone present at the end of the assay it is necessary to include an incubation to which excess antibody can be added at the end of the normal incubation period. This antibody must be sufficient to combine all the labelled and unlabelled insulin present. At this high concentration reaction is relatively rapid and therefore only a short further incubation is required to obtain maximum binding. In this way the proportion of the labelled antigen which is immunologically inactive at the end of the incubation can be calculated and if it differs from that in the standard incubation a correction may be applied.

The ideal solution to the problem of incubation damage is to avoid it and various steps may be taken in this direction:

1. Use of a stable labelled hormone. Some preparations are much more susceptible to damage than others and this property of the labelled hormone is important in assessing its use in radio-immunoassays.

2. Reduction of length of incubation of labelled hormone in the presence of plasma. This can be achieved by working at the highest concentration of the reagents which gives an adequate sensitivity and by adding the labelled hormone last and for as short a period as possible.

3. Working at the highest possible dilution of plasma. To a certain extent measures 2 and 3 are mutually exclusive, i.e. the more sensitive the assay, the less plasma required but the longer the incubation period must be.

4. Plasma damage may be due to proteolytic enzymes and -SH containing compounds both of which may be inactivated.

5. Damage increases with the temperature of incubation and therefore low incubation temperatures are to be preferred.

Immunological versus Biological Activity

It is theoretically possible that derivatives of hormones possessing biological and not immunological activity or vice versa, may exist in plasma. Antibodies to ACTH have been shown to react most strongly with the part of the molecule which is not essential for biological activity. Biological insulin-like activity in plasma exceeds immunological insulin-like activity and is not neutralized by an insulin antiserum. The physiological significance of the former activity is not known. For a provocative, if partisan, discussion of these problems a recent review of Berson & Yalow (1965) may be consulted. *A priori* there is no good reason to suppose that an *in vitro* adipose tissue bioassay which is known to be non-specific is likely to provide a more valid estimate of plasma insulin concentration than an immunoassay. It would be most satisfactory if both types of assay could be shown to agree but very few bioassays possess the necessary sensitivity, specificity and precision for them to provide a criterion by which to judge the validity of immunoassays. There are a number of other criteria by which the validity of immunoassays of plasma may be judged:

1. Abolition of activity by removal of the source of the peptide.
2. Parallelism between the effects of dilution of plasma and standard hormone.
3. Recovery of hormone added to plasma.
4. Changes in immunological activity which agree with those predictable on physiological and pathological grounds.
5. Ability of the antiserum employed to neutralize the biological activity of the hormone in an (often insensitive) bioassay.

Hormones for which Assays have been Described

The speed with which new immunoassays are being developed insures that any list such as is given below becomes out of date rapidly. However, the list does give an indication of the general applicability of the methods described.

Consideration of and reference to the following assays is given in the reviews of Greenwood (1967) and Potts *et al* (1967) to which the reader is referred for the original references: insulin, human growth hormone, glucagon, ACTH, parathormone, thyrotrophic hormone, luteinizing hormone, placental lactogen; human chorionic gonadotrophin, angiotensin, rat growth hormone, porcine growth hormone, bradykinin and oxytocin.

Additional accounts of the assays of the following hormones are available: human growth hormone (Morgan 1966a; Lazarus & Young 1966; Lau, Gottlieb & Herbert 1966); human growth hormone and insulin assayed simultaneously (Morgan 1966b); human chorionic 'growth hormone-prolactin' (Lau, Gottlieb & Herbert 1966); glucagon (Lawrence 1966), ACTH (Demura *et al* 1966);

parathormone (Berson & Yalow 1966b), thyrotrophic hormone (Lémarchand-Béraud & Vannotti 1965), luteinizing hormone (Bagshaw, Wilde & Orr 1966; Midgley 1966; Midgley & Jaffe 1966, Odell, Ross & Rayford 1966); human chorionic gonadotrophin (Bagshawe, Wilde & Orr 1966; Midgley 1966;) follicle stimulating hormone (Faiman & Ryan 1966) and α-melanocyte stimulating hormone (Ross, Odell & Goodfriend 1966; Goodfriend Ross & Schalch 1966); angiotensin and bradykinin (Goodfriend, Ross & Schalch 1966).

SUMMARY AND CONCLUSIONS

Radio-immunoassays which combine the specificity of the antigen-antibody reaction with the sensitivity of radioisotope detection have provided assays for many of the peptide hormones present in plasma. These assays have been considered in relation to other immunoassays of hormones and other biologically active substances. It has been shown that all immunoassays depend upon the detection or estimation of non-antibody bound antigen, non-antigen bound antibody or the antigen-antibody complex. The technical details of the individual methods are determined by the component estimated. The estimation of a biologically active antigen may involve the estimation of the biological activity. Provided the latter is neutralized by combination with antibody the estimation of biological activity will distinguish between antibody and non-antibody bound antigen. Non-antigen bound antibody may be detected by reaction with antigen-coated red cells. The antigen-antibody complex may be estimated by the estimation of a precipitate, complement fixation or by using a labelled antigen.

The radio-immunoassay of hormones has been considered in some detail with particular reference to the production of antisera, preparation of high specific activity labelled hormone and separation of non-antibody bound hormone from antibody-bound hormone. The differences between various radio-immunoassay procedures relate mainly to differences in the methods of separation. Certain problems arising in the application of radio-immunoassays to the assay of hormones in plasma have been described. Inadequate sensitivity remains an obstacle to the assay of ACTH and parathormone under some circumstances and damage to labelled hormone during assay can produce errors in the estimation. The criteria to be applied in the assessment of the validity of results have been presented.

The range of hormone radio-immunoassays has been shown by reference to the original descriptions.

It is concluded that many radio-immunoassay procedures for the assay of hormones in plasma are firmly established. They have already allowed a rapid increase in the understanding of the physiological and pathological role of hormones and hormone-hormone interactions. Further development and wider application of the methods may be predicted with confidence.

REFERENCES

BAGSHAWE K.D., WILDE C.E. & ORR A.H. (1966) Radioimmunoassay for human chorionic gonadotrophin and luteinizing hormone. *Lancet* **i,** 1118–21.

BERSON S.A., YALOW R.S., BAUMAN S.A., ROTHSCHILD M.A. & NEWERLY K. (1956) Insulin-I^{131} metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J. clin. Invest.* **35,** 170–90.

BERSON S.A. & YALOW R.S. (1965) Some current controversies in diabetes research. *Diabetes* **14,** 549.

BERSON S.A. & YALOW R.S. (1966a) Iodoinsulin used to determine specific activity of iodine^{-131}. *Science* **152,** 205–7.

BERSON S.A. & YALOW R.S. (1966b) Parathyroid hormone in plasma in adenomatous hyperparathyroidism, uremia and bronchogenic carcinoma. *Science,* **154,** 907–9.

BRODY S. (1966) Immunoassay of human chorionic gonadotrophin. *Acta. Endocr.* **52,** 113–29.

CINADER B. (1963) Ed. Antibody to Enzymes—a three-component system. *Ann. N.Y. Acad. Sci.* **103,** 493–1154.

DEMURA H., WEST C.D., NUGENT C.A., NOKAGAWA K. & TYLER F.H. (1966) A sensitive radioimmunoassay for plasma ACTH levels. *J. Clin. Endocr.* **26,** 1297–302.

FAIMAN C. & RYAN R.J. (1966) Radioimmunoassay for human follicle stimulating hormone (FSH). *J. Lab. clin. Med.* **68,** 872.

GLOVER J.S. & SHEPHERD B.P. (1966) Determination of iodine^{-131} specific activity by protein iodination. *Nature* (*Lond.*) **212,** 285–6.

GOODFRIEND T., ROSS G.T. & SCHALCH D.S. (1966) Radioimmunoassay of angiotensin, bradykinin and α-melanocyte stimulating hormone. *Clin. Res.* **14,** 332.

GREENWOOD F.C. (1967) Immunological procedures in the assay of protein hormones. In *Modern Trends in Endocrinology,* 3rd series, p. 288, ed. H. GARDINER-HILL. London: Butterworth.

HALES C.N. & RANDLE P.J. (1963) Immunoassay of insulin with insulin-antibody precipitate. *Biochem. J.* **88,** 137.

HUNTER W.M. & GREENWOOD F.C. (1962) Preparation of iodine^{131} labelled human growth hormone of high specific activity. *Nature* **194,** 495–6.

KABAT E.A. & MAYER M.M. (1961) *Experimental Immunochemistry,* 2nd ed. Springfield, Illinois, U.S.A.: Charles C. Thomas.

LAU K.S., GOTTLIEB C.W. & HERBERT V. (1966) Preliminary report on coated charcoal immunoassay of human chorionic 'growth hormone-prolactin' and growth hormone. *Proc. Soc. exp. Biol. Med.* (1966) **123,** 126–31.

LAWRENCE A.M. (1966) Radioimmunoassayable glucagon levels in man: effects of starvation, hypoglycaemia and glucose administration. *Proc. Nat. Acad. Sci.* **55,** 316–20.

LAZARUS L. & YOUNG J.D. (1966) Radioimmunoassay of human growth hormone using ion exchange resin. *J. clin. Endocr.* **26,** 213–18.

LÉMARCHAND-BÉRAUD T. & VANNOTI A. (1965) A radioimmunoassay for the determination of thyroid stimulating hormone. *Experientia* **21,** 353–6.

MIDGLEY A.R. (1966) Radioimmunoassay: a method for human chorionic gonadotrophin and human luteinizing hormone. *Endocr.* **79,** 10–18.

MIDGLEY A.R. & JAFFE R.B. (1966) Human luteinizing hormone in serum during the menstrual cycle: determination by radioimmunoassay. *J. Clin. Endocr.* **26,** 1375–81.

MOLONEY P.J. & COVAL M. (1955) Antigenicity of insulin. Diabetes induced by specific antibodies. *Biochem. J.* **59,** 179–85.

MORGAN C.R. (1966a) Human growth hormone immunoassay: two-antibody method using I^{125} tracer. *Proc. Soc. exp. Biol. Med.* **121,** 62–4.

MORGAN C.R. (1966b) Immunoassay of human insulin and growth hormone simultaneously using I^{131} and I^{125} tracers. *Proc. Soc. exp. Biol. Med.* **123,** 230–3.

ODELL W.D., ROSS G.T. & RAYFORD P.L. (1966) Radioimmunoassay for human luteinizing hormone. *Metab.* XV, 287–9.

OLIVER G.C. (Jr), BRASFIELD D., PARKER B.M. & PARKER C.W. (1966) A sensitive radioimmunoassay for digitalis. *J. Lab. clin. Med.* **68,** 1002–3.

OLOVNIKOV A.M. & GURVICH A.E. (1966) Immunization with protein-cellulose co-polymer (immunosorbent). *Nature (Lond.)* **209,** 417–19.

POPE C.G. (1963) Development of knowledge of antitoxins. *Brit. Med. Bull.* **19,** 230–4.

POTTS J.T. (Jr), SHERWOOD L.M., O'RIORDAN J.L.H. & AURBACH G.D. (1967) *Radioimmunoassay of Polypeptide Hormones.* In *Advances in Internal Medicine,* ed. I. SNAPPER & W.E. DOCK. Vol. 13.

ROSS G.T., ODELL W.D. & GOODFRIEND T. (1966) Radioimmunoassay for alpha melanocyte stimulating hormone. *Proc. Soc. exp. Biol. Med.* **123,** 218–20.

SMITH L. (1966) Species variation in the amino acid sequence of insulin. *Amer. J. Med.* **40,** 662–6.

STAVITSKY A.B. & ARQUILLA E.R. (1953) Estimation of insulin and antibodies to insulin *in vitro* by haemagglutination and haemolysis of insulin-treated red cells and inhibition of these reactions. *Fed. Proc.* **12,** 461.

TASHJIAN A. H. (Jr), LEVINE L. & MUNSON P.L. (1964) Immunoassay of parathyroid hormone by quantitative complement fixation. *Endocrinol.* **74,** 244–54.

THOMPSON K.W. (1941) Antihormones. *Physiol. Rev.* **21,** 588–631.

WARDLAW A.C. & MOLONEY P.J. (1961) The assay of insulin with anti-insulin and mouse diaphragm. *Canad. J. Biochem. Physiol.* **39,** 695–712.

WASSERMAN P. & MIRSKY I.A. (1942) Immunological identity of insulin from various species. *Endocrinol.* **31,** 115–18.

WIDE L. & PORATH J. (1966) Radioimmunoassay of proteins with the use of Sephadex-coupled antibodies. *Biochim. Biophys. Acta,* **130,** 257–60.

WOLSTENHOLME G.E.W. & CAMERON M.P. (1962) Eds. *Immunoassay of Hormones.* Ciba Found. Colloquium on Endoc. Vol. 14. London: Churchill.

YALOW R.S. & BERSON S.A. (1959) Assay of plasma insulin in human subjects by immunological methods. *Nature (Lond.)* **184,** 1648.

YALOW R.S. (1966) Communicated to the Euratom conference on problems connected with the preparation and use of labelled proteins in tracer studies, Pisa, January, 1966.

SECTION II

PHYSIOLOGICAL SYSTEMS OPERATING IN THE ALLERGIC STATE

CHAPTER 11

BIOLOGY OF THE IMMUNE* (ALLERGIC) RESPONSE

J.F.A.P.MILLER

* In this chapter Dr Miller has used the words 'immune' 'immunity' in the broad sense acceptable to many immunologists. In many of the situations the editors, by preference, would have used the words 'allergy' and 'allergic'.

Immunological tolerance
Experimental induction of immunological tolerance: Maintenance and breakdown of immunological tolerance: Summary and conclusions.

Regulation of the Immune Response
Genetic factors: Anatomical factors: Physiological factors: Immunological factors.

INTRODUCTION

The immune system is a multicomponent organization in which each component is specialized to deal adequately with one or more of the many challenges which confront the system. The activities carried out by this organization can be broadly divided into two main groups: humoral antibody responses and sensitized cell-mediated reactions.

Humoral Antibody Responses

These responses are effected by antibody molecules or immunoglobulins which are synthesized by immunologically activated cells. There are at least five classes and several subclasses of immunoglobulins in man (Chapter 13), the three major classes being known as IgM, IgG and IgA.

The complex of cells in the IgM system is rapidly deployed and immediately turns on the production of large numbers of molecules extremely efficient in binding to particulate antigens such as bacteria and erythrocytes, but not so efficient in binding to soluble antigens such as toxins. The cells do not take part in long-continued synthesis of the antibody molecules and do not generally participate in organizing the machinery responsible for immunological memory.

Within the IgG system there is a progressive change in the quality of the antibody molecules produced, so that later in the response more efficient molecules are produced, the binding affinity between the antibody and the antigen having increased some 10,000-fold. The antibodies can bind to soluble antigens and are produced for much longer periods of time. The development of a complex responsible for persisting immunological memory is organized.

The cells of the IgA and perhaps IgE (Ishizaka *et al* 1966) system produce molecules capable of binding to skin and mucous membranes. After interaction with antigen, histamine and other pharmacological agents are released locally. It is thus possible that this system plays a role in defending exposed body surfaces against infiltration by microorganisms.

Sensitized Cell-Mediated Reactions

Immunological responses carried out by sensitized cells in the absence of circulating antibody include delayed sensitivity, transplantation immune reactions

and various autoimmune phenomena. These reactions can be successfully transferred from sensitive to normal animals by cells (generally living cells) but not by serum. The cellular systems capable of carrying out these activities may, as pointed out by Thomas (1959) and elaborated by Burnet (1965), form part of a surveillance system the function of which may be to eliminate cells arising as a result of somatic mutation, e.g. neoplastic cells.

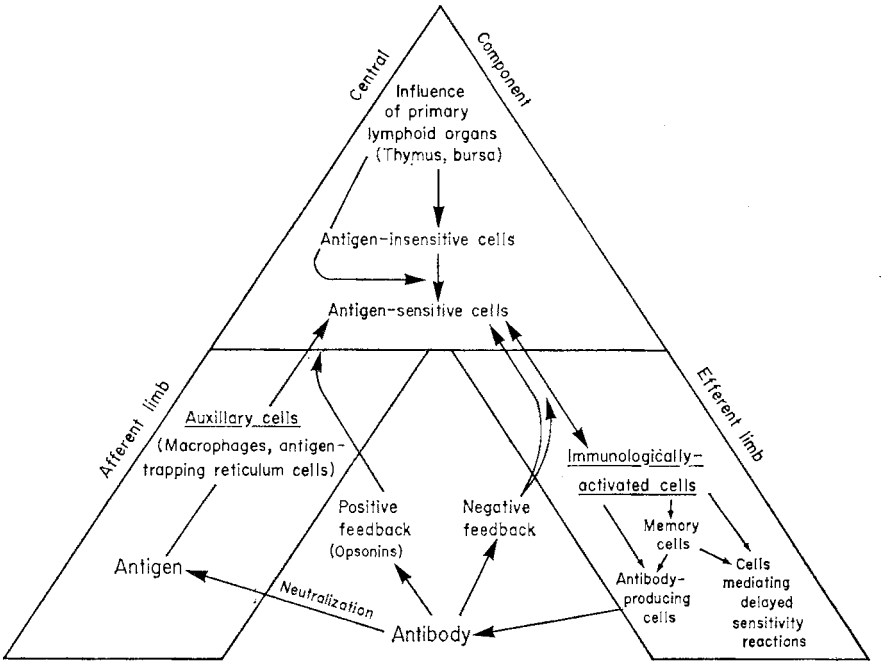


FIG. 11.1. A panoramic view of the immune response—a multitudinous series of complex events integrated in time and space in a precise orderly sequence.

COMPONENTS OF AN IMMUNE RESPONSE

An immune response represents the integration in time and space of a multitudinous series of complex events. These can be arbitrarily divided into three main groups: the afferent limb, the central component and the efferent limb (Fig. 11.1).

1. The afferent limb is concerned with the role of antigen in the inductive phase of the response. The points to be considered here are: the movement, distribution, catabolism and fate of antigen, the role of certain cell types in the 'capture' and 'processing' of antigen and the mechanism by which antigen, or processed antigen, triggers off those immunologically competent cells which are fully qualified to initiate the response to that antigen.

2. The central component deals with the population dynamics of those cells competent to respond, their origin, distribution, identity, life span, recruitment, diversity and heterogeneity.

3. The efferent limb is concerned with the fate of the responsive cells once they have been triggered off or activated by antigen, their differentiation and proliferation to effector cells and the activities of the different immune factors produced by the effector cells in regulating the immune response by acting on the afferent limb (e.g. increased selective localization of antigen by opsonins) and on the central component and efferent limb (feedback inhibition of antigen-triggered immunologically competent cells).

THE ROLE OF ANTIGEN

Antigen is the stimulus which impinges upon a responsive cell and triggers off in it a complex series of events—differentiation, proliferation and synthesis of specific antibodies. This relationship between the stimulus and the response immediately raises many questions. Does the antigen instruct the cell to carry out its new synthetic activities and, if so, how are these instructions delivered? Alternatively, does the antigen act as an inducer bringing out a potentiality latent in the responding cell? These questions have not been answered but they have been responsible for the formulation of a multitude of mutually contradictory theories concerning the cellular basis of the immune response (reviewed by Fischer 1964). The recapitulation of these theories in detail would serve little useful purpose. In brief, they can be divided into two groups: instructive and elective. In the former theories, specific antibody synthesis represents a unique modification of the synthesis of natural proteins for which information is supplied by the antigen injected. Such mechanisms as may be involved seem untenable in the light of present knowledge of the genetic control of protein synthesis gained from the science of molecular biology. In elective theories, all the genetic information necessary for subsidizing the production of antibodies to all possible antigenic determinants is resident in the responsive cells. The genetic diversity for this information may be present in the zygote (having been acquired by a process of germline mutation during evolution) or it may arise *de novo* by somatic mutation in each individual. The responsive cells may be either multipotential, i.e. possess the capacity to respond to any antigen or, as postulated by the clonal selection theory (Burnet 1959), they may be clonally individuated, each clone being restricted in its capacity to respond to one or only a few of the possible antigenic determinants.

Many other questions concerning the relationship between the antigen and the response may be raised. How is the triggering action brought about? Must the antigen be processed to an appropriate molecular form? Do the molecules of antigen act on the cell membrane by distorting some specific

'sentinel site' which then fires off an appropriate operon in the genetic apparatus of the nucleus? Or do the antigenic molecules penetrate the cell membrane to act at some intracytoplasmic or intranuclear site? Must the antigen persist at some critical site in the new generations of antibody-forming cells? A precise and detailed knowledge of the cellular and subcellular distribution of antigen and of its processing or catabolism during the inductive phase of the immune response appears to be necessary if we are to attempt to answer some of the above questions.

Movement and Distribution of Antigen

Various systems to study antigen distribution have been developed. A useful one is the flagellar antigen system studied by Nossal and his colleagues (reviewed by Nossal & Abbot, 1966). A single low dose (e.g. 10 μg) of flagellar antigens will induce antibody formation uniformly without the use of adjuvants. The antigens can be labelled with carrier-free radioactive iodine (I^{125}): when injected subcutaneously enough antigen reaches and persists in the draining lymph node to provide heavy labelling visible both by light and electronmicroscopic autoradiography. When injected intravenously, similar considerations hold for the spleen.

Localization Patterns in the Lymph Nodes

In the lymph nodes, antigen was localized predominantly in two sites: in the macrophages of the medullary sinuses and in the lymphoid follicles in the cortex. (These follicles, in unstimulated animals, consist essentially of lymphocytes, reticular cells and a few 'tingible' body macrophages.) The heavy labelling in electronmicroscopic autoradiographs was associated in the follicles, with the cell membrane of fine reticular processes of 'dendritic' reticular cells. The cytoplasm of these cell processes was as a rule featureless and only occasionally contained a few mitochondria, lysosomes and protolysosomes in marked contrast to the cytoplasm of the macrophages in the medullary sinuses. Small lymphocytes in the follicles were usually free from label but when clustered around heavy antigen depot an occasional lymphocyte had small amounts of label in its nucleus.

Localization Patterns in the Spleen

After an intravenous injection of labelled flagellar antigen, a highly characteristic sequential movement pattern of label was observed. In a few minutes, label was seen scattered diffusely throughout the red pulp and the marginal zone and then was found in the white pulp. After one hour, the label was mostly concentrated in the marginal zone and after two hours, it moved across the marginal sinus into the white pulp where it was localized in the lymphoid

follicles among the cuff of small lymphocytes on the outer aspect of the germinal centre. It never penetrated the area of white pulp immediately surrounding the central arteriole. By four hours, the label had virtually disappeared from the red pulp and the marginal zone and was strictly localized to the germinal centre cap region. These sequential changes are thought to result from the purposeful movement of discrete antigen-carrying cells.

Different Localization Patterns Among Different Antigens

Medullary macrophages ingested all antigens, strong or weak. Minimally antigenic materials such as insulin or gelatin showed no detectable follicular localization. Moderately antigenic materials as heterologous serum proteins or ferritin showed some follicular localization but this was not as intense as that obtained with maximally antigenic proteins such as flagella. Hence the more immunogenic a protein the higher was the intensity of follicular localization.

Effects of Pre-immunization on Antigen Localization

Active or passive immunization caused a marked acceleration of the process of follicular localization. An increase in the intensity of this localization was seen only with antigens weaker than flagellar antigens. Pre-immunization did not increase antigen localization in the medullary macrophages. The results of pre-immunization are consistent with the notion that opsonic factors play a major role in the follicular antigen trapping mechanism.

Presence of Antigen in the Developing Antibody-Forming Cells

Antibody-producing blasts, could be detected in single cell suspensions prepared from lymph nodes 3 days after antigen challenge. The antigen content of these blasts and their progeny, the primitive and mature plasma cells, was examined by autoradiography of single cells. Some lymph node blasts and mature plasma cells in the IgM phase, contained labelled antigen either as a tuft over the cell surface or as a depot over or in the nucleus. The proportion of such labelled cells was higher in the early part of the response to a second challenge with the antigen. However, no antigen was ever found in antibody-producing blasts, harvested from the thoracic duct lymph. Furthermore, in only a minority of the plasma cells at the height of the IgG response was macromolecular labelled antigen detectable. One conclusion is clear: the fact that numerous plasma cells were unlabelled suggests that antigen does not persist in the progeny of the early antibody-producing blasts. Antibody specificity is thus not acquired through the continuing persistence of antigen acting as a direct template to guide the folding of the globulin chains. The presence of antigen tufts or depots in some

of the cells in the early phase of the response may be an epiphenomenon bearing no relationship to the induction of antibody formation. On the other hand, the possibility has not been excluded that all blasts may have contained a critical concentration of antigen early in the inductive phase, this concentration having fallen to below the threshold of detectability when the first antibody-producing blasts were examined by autoradiography.

Cells Involved in Antigen Capture and Processing

Frequent associations between antibody-producing cells and antigen-capturing cells, have been noted. The work on antigen localization indicates that lymphocytes can become intimately associated with the antigen-loaded dendritic processes of follicular reticular cells. Lymphocytes have been observed adhering to the surface of macrophages isolated from a regionally stimulated lymph node, not from the contralateral site. Cytoplasmic bridges between lymphocytes and macrophages in immunized lymph nodes have been described in one report. These cellular associations, together with the finding that antibody-producing cells need not contain antigen and that they are not derived from phagocytic cells, suggest that the induction of an immune response requires the concerted action of two cell types: one which processes the antigen and one which is triggered off by the processed antigen. It is not known, however, whether this generalization can be applied to all classes of immunological responses.

Role of the Macrophage

After entrance in the circulation or lymph, antigen is taken up by phagocytic cells of the reticuloendothelial system. The cell membrane of the macrophage becomes invaginated to create a phagocytotic vacuole or phagosome. Golgi vesicles or protolysosomes cluster around the phagosome and fuse with it. Next, lysosomes discharge their enzyme contents into the phagosome creating a complex phagolysosome. Digestion takes place and the antigen is catabolized into smaller fragments which become associated with ribosomes (Garvey & Campbell 1957). Finally, telolysosomes are found in which digestive enzymes are conserved and able to act again in another phagocytotic cycle (Gordon *et al* 1965). In immunized animals, phagocytosis is facilitated by opsonins.

The purpose of antigen phagocytosis may be several fold:

1. Antigen concentration may have to be reduced to subparalytic levels to prevent paralysis of cells capable of responding immunologically. This will be discussed later under the heading of 'Immunological Tolerance'.

2. Catabolism of antigen may be an essential step for immunization. Consistent with this notion are the following observations: (*a*) different preparations of one antigenic protein which have the same immunological specificity can

influence the chronology of the sequential pattern of the types of antibodies produced (Uhr 1964), (*b*) amino acid polymers, must be metabolized by cells in order to be antigenic (Maurer 1963), (*c*) only living macrophages are capable of processing antigens in such a way as to induce antibody production in responsive cells. Killed macrophages containing antigens do not induce immunity. Furthermore, responsive cells can be triggered off only if they are exposed to living macrophages before a critical period of time (6 hours) has elapsed after the macrophages have ingested the antigens. No induction of antibody production takes place when the responsive cells are exposed to macrophages after this period (McCulloch 1967).

Hence, phagocytosis of antigen appears to be required in order to provide a highly effective immunogenic stimulus to the responding cell.

3. There may be a transfer of specific information between the macrophage and the immunologically competent cell. Fishman & Adler (1963) have shown that an extract of bacteriophage antigen-containing peritoneal macrophages could induce antibody formation in a suspension of lymph node cells cultured *in vitro*. The extracted material was inactivated by ribonuclease. Other workers (Friedman, 1964; Cohen & Parks 1964) have shown that RNA prepared from immunized cells could transfer immune capacity to non-immune cells. Whether such a transfer takes place *in vivo* is unknown. It has been hypothesized that the cytoplasmic bridges sometimes observed between lymphocytes and macrophages could provide a mechanism for the transfer of immunogenic complexes (Schoenberg *et al* 1964). One interpretation of these experiments is that a specific RNA, free of antigenic determinants, 'instructed' lymphoid cells to synthesize antibody. If this is true, it has important implications with respect to the cell type acting as a 'recognition agent': if antigen-free RNA (containing the instructions for the synthesis of specific antibodies) is transferred from macrophage to responsive cell, the latter may play only a passive role. Experiments with populations of lymphocytes specifically tolerant to an antigen have indicated, however, that the specific reactivity must lie in the lymphocytes (Gowans & McGregor 1965). This is more consistent with the view that the lymphocytes act as recognition agents, the macrophages simply providing them with an effective antigenic stimulus. In fact, some experiments have shown that the RNase-sensitive material consists of a complex of RNA with some residue containing antigenic determinants and that this complex is more immunogenic than is the native antigen (Askonas & Rhodes 1965).

Role of the Dendritic Reticulum Cell

Nossal has drawn attention to the differences in the subcellular distribution of antigen in the follicles and elsewhere. Phagocytosis of antigen in the medulla appears similar to the process which occurs in reticuloendothelial cells all over the body, for instance, the Kupffer cells of the liver. On the other hand, follicular

localization exhibits some unique features. The antigen is captured by specialized dendritic macrophages and is retained in high concentration on the surface of the cell membrane of the fine processes of these cells. The scarcity of cytoplasmic inclusions such as lysosomes or protolysosomes in the processes of these cells suggests that antigen may not be subjected to digestion as in the phagocytes of the medulla, but may persist undenatured in a situation which is highly accessible to any adjacent lymphoid cells. This arrangement may have some special significance in the triggering of potential antibody-producing cells during certain stages of the immune response.

From phylogenetic studies on the toad (Diener & Nossal 1966) and ontogenetic studies on rat (Williams & Nossal 1966) it is known that IgM immunoglobulin synthesis can take place in the absence of the follicular dendritic antigen-retaining web. Furthermore, antibody production to horse gammaglobulin is unimpaired in rabbits whose follicles have been destroyed by X-irradiation (Keuning *et al* 1963). It seems therefore that follicular localization may not be an essential step for the most primitive type of immune response (IgM synthesis) but may be a necessary one for the development of the secondary immune response and immunological memory.

Role of the Small Lymphocytes

No role has yet been assigned to macrophages in the processing of antigen for the induction of transplantation immunity. The interaction between a responsive cell and antigen may occur within the graft (Medawar 1958). When thoracic duct lymphocytes were perfused through the kidney of a foreign recipient they became capable of conferring upon their donor sensitivity to a skin homograft from the foreign recipient (Strober & Gowans 1965). It seems, therefore, that an interaction between blood-borne lymphocytes and homograft antigens is possible but the mechanism by which this is achieved is not clear.

IMMUNOLOGICALLY COMPETENT CELLS

The term immunological competence refers to the present status of a cell which, although not actually engaged in an immunological response, is nevertheless fully qualified to undertake such a response when appropriately stimulated by antigen. Immunologically *competent* cells are thus distinguished from immunologically *activated* cells which carry out the response. In the words of Sir Peter Medawar (1963a) 'to describe an immunologically activated cell, a cell actually doing something, as immunologically competent strikes me as supererogatory, like describing an aircraft already overhead as competent to engage in flight'. This distinction between competence and actual performance is important: as far as performance is concerned the evidence is that an antibody producing cell (or immunologically *activated* cell) can produce, in general, no more than one

antibody and rarely two. It does not necessarily follow that the precursor of this cell—the immunologically *competent* cell—must itself be restricted in its capacity to respond to only one, or at the most, two antigenic determinants.

The term 'antigen-sensitive cell' or 'antigen-responsive cell' has been used to refer to those cells which respond to antigen not by the formation of antibody but by the formation of a large number of antibody-producing cells through repeated cell divisions associated with progressive differentiation (Kennedy *et al* 1966). The evidence that antigen-sensitive cells are not antibody-producing cells is clear from experiments in which normal mouse spleen cells were transferred together with sheep erythrocytes into heavily irradiated mice. Individual haemolysin-producing cells present in a population of spleen cells can be detected by plating the cells in agar together with sheep erythrocytes and incubating them with complement. Each cell producing antibody will release some of it into the surrounding agar lysing the erythrocytes and causing the formation of a plaque (Jerne & Nordin 1963). Estimations have shown that there were not more than 100 haemolysin-producing cells or plaque-forming cells in the spleens (one spleen has from 1 to 2×10^8 nucleated cells) of normal unimmunized animals and that pre-irradiation with doses as high as 700 r did not decrease this 'background' count of plaque-forming cells. When normal spleen cells and sheep erythrocytes were injected into lethally irradiated mice there appeared within 4 to 5 days a number of clusters of haemolysin-producing cells in the spleens of the recipients. These clusters could be detected by their ability to produce large foci of haemolysis in a layer of agar containing sheep red cells. The relationship between the number of nucleated cells injected and the average number of haemolytic foci that resulted was a straight line extrapolating to the origin. Each cluster therefore resulted from the localization in the spleen of a single entity (a single cell) which was randomly distributed in the injected cell population and which was capable of responding to the antigen by proliferating to form a progeny of haemolysin-producing cells. By using this method, it was estimated that the average normal spleen contains about 1000 such cells sensitive to antigen (Kennedy *et al* 1966; Playfair *et al* 1965). Since the average spleen of unstimulated mice contains no more than 100 plaque-forming cells, it can be concluded that the majority, if not all of the 1000 antigen-sensitive cells do not themselves produce antibody. Strictly speaking, therefore, antigen-sensitive cells or antigen-responsive cells are a class of immunologically competent cells.

The Small Lymphocytes

Round cells with a high nucleocytoplasmic ratio and very scanty cytoplasm, measuring 5–8 μ in diameter in smears, qualify morphologically for the name 'small lymphocyte'. They are the major cellular constituents of lymphoid tissues. They are widely distributed throughout the body but are found particularly in lymph nodes, in the gut-associated lymphoid tissues (tonsils, adenoids,

appendix, Peyer's patches), spleen, thymus and bone marrow. They comprise a large percentage of the leucocytes normally found in blood and peritoneal fluid and 95% of the lymphocytes in the major lymph channels. There is now abundant experimental evidence to indicate that small lymphocytes as defined by size and morphology are a heterogenous collection of cells with different origins, functions, fates and life spans. For convenience, small lymphocytes can be grouped into various compartments (Fig. 11.2), but it must be borne in mind that a functional heterogeneity exists even in the cells within any one compartment.

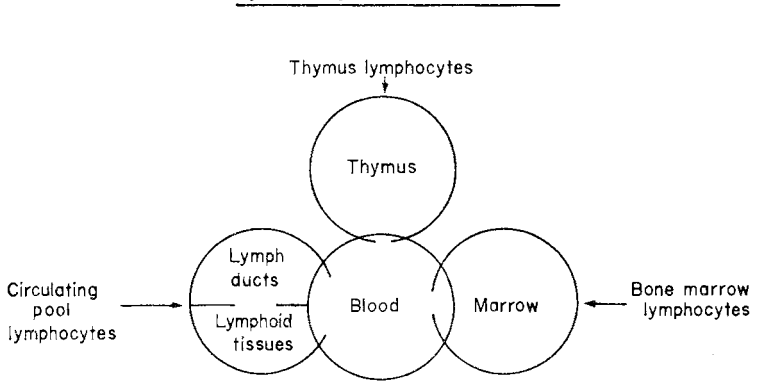


FIG. 11.2. Lymphocyte compartments.
Lymphocytes are distributed widely throughout the body and circulate in the blood and lymph. They are heterogeneous in origin, function and life span and may be grouped, for the sake of convenience, into various 'compartments' as shown here.

BONE MARROW LYMPHOCYTES

In some species, notably the rodents, lymphocytes form a considerable proportion of the cells in bone marrow. Autoradiographic data has indicated that many of these cells are formed *in situ* in the marrow, the entire marrow small lymphocyte population being renewed in 3 days or less. Lymphocytopoiesis in the marrow thus occurs on a much more extensive scale than had hitherto been considered. Significant numbers of marrow small lymphocytes migrate out of the marrow into the blood stream by passing between the cells of the sinusoidal endothelium (not through the cells as is the case in the post-capillary venule of the lymph nodes). (Yoffey *et al* 1965).

Populations of marrow cells containing small lymphocytes have in many experimental systems failed to produce any evidence for a direct participation of the marrow small lymphocyte in immune responses. The marrow, however, does contain cells capable of repopulating the thymus, spleen and lymph nodes,

as well as the myeloid centres of lethally irradiated animals (Ford *et al* 1956; Ford & Micklem 1963). A relationship has been established between marrow small lymphocytes and erythropoiesis though not with granulocytopoiesis. In other experiments, it was shown that the marrow was an important source of small round cells which emerged from the blood in sites of acute inflammation and gave rise to macrophages at these sites. These experiments hint at the possibility that bone marrow small lymphocytes may function as haemopoietic stem cells, as originally postulated by Maximow (1909) and upheld by Yoffey (1952). Since, however, haemopoietic stem cells form only about 0.1% of the nucleated cells in bone marrow, any attempt to correlate stem cell capacity with a particular morphological cell type, must remain inferential, until methods become available to increase considerably the proportion of stem cells in a given population.

Thymus Lymphocytes

The thymus is the organ with the highest rate of production of small lymphocytes. Although it makes up only 10–20% of the total lymphoid mass in the mouse, it produces some 50–70% of all the new lymphocytes. Radioautographic data in mice has indicated (*a*) that small lymphocytes arise from the asymmetrical division of primitive lymphoid cells (probably the medium lymphocytes), (*b*) that lymphoid cell division is very rapid (large lymphocytes 6.8 hr, medium lymphocytes 8.2 hr) but probably no more rapid than the rate of division of cells in lymph nodes under conditions of maximal antigenic stimulation, (*c*) that the mean intrathymic life span of the majority (90–95%) of small lymphocytes is 3–4 days. The slower rate of production of small lymphocytes in lymph nodes and spleen is probably due to the smaller percentage of primitive cells in these tissues (Metcalf 1966). The most outstanding feature of thymus lymphopoiesis is that, unlike lymphocyte production in lymph nodes and spleen, it is independent of antigenic stimulation, e.g. it is as intense in the thymus of the germfree animal as in that of the conventional animal (Gordon 1959). The pattern of lymphopoiesis is the same in the normal thymus as in thymus implants and it is not affected by the amount of thymus tissue present in the host animal (e.g. after thymus grafting and after partial thymectomy) nor by the age of the host. Thymus lymphopoiesis, therefore, appears to be intrinsically regulated—presumably by the epithelial elements of the thymus (Metcalf 1964).

The primitive lymphoid cells of the thymus may originate either from cells migrating into the thymus from the circulation or from intrinsic cells. There is some evidence that during embryonic life epithelial cells may differentiate to thymus lymphocytes. However, in postnatal life, there is unequivocal evidence that the thymus receives cells from the circulation which subsequently behave in the thymus as typical thymus lymphocytes. Nothing, however, is known as to the identity of the cell type which migrates into the thymus.

What is the fate of the lymphocytes produced in the thymus? Theoretically they can die within the organ or emigrate. Evidence of migration of thymus lymphocytes has come from various studies employing labelling with tritiated thymidine or with chromosome markers. These studies indicate that only a few of the cells produced in the thymus of the adult rodent seed the host tissues and hence that the vast majority of thymus small lymphocytes are destroyed within the organ after a short life span of a few (3) days. This raises major questions such as what are the functions of the short-lived lymphocytes and what is the purpose of most of the intense thymus lymphopoiesis? These questions still remain unanswered. It has been suggested that short-lived thymus lymphocytes may act as trephocytes giving up their DNA and perhaps other nutrients to newly-formed cells. However, all attempts to establish a trophic function for thymus lymphocytes have been unsuccessful (e.g. Metcalf 1964).

The few cells which migrate out of the thymus may conceivably be those with a long-life span and may represent an important contribution to the pool of small lymphocytes outside the thymus. Their exact functions are unknown. Suspensions of thymic lymphocytes, however, can perform many of the same immunological reactions as suspensions of lymphocytes from lymph nodes, spleen and thoracic duct lymph, although they are quantitatively much less efficient. These quantitative differences may depend on the presence in the thymus of a much larger population of immunologically immature cells than is the case in the other lymphoid tissues.

In summary, the available evidence indicates that the thymus recruits cells from the bone marrow via the blood stream and that these cells are precursors for the greater part of the lymphopoiesis which occurs within the thymus. A large proportion of the small lymphocytes produced in the organ are short-lived and die without emigrating. Their function is unknown and it has been surmised that they act as trephocytes. A small proportion of the thymus small lymphocytes are long-lived and may be exported from the organ thus contributing to the pool of small lymphocytes outside the thymus (Fig. 11.3).

Circulating Pool Lymphocytes

In contrast to the situation in bone marrow, none of the lymphocytes found in lymph nodes and in lymphatic channels can function as general haemopoietic stem cells. The small lymphocytes in thoracic duct lymph are neither newly-formed cells nor end cells but recirculating cells (Gowans and Knight 1964). After entering the blood stream from the thoracic duct, these cells migrate through the endothelial cells of the post-capillary venules to enter the lymph nodes, traverse the lymphocytic fields to leave via the efferent lymphatics eventually to reach the thoracic duct and hence the blood. They pass through the periarteriolar lymphocyte sheaths of the spleen but do not normally enter the thymus. For convenience, they may be referred to as cells of the circulating

pool. The majority (95%) of these cells have a long life span, of the order of months in rodents (Little *et al* 1962) and years in man (Buckton & Pike 1964). Their function is in the mediation of immune responses.

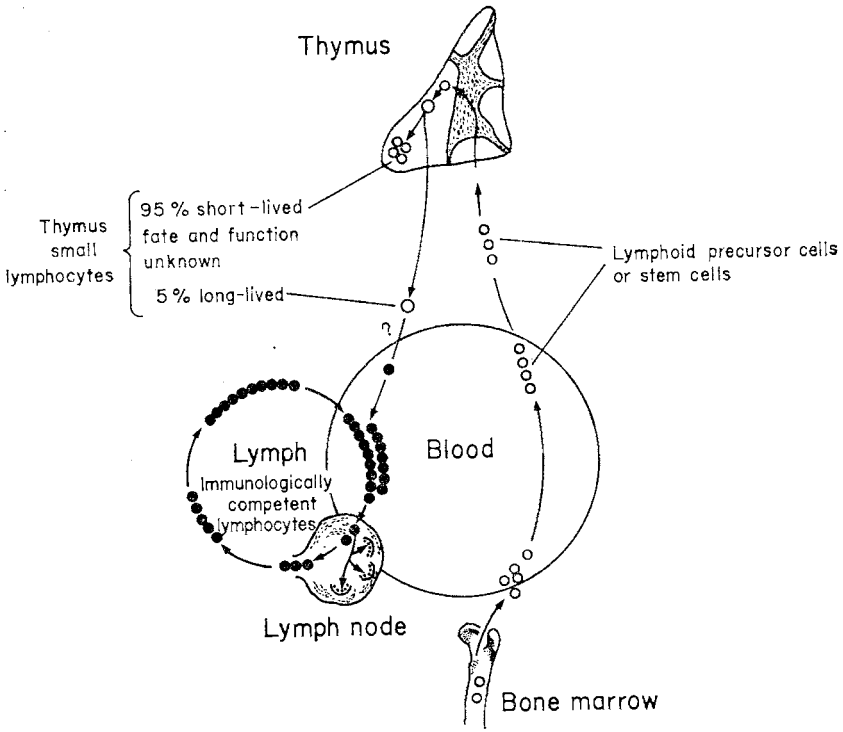


FIG. 11.3. Cellular migration streams in the lymphoid system.
The stem cell precursors of lymphoid cells probably originate in bone marrow and migrate via the blood stream to the thymus where they differentiate to thymus lymphocytes. Ninety-five per cent of these cells are short-lived and their fate and functions are unknown. They probably die within the organ without emigrating. About 5% of the small thymus lymphocytes are long-lived and may possibly emigrate to join the pool of cells outside the thymus.

Evidence Linking Circulating Pool Lymphocytes to Immunity

There is considerable evidence, both direct and indirect, that the lymphocytes of the circulating pool are immunologically competent cells.

Immunological processes of all types appear to be limited to vertebrates. The hagfish, the most primitive representative of the living vertebrates, exhibits none of the characteristic vertebrate immune responses and has no organized

lymphoid tissue and no recognizable thymus tissue. The primitive cyclostome fishes, the lampreys, have some lymphoid follicles and an epithelial thymus with some lymphoid cells and are capable of producing feeble antibody responses. In all the more highly developed fishes and higher vertebrates, the thymus differentiates from a primary epithelial rudiment to a lymphoid structure, the spleen shows organized lymphoid nodules and all types of immune responses can be effected. These phylogenetic studies suggest that the development of the immune mechanism was dependent on the evolution of the thymus and organized lymphoid structures (Good & Papermaster 1964).

The time at which a developing vertebrate can first react immunologically coincides with the period at which lymphocytes can first be identified in the circulation (reviewed by Miller 1966). For example, tadpoles develop the capacity for rejecting skin grafts at the very time (40 to 50 days post-hatching) when small lymphocytes make their appearance and the immature opossum in the pouch can produce antibody as soon as, but not before, lymphocytes can be seen in the blood. The development of the capacity to effect immune reactions thus coincides, both in phylogeny and ontogeny, with the appearance of circulating lymphocytes and organized lymphoid structures.

A variety of procedures is available to deplete animals of lymphocytes (reviewed by Gowans & McGregor 1965). Included among these are thymectomy (*vide infra*), draining away lymph for several days by a thoracic duct fistula, exposure to ionizing irradiation and the administration of chemical agents (e.g. cortisone) which damage or kill lymphocytes. Such lymphocyte-depleted animals have an impaired capacity to undertake primary immune responses—both reactions against grafts and humoral antibody responses. Immunological capability can be restored to depleted animals by injecting lymphoid cells and by inocula consisting almost exclusively of small lymphocytes. The fact that normal lymphoid cells can correct immunological defects provides only circumstantial evidence for the complicity of lymphocytes as immunologically competent cells capable of initiating immune reactions. The lymphocyte-depleted animal may have lost its power to respond, not through the loss of immunologically competent cells, but through the loss of cells serving some non-specific trophic or other ancillary function in the immune response. The fact, however, that lymphocytes from rats made immunologically tolerant to sheep erythrocytes (in contrast to lymphocytes from normal rats) failed to restore the response of X-irradiated rats to sheep erythrocytes, strongly suggests that lymphocytes mediate immune reactions by acting as immunologically competent cells rather than as trephocytes or other type of auxilliary cells. Vinblastine, a mitotic poison which exerts its killing effect at mitosis, is a potent inhibitor of the primary immune response when given after, but not before, antigen (Syeklocha *et al* 1966). Antigen-sensitive cells are thus non-proliferating cells and may well belong to the class of 'circulating pool' small lymphocytes.

There is as yet no direct evidence that the small lymphocyte is the ancestor of the antibody-producing plasma cell but the indirect evidence that it may be is very strong (*vide supra*). There is, however, direct evidence that the immunologically competent cells in the thoracic duct lymph and blood of rodents, which can initiate transplantation immune reactions, such as graft-versus-host reactions and skin homograft rejection, are the 'circulating pool' small lymphocytes.

TABLE II.1

Differences between primary and secondary lymphoid organs

	Primary Lymphoid Organs	Secondary Lymphoid Organs
Organs	Thymus (all vertebrates) Bursa of Fabricius (birds) ? Peyer's patches (mammals)	Lymph nodes Spleen Some of the lymphoid tissue along the alimentary canal
Origin of anlage	Ecto-endodermal junction	Mesoderm
Lymphoid Development	Early embryonic (or larval) life	Late in foetal life or after birth
Persistence	Involute in adult life	Persists throughout life
Lymphocytopoiesis	Marked; independent of antigenic stimulation	Low; totally dependent upon antigenic stimulation
Plasmacytopoiesis and germinal centre formation	Little or none even after antigenic challenge	Characteristically occurs after antigenic challenge
Effect of early removal	1. Deficient 'circulating pool' lymphocytes 2. Early defects in immune capacity	Insignificant
Effect of late removal	1. Fall in population of 'circulating pool' lymphocytes 2. Late defects in immune capacity (after months or years)	Inconsistent and usually not severe. Some immune defects occur depending upon experimental system used

Radioautographic studies have shown that under the impact of foreign histocompatibility antigens, a fraction of the cells present in inocula rich in small lymphocytes enlarged to pyroninophilic cells: these subsequently divided to produce a progeny of lymphoid cells of progressively decreasing size. The sequence small lymphocyte → large pyroninophilic cell → large and medium lymphoid cells → small lymphocytes suggests the existence of two functional

classes of small lymphocytes: the former are uncommitted, immunologically competent cells potentially capable of initiating a response; the latter are committed immunologically activated effector cells possessing the machinery for carrying out a specific type of response (Gowans & McGregor 1965). A transformation of small lymphocytes into large 'blast' cells can be induced by phytohaemagglutinin and certain antigens in tissue culture; it is not known, however, to what extent an immunological reaction is involved in this *in vitro* system.

The Role of the Thymus and Bursa in the Genesis of Immunologically Competent Cells

Experimental work performed in recent years and reviewed below and elsewhere in greater detail (Miller & Osoba 1967) has indicated that the thymus and analogous organs, such as the bursa of Fabricius in birds, play a crucial role in the normal development of cells that will subsequently be called upon to participate in immune reactions. The thymus and bursa differ from other lymphoid organs in their origin and structure, in their reaction to antigenic stimuli and in the effect of their removal (Table 11.1). The anatomical and functional defects occurring in the lymphoid tissues following the early removal of the thymus and bursa have suggested that there are primary lymphoid organs that control the development of cells found in other lymphoid tissues.

Effects of Thymectomy and Bursectomy

In general, the effects of thymectomy on the immune system can be summarized as follows:

1. Fall in the population of some classes of small lymphocytes.
2. Defects in the capacity to produce some types of immune responses.
3. Increased susceptibility to infections.

The thymus exerts some influence on the level of circulating small lymphocytes. Thymectomy is followed by a fall in the number of small lymphocytes in the blood and a decline in the population of these cells in the lymphoid tissues but generally not in the bone marrow. In the lymphoid tissues of neonatally thymectomized mice, the areas depleted of lymphocytes correspond to the areas traversed by the cells of the circulating pool, *viz.* the lymphocytic fields in the lymph nodes and the periarteriolar lymphocyte sheaths in the spleen. In fact, neonatal thymectomy depresses the population of cells in the circulating pool to a very marked extent. Thus, when the thoracic duct of 6-week-old mice thymectomized at birth was cannulated, the output of lymphocytes during the first 24 to 48 hours of drainage was only 1% of that of neonatally sham-thymectomized control mice of the same age and strain (Fig. 11.4). Furthermore, the percentage of small lymphocytes in the lymph was less in the thymectomized mice than in the controls. There was therefore both a reduction in the

numbers of circulating small lymphocytes and an increase in the ratio of large to small cells. In contrast to the lack of small lymphocytes, plasma cells are not deficient in thymectomized animals.

Since circulating small lymphocytes are immunologically competent cells, it can be expected that thymectomized animals show defects in immune performance. This was found to be the case and in general it can be said that thymectomy impaired immune reactions mediated by small lymphocytes such as transplantation immune reactions and delayed sensitivity reactions. The humoral antibody

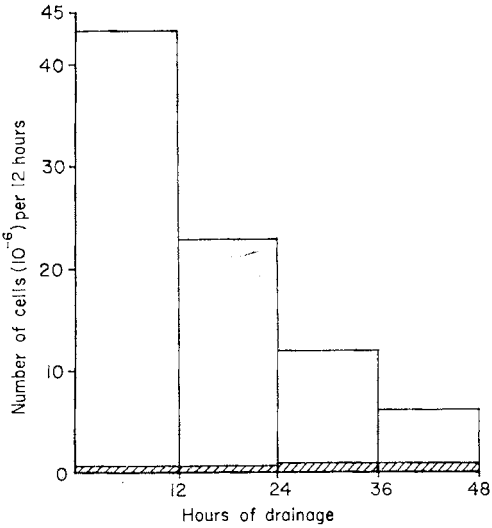


FIG. 11.4. Output of thoracic duct lymphocytes from young mice thymectomized and sham-thymectomized at birth.
The number of lymphocytes drained from the thoracic duct at 12-hourly intervals is shown in these histograms: unshaded blocks represent values from control mice; shaded blocks represent values from neonatally thymectomized mice. (From Mitchell G.F. & Miller J.F.A.P., 1967).

responses to many antigens (with the exception of a few antigens such as sheep erythrocytes, salmonella flagella, etc. in the mouse or rat) were less affected and immunoglobulin production was not impaired. These responses are mediated by plasma cells, which are not deficient in thymectomized animals. There are, therefore, two systems: one, thymus-dependent, composed of the thymus and circulating small lymphocytes and associated with the expression of cellular immunity as in delayed sensitivity and transplantation immune reactions; the other, thymus-independent, composed of plasma cells and follicles and associated with the production of immunoglobulins and humoral antibodies

(Fig. 11.5). The most impressive evidence for the existence of this thymus-independent system comes from work in chickens. If the bursa of Fabricius, an organ analogous to the thymus but situated near the cloaca, is removed, immediately after hatching, or if its development *in ovo* is suppressed by the injection of nortestosterone, the bursaless chickens are unable to produce normal antibody reponses. The immunological defects of bursaless chickens can be accentuated by sublethal irradiation. All chickens without a bursa lack IgG and when bursectomy is followed by sublethal irradiation the birds also lack IgM. The equivalent of the bursa has not yet been found in mammals. It has been suggested that some of the gut-associated lymphoid tissues such as the Peyer's patches and appendix

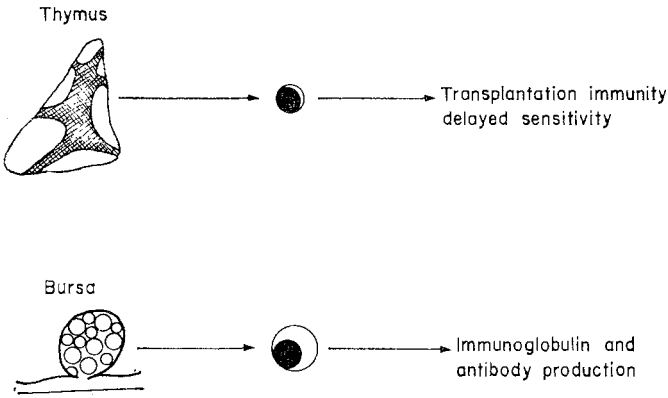


FIG. 11.5. Dissociation of immunological responsiveness.
The thymus is mainly responsible for the development of the 'circulating pool' small lymphocytes, which are competent to initiate cell-mediated immune responses. The bursa of Fabricius in birds (and its mammalian equivalent yet unidentified) is responsible for the development of some antigen-sensitive cells capable of responding to antigen by proliferation and progressive differentiation to antibody producing cells which synthesize immunoglobulins and specific antibodies.

may function as the equivalent of the bursa but this has not been proved unequivocally.

The extent of the immunological defects of thymectomized animals depends on factors such as the age at thymectomy, the age at challenge, and the species and strain of animals used. The majority of neonatally thymectomized mice of many strains failed to reject foreign skin grafts when grafted from 3 days to 5 weeks after thymectomy. In some cases, skin grafts from distantly related donors and even from rats failed to be rejected. The grafts grew luxuriant tufts of hair and many persisted indefinitely. Similar observations have been made in other species such as rats and hamsters. Neonatal thymectomy in mice was also

associated with an impairment of the capacity to produce cells in the spleen capable of haemolysing sheep red cells ('plaque-forming cells'). This impairment was extreme when neonatally thymectomized mice were challenged during the first 3 weeks of life. When challenged later, the mice were capable of giving some response although the magnitude of the response was less than that of control sham-thymectomized mice. These results suggest that neonatally thymectomized mice can develop immunological capacity with respect to sheep red cell antigens, but this development is delayed. Whether this is dependent on a system other than that governed by the thymus is not known.

Thymectomy in adult life has exerted no immediate significant defects on humoral antibody production nor on the capacity of the animals to perform transplantation immune reactions. However, the adult thymus still continues to influence immune capacity. Thus, for instance, when the lymphoid system is damaged or destroyed by ionizing irradiation, the anatomical and functional regeneration of this system was in part thymus–dependent. Furthermore, adult thymectomy by itself, was followed 6 to 9 months later by an impaired capacity to respond to a new antigen. Presumably the adult thymus influences the development of an adequate population of long-lived immunologically competent small lymphocytes. Only when this pool has been depleted, as a result of the limited life-span of its cells, do defects in immune capacity become apparent.

In some strains of mice neonatal thymectomy was followed from 1 to 3 months later by the development of a wasting disease very similar to the wasting syndromes which occur in graft-versus-host conditions. Evidence of reticulo-endothelial cell hyperplasia and necrotic lesions in the liver and haemopoietic tissues was seen in these mice. Furthermore, thymectomized mice were more susceptible to infection by hepatotrophic viruses. Autoimmune phenomena, such as the development of a positive Coombs test were found in thymectomized mice, but it is not clear to what extent these occur as a result of environmental influence (? viruses) to which the mice, because of their immunological deficiencies, are susceptible. Although wasting syndromes did occur after graft-versus-host reactions in germfree mice, neither wasting nor proliferative and destructive lesions were found in neonatally thymectomized germfree mice. Immune defects were evident in the germfree thymectomized mice although the extent of the impairment was less than that of conventional thymectomized mice. These studies indicate that the susceptibility of neonatally thymectomized mice to infection is increased.

Reconstitution of Thymectomized and Bursectomized Animals

A suspension of spleen or lymph node cells was effective in restoring immune mechanisms and preventing wasting disease when injected into recipients of the

same strain within a week after neonatal thymectomy. Discriminant spleen assays performed in such mice showed that the restoration of immune capacity was attributable to the donor cells.

Many investigators have found that intravenously injected thymus cells are much less effective than the same number of spleen and lymph node cells in correcting the immunological defects of neonatally thymectomized mice. Only huge numbers of thymus cells could restore immunological capacity. Whether this reflects a lower percentage of immunologically competent cells in the population of thymus lymphocytes, the short life span of a large proportion of this population (*vide supra*), or differences in the distribution of injected spleen and thymus cells is not known. Bone marrow cells failed to restore immune capacity when given to neonatally thymectomized mice. Likewise, neither foetal liver cells nor marrow cells in doses of up to 40×10^6 nucleated cells per mouse could restore transplantation immune reactions in thymectomized irradiated mice. Spleen cells from normal mice (but not from neonatally thymectomized donors) restored the capacity of thymectomized irradiated mice to reject skin homografts. These experiments suggest that there are in spleen and lymph nodes, but not in thymus and bone marrow, many cells which are fully capable of restoring immune mechanisms in thymectomized mice and hence which are presumably immunologically competent. It is very likely that these cells form part of the pool of recirculating small lymphocytes. Indeed when pure suspensions of lymphocytes recovered from the thoracic duct lymph of normal mice were inoculated into neonatally thymectomized mice, these mice immediately became capable of performing immune reactions as normal mice.

Syngeneic or allogeneic thymus tissue grafted in a subcutaneous site or under the renal capsule restored to neonatally thymectomized mice or thymectomized irradiated mice the capacity to effect transplantation reactions. The majority of cells multiplying in the lymphoid tissues and thymus implant itself were shown, by means of a chromosome marker method, to be of host origin and hence could not have been the direct descendents of cells of the thymus donor strain. Discriminant spleen assays showed that host cells were responsible for immunological reactivity in the restored mice. However, in some cases thymus-derived cells were found in the spleen and lymph nodes of thymectomized mice and there was a transient but well-defined increase in the proportion of these cells after antigenic stimulation. The significance of these cells in the immune reaction is unknown.

In order to test whether a direct cellular transfer between thymus implant and thymectomized host was crucial for the restoration of immune capacity, thymus was placed in millipore diffusion chambers in the peritoneal cavity of young mice thymectomized at birth. The walls of the chambers were impermeable to cells, and yet the mice were restored in their capacity to react immuno-

logically. It appears therefore that a humoral thymus mechanism must be operative in the chain of events leading to the establishment of a functioning immunological system. Thymus extracts, however, have to date failed to restore thymectomized mice.

In bursectomized chickens, both bursal grafts and bursal tissue in diffusion chambers restored antibody-producing capacity.

In summary, the immunological defects of thymectomized rodents could be corrected either by injecting lymphoid cells obtained from the circulating lymphocyte pool (spleen, lymph nodes, thoracic duct lymph) of normal adult animals of the same strain, or by grafting thymus tissue. Thymus graft-derived cells appeared in the lymphoid tissues of the host and their numbers increased transiently after specific antigenic stimulation. When, however, the thymus was enclosed in a chamber the walls of which were impermeable to the passage of cells, the host's immune capacity was restored to normal thus suggesting that some of the action of the thymus on immune competence may be mediated by means of a humoral mechanism.

Possible Mechanisms of Action of Thymus and Bursa

The exact mechanism by which the thymus and bursa exert their influence during immunogenesis is unknown. It is unlikely, however, that the thymus exerts any effect on the population of immunologically competent cells or antigen-sensitive cells once they have been produced. Thus, for instance antigen-sensitive cells injected into neonatally thymectomized mice responded to antigen by a burst of proliferation and differentiation; the extent of this response was similar to that obtained when antigen-sensitive cells were exposed to the same antigen in the normal animal. This strongly suggests that antigen does not require the help of any thymus factor to cause proliferation and differentiation of antigen-sensitive cells (Weiss *et al* 1967).

The long delay in the regeneration of the capacity to produce an immune response following radiation damage stands in sharp contrast to the speedy regeneration occurring in other cellular systems. Thus, for instance, an animal's capacity to respond to sheep erythrocytes returns 3 to 4 weeks after doses of 500 to 700 rads (Taliaferro *et al* 1964). In contrast, the haemopoietic colony forming ability lost after 400 rads is completely replaced within 7 to 8 days by extensive proliferation of the surviving cells (Till & McCulloch 1964). It is thus probable that antigen-sensitive cells do not proliferate in the absence of antigen and that new antigen-sensitive cells are the progeny of more primitive antigen-insensitive precursors, i.e. of cells which proliferate independently of antigenic stimulation. Since lymphocytopoiesis in the thymus, unlike lymphocytopoiesis elsewhere, is quite independent of antigenic stimulation and since the thymus is required for the anatomical and functional regeneration of the

immune system after damage by irradiation, it would appear that the precursors of the antigen-sensitive cells are to be found in the population of thymus lymphocytes.

Various experiments suggest that there are thymus humoral factors stimulating lymphocytosis (Metcalf 1956) and inducing the differentiation of immunologically competent cells (Osoba & Miller 1963). The lymphocytosis-stimulating

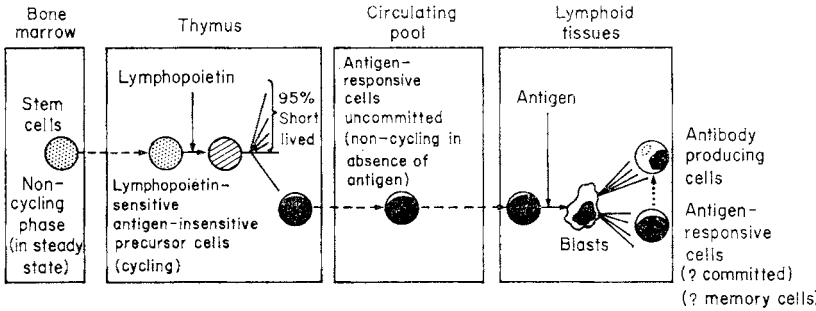


FIG. 11.6. The pathway of lymphoid differentiation to illustrate the cellular targets of thymus lymphopoietin and antigen.

In the bone marrow, there is a self-maintaining population of cells, the stem cells, from which other cell lines may originate. In the normal organism the stem cell population is in a steady state, i.e. they maintain their own numbers, in spite of a steady rate of cell removal for the purpose of differentiation, a process which primarily affects those stem cells in a non-cycling phase (resting phase, G_0).

A thymus-dependent mechanism is responsible for the irreversible differentiation of stem cells to lymphoid-precursor cells. This differentiation is probably mediated by a thymus humoral factor, termed lymphopoietin. Lymphopoietin-sensitive cells are antigen-insensitive but undergo proliferation associated with progressive differentiation to give rise to a progeny of cells, some of which (probably less than 5%) become antigen-sensitive cells.

The bulk of the antigen-sensitive cells produced forms part of the circulating pool. They are non-cycling cells but can respond to appropriate antigens within the microenvironment of the peripheral lymphoid tissues (e.g. lymph nodes and spleen). Their response to antigen is characterized by the production of (1) cells which undergo proliferation associated with progressive differentiation to antibody-producing cells and (2) more antigen-sensitive cells. Whether these are identical to the original parent antigen-sensitive cell or whether they differ from the parent cell in their degree of commitment is unknown.

factor (LSF) of Metcalf may conceivably act by promoting the proliferation of antigen-insensitive thymus lymphoid cells. It is unlikely that it promotes the proliferation of immunologically competent cells outside the thymus since, as mentioned above, antigen-sensitive cells do not proliferate in the absence of antigen but can do so following antigenic stimulation even in the absence of the thymus. Experiments on the restoration of immunological capability in neo-

natally thymectomized mice by thymus enclosed in millipore diffusion chambers have suggested that the thymus may produce a 'competence-inducing factor' (CIF) to allow the differentiation of antigen-insensitive precursors. In the normal animal this step may be initiated within the thymus environment, but under certain experimental conditions cells outside the thymus may be influenced. It is conceivable that both LSF and CIF are one and the same factor which could adequately be termed 'lymphopoietin'. Such a factor would trigger off the proliferation of antigen-insensitive (but lymphopoietin-sensitive) lymphoid precursor cells (?haemopoietic stem cells) and their progressive differentiation to antigen-sensitive (but lymphopoietin-insensitive) cells (Fig. 11.6).

FATE OF RESPONSIVE CELLS

Following contact with antigen, or processed antigen, the antigen-sensitive cells differentiate and proliferate to give rise to effector cells which carry out the immune reaction. Whether a responsive cell can become primed by antigen without undergoing proliferation has not been unequivocally established. Cell replication does take place at some stage after antigen triggering and the evidence for this comes from various sources:

1. Histological and autoradiographic studies have shown that cell division occurs following antigenic stimulation (Nossal 1962).

2. The radiation sensitivity of the ability of mice to produce antibody-forming cells in response to an antigen is very similar to that found *in vivo* and *in vitro* for the ability of cells to proliferate (Puck & Marcus 1956; McCulloch & Till 1962).

3. Following transfer of lymphoid cells into heavily irradiated recipients, single antigen-sensitive cells divide repeatedly in response to antigen to form discrete clusters of antibody-producing cells (Kennedy *et al* 1966).

Cell replication is associated with progressive differentiation of a fraction of the replicating cells to effector cells (detectable as antibody-producing cells). The effect of antigen may be not only to produce a large number of effector cells but also to increase the number of antigen–sensitive cells themselves. Whether these new antigen–sensitive cells differ from their original ancestors in their degree of commitment and their susceptibility to immunological feedback inhibition and whether they form part of the machinery responsible for immunological memory has not been established experimentally.

There is a polydiversity of the effector cells: separate sets of cells, generally members of the plasma cell series and large lymphoid cells, are engaged in synthesizing the various molecular species and subspecies of immunoglobulins; other types of cells, small lymphocytes and 'immune macrophages', carry out delayed sensitivity and transplantation immune reactions.

Depending on the nature and physical state of antigen, the dose given, the

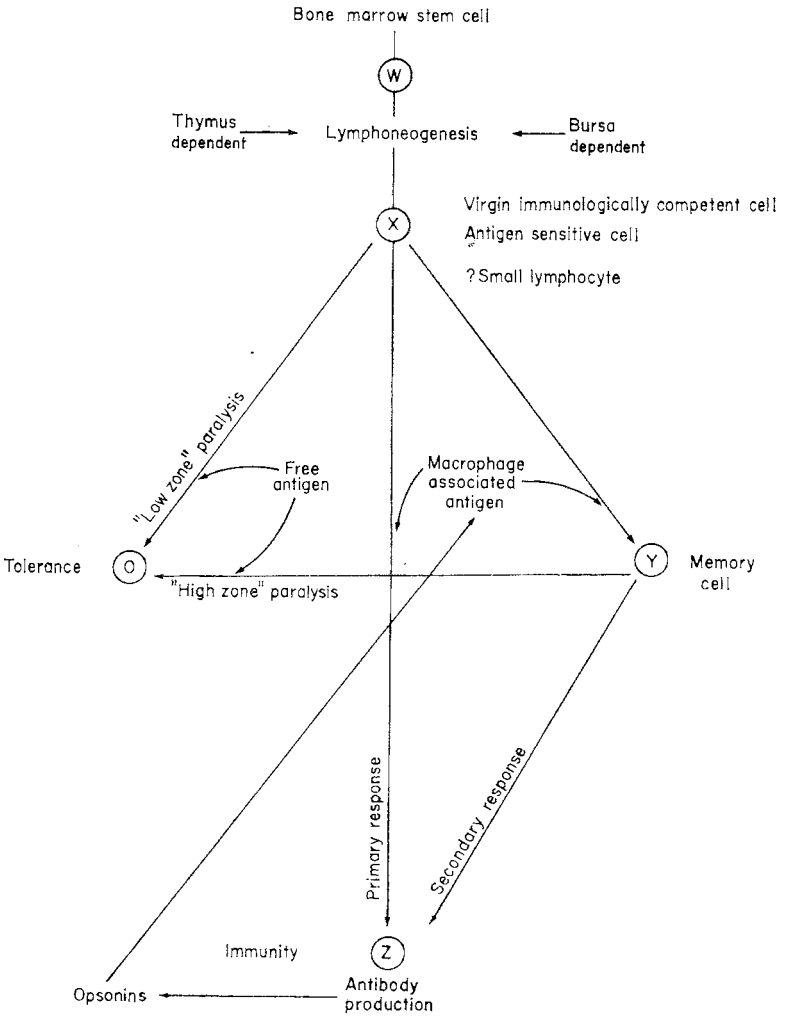


FIG. 11.7. Pathways available to immunologically competent cells (X) following contact with antigen.

Depending on whether contact is made with 'free' antigen or 'macrophage-processed' antigen the responsive cell may be 'paralyzed' (O) or may undergo proliferation associated with progressive differentiation to immunologically activated cells: antibody-producing (Z) cells or memory cells (Y) responsible for the anamnestic or secondary response (see text); (partly after Nossal (1965)).

manner in which it is presented and the species and age of the host, several pathways are available to a responsive cell (X): it can be driven towards antibody production (Z), towards memory cell production (Y) or towards non-reactivity, paralysis or tolerance (O) (Fig. 11.7).

Primary Immune Response

A primary immune response occurs when 'virgin' immunologically competent cells encounter antigen for the first time.

Antibody-Producing Cells

When an antigen is injected for the first time into a normal animal, a fraction of the antigen–sensitive cells differentiate within a few hours to primitive blast cells which divide and differentiate further to give rise to members of the plasma cell series. The blasts divide every 8 to 10 hr and undergo a total of eight to nine sequential mitotic divisions (Nossal 1962; Urso & Makinodan 1963). During differentiation there is an increase in the amount and organization of the endoplasmic reticulum and an increase in intracellular immunoglobulin. The various cell stages are arbitrarily divided into plasmablasts, immature plasma cells and mature plasma cells.

The plasmablasts produce the first detectable antibody. In the case of particulate antigens, such as whole bacteria or viruses, this antibody is of the IgM class. (Less commonly, with certain soluble antigens, the first antibody detected is of the IgG class (Nossal, Ada & Austin 1963)). However, it must be remembered that different assay systems may yield different results. For instance, natural antibodies to Gram-negative bacteria have recently been shown by immunofluorescent methods to reside within both the IgG and IgM class (Cohen & Norins 1966).

A number of plasmablasts producing IgM antibody mature to plasma cells and die without ever having produced any other class of immunoglobulins. Single cell studies (Nossal *et al* 1964) have indicated, however, that a switch from IgM occurs in individual cell lines as they mature from plasmablasts to plasma cells. In some systems, when the antigen dose is minute, IgM antibody formation occurs without a subsequent IgG phase (Uhr 1964). The IgM to IgG switch does not occur in young animals nor in lower vertebrate species such as toads (Diener & Nossal 1966; Williams & Nossal 1966) indicating that the appearance of IgG is a late and sophisticated step in both phylogeny and ontogeny.

The plasma cells are highly specialized non-dividing end cells. There is no evidence that they can perform any function other than antibody synthesis. Studies of plasma cell tumours in mice (Potter 1962) indicate that a single clone of plasma cells produces only one molecular species of immunoglobulins. Single cell studies on normal plasma cells (Nossal & Makela 1962) have generally

shown that a single cell can produce antibody of only one combining specificity. Many plasma cells die after a short life span of less than 1 week. Less than 1% live for many months and, because they possess a stable messenger RNA, they are capable of continuous antibody synthesis (Miller III, 1964).

Cells Producing Delayed Sensitivity and Transplantation Immune Reactions

Circulating cells are responsible for the cellular infiltrate seen in delayed sensitivity reactions. The identity of the circulating 'hypersensitive' cells has not been unequivocally established: radioautographic studies suggest that they may be small or 'medium-sized' lymphocytes which are the progeny of dividing large pyroninophilic cells (Turk & Stone 1963). Cell transfer studies, in which either donor or host cells were labelled with tritiated thymidine, have led to the suggestion that interaction between sensitized cells and antigen causes the release of some factor involving non-sensitized host lymphoid cells which become the predominant cells in the lesions (McCluskey *et al* 1963). This factor may be a non-specific factor aggregating cells (Nelson & Boyden 1963) or a specific factor of the nature of cytophilic antibody (*vide infra*) passively sensitizing normal lymphoid cells (Boyden 1964).

In transplantation immune reactions there is direct evidence that a fraction of the immunologically competent small lymphocytes transform into large pyroninophilic cells which divide to give rise to a progeny of lymphoid cells of progressively decreasing size. Electron micrographs of the large pyroninophilic cells showed abundant ribosomes but little or no endoplasmic reticulum: these cells are thus quite distinct from plasma cells.

It seems, therefore, that the same series of cellular events takes place in both delayed sensitivity reactions and in the primary response to histocompatibility antigens: responsive cells (small lymphocytes in the case of transplantation immune reactions) transform to large pyroninophilic cells which divide eventually to give rise to 'sensitized' or 'committed' small lymphocytes.

Macrophages are present in inflammatory exudates in delayed sensitivity and are a prominent cell type in graft-versus-host reactions and in certain types of graft rejections. Are macrophages effector cells in cell-mediated immune reactions? It seems that macrophages, like small lymphocytes are a heterogeneous population of cells with different origins depending on the stimulus which provoked their formation. Experiments employing tritiated thymidine labelling techniques showed that in rats, blood monocytes and inflammatory exudate macrophages were derived, not from 'circulating pool' small lymphocytes, but from rapidly and continuously proliferating precursors in bone marrow (Volkman & Gowans 1965). On the other hand, experiments with chromosomally-marked cells in mice, gave convincing evidence that during the graft-versus-host reaction produced by an injection of allogeneic thoracic duct lymphocytes, the

dividing phagocytic cells in the liver were derived from a cell type in the inoculum, quite possibly the 'circulating pool' small lymphocyte (Howard *et al* 1966). Under certain conditions, therefore, the macrophage may inherit a kind of immunological committment from its small lymphocyte ancestor. In mice immunized with sarcoma I ascites tumour cells, peritoneal macrophages were capable of adhering specifically to appropriate target cells *in vitro* and producing specific immunological injury which had no dependence on humoral antibody (Granger & Weiser 1966). It is not quite clear whether cytophilic antibody plays a part in this reaction.

The manner in which sensitized cells ('sensitized' small or medium lymphocytes and 'immune' macrophages) inflict tissue damage has not been established in detail. The reactions can occur in the absence of demonstrable circulating antibody. It is thought, however, that antibody molecules are produced by the sensitized cells and located at the cell surface where they can interact with antigens present outside the cell. In graft rejection and in certain delayed hypersensitivity situations the close adherence of effector to target cells is a common feature. It has been shown in some experimental systems that a marked aggregation of sensitized lymphoid cells on target cells regularly precedes cytotoxicity. When, however, aggregation of non-sensitized lymphoid cells was produced artificially by heterologous antibodies or phytohaemagglutinin, non-sensitized lymphoid cells were as effective as sensitized cells in killing incompatible target cells. Furthermore, following aggregation of incompatible ('foreign') target cells with lymphoid cells incapable of reacting immunologically (e.g. in parental to F_1 situations or when the lymphoid cells were preirradiated) a cytotoxic effect was just as marked. Aggregation of target cells with compatible syngeneic lymphoid cells had no effect. It was suggested that cell damage was primarily caused by contact between antigenically distinct lymphoid cells and target cells and this phenomenon was termed 'allogeneic inhibition' (Möller & Möller 1966). The sensitization of cells (active and passive) may thus be an essential step whereby antibody bound to the cell membrane ensures that the cell establishes close contact with the antigen or with the antigenically distinct tissue cells. This contact may cause the release of lysosomal enzymes present in stimulated lymphocytes and adherent macrophages thus producing the damage.

Histophysiology of the Primary Immune Response

The development of plasmacellular and follicular centre reactions during a primary response has been studied in the regional lymph node following a single injection of antigen (e.g. van Buchem 1962) and in the spleen after a *single* intravenous injection of antigen (e.g. Langevoort 1963). The anatomical terms referred to are illustrated in Figs. 11.8 and 11.9. Within 24 hours after the injection of antigen, plasmablasts appeared first in the lymphocytic fields in the case of the regional lymph nodes and in the periarteriolar lymphocyte sheaths in

the white pulp in the case of the spleen. These cells were scattered between the lymphocytes which normally lie in these areas. From the 2nd to the 5th day

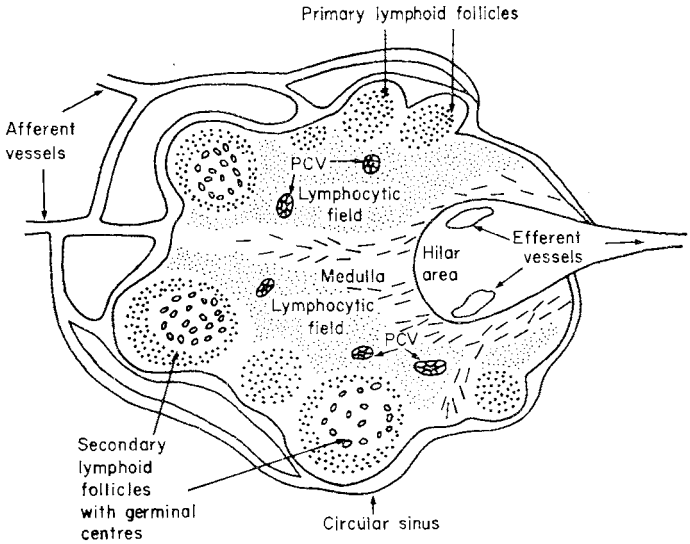


FIG. 11.8. Schematic diagram illustrating the structure of the lymph node. The node consists essentially of a circular lymph sinus immediately deep to the capsule, a cortex and a medulla. The cortex contains the bulk of the lymphoid tissue in which can be distinguished the lymphocytic fields and the lymphoid follicles. In the lymphocytic fields, diffuse cortex or paracortical area, small lymphocytes lie without any obvious arrangement and, in the unstimulated node, are only occasionally accompanied by primitive pyroninophilic blast cells. The postcapillary venules (PCV) with their characteristic cuboidal endothelial cells, lie in these fields: through these endothelial cells the circulating pool lymphocytes pass from the blood stream into the lymphocytic fields. Following primary antigenic stimulation, plasmablasts or large pyroninophilic cells make their first appearance in the fields. The lymphoid follicles are densely packed collections of small lymphocytes separated from the circular sinus by only two to three cells. In the unstimulated animal there are no blast cells in the follicles and no germinal centres. Five days after antigenic stimulation large blast cells have collected in the follicles forming typical germinal centres. The medulla consists essentially of lymph sinuses lined by phagocytic cells and cords which contain only a small number of plasma cells in the unstimulated animal and a few scattered mast cells. A relatively acellular loose tissue in the middle of the medulla (hilar region) contains efferent vessels. After antigenic stimulation plasma cells accumulate in the medulla.

large numbers of immature plasma cells appeared, presumably as a result of the proliferation and differentiation of the blasts. In the lymph nodes, large numbers of these cells seemed to be released from the fields into newly formed sinuses and

drained away into efferent lymph. A smaller number of immature plasma cells remained in the concomitantly formed medullary cords where they completed their differentiation into plasma cells. It has been suggested that the blasts released from the lymph nodes via the efferent lymphatics might propagate the immune response by colonizing other lymph nodes and lymphoid

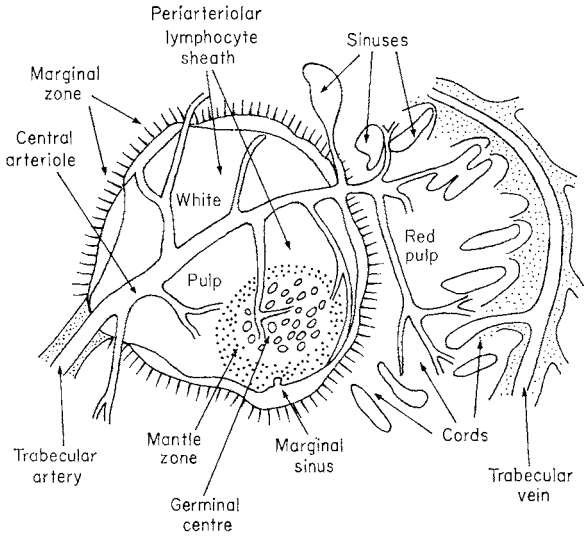


FIG. 11.9. Schematic diagram illustrating the structure of the spleen.
The spleen consists essentially of two areas: white pulp and red pulp. The white pulp contains the lymphoid tissue—an expanded periarteriolar lymphocyte sheath containing lymphoid follicles. It is bound by a marginal sinus beyond which lies the marginal zone, a fine reticulum meshwork separating the white pulp from the red pulp and containing macrophages and lymphoid cells. The red pulp is composed of cords and sinuses, the cords frequently showing prominent arterioles surrounded, in the animal exposed to antigens, by one or more layers of plasma cells. Following antigenic stimulation plasmablasts first appear among the lymphocytes of the periarteriolar lymphocyte sheaths. The follicles are separated from the rest of the white pulp by a mantle zone of small lymphocytes. In the unstimulated animal, the follicle has a pale inactive centre and no blast cells: these accumulate 3 to 4 days after antigenic stimulation, eventually forming a typical germinal centre (partly after Leon Weiss 1964).

organs where they could proliferate and differentiate to give rise to more antibody-producing cells (Morris 1966). In the spleen, the plasmablasts and immature plasma cells apparently moved to the periphery of the periarteriolar lymphocyte sheaths at the border of the red pulp. Many of these cells presumably entered the blood stream via the sinuses. Some mature plasma cells

remained in the red pulp. The follicular centre reaction in both the lymph nodes and the spleen started on the 3rd day after antigen administration. The first change was the appearance of large numbers of blasts many of which were dividing. By the 5th day typical germinal centres had developed; dividing large and medium-sized lymphocytes were interspersed among phagocytic cells with tingible bodies. The follicles enlarged considerably during the subsequent 5–10 days after which the reaction gradually subsided. In the rabbit, total body irradiation with 450 rads destroyed the lymphoid follicles in both lymph nodes and spleen but did not damage the lymphocytic fields and periarteriolar lymphocyte sheaths. An essentially normal plasmacellular reaction took place in these fields following the administration of antigen either a little before or soon after irradiation. It was thus concluded that the initiation of antibody response could be effected entirely by cells developing in the fields and sheaths and that the follicular centre reaction did not essentially contribute to the initial antibody produced (Keuning *et al* 1963).

The changes which occur in the regional lymph nodes following the application of sensitizing chemicals to the skin have been studied by Turk and his associates (Turk & Stone 1963). There occurred a progressive enlargement of the lymphocytic fields of the cortex ('paracortical area' in Turk's terminology), due to the accumulation of large pyroninophilic cells, reaching a maximum on the 4th day after sensitization, the day before the animals became sensitive. The follicles were unaffected during sensitization and germal centres were irregularly produced within the follicles after 6 days in marked contrast to their regular formation between the 3rd and 5th day during a primary antibody response. The large pyroninophilic cells filling the lymphocytic fields were shown by labelling methods to give rise not to plasma cells but to small lymphocytes. This sequence of events is similar to that which takes place in the lymph nodes draining a skin homograft (Scothorne 1957) and in the white-pulp of the spleen during graft-versus-host reactions (Gowans & McGregor 1965): in both these situations large pyroninophilic cells accumulate in the lymphocytic fields and periarteriolar lymphocyte sheaths and divide to give rise to progressively smaller lymphoid cells and eventually to small lymphocytes. There is no germinal centre development at all.

Secondary Immune Response and Immunological Memory

An enhanced antibody response occurs characteristically following the reinjection of a specific antigen. This phenomenon of enhanced reactivity, designated as the secondary or anamnestic response, is dependent upon the existence of a special population of cells which carry the property of immunological memory—the memory cells (Y cells).

Differences Between the Primary and Secondary Immune Responses

An animal's response to a second dose of antigen exhibits certain features which differentiate it from the response to a first dose of antigen:

1. The two responses have different dose-response kinetics (e.g. Nossal, Austin & Ada 1965). In the primary response antibody production is very prolonged; in the secondary response, 'excess' antibody (that produced in excess of the mean primary titre expected from the dose used as the secondary stimulus) is produced for only a short time (4 weeks or less). Furthermore, the curves of peak antibody titres: antigen dose differ for both responses.

2. In the primary response an IgM phase generally precedes the appearance of IgG antibody; in the secondary response, with appropriate doses of antigen, a brisk appearance of both IgM and IgG occurs. Many plasmablasts early in the secondary response thus form IgG *ab initio* without having to go through the IgM phase as in the early primary response.

The capacity to produce a primary response is abolished by certain treatments which do not affect the secondary response: for instance, secondary responses can be elicited in animals depleted of 'circulating pool' lymphocytes by X-irradiation, lympholytic drugs and chronic thoracic duct drainage. Furthermore, passively administered antibody inhibits primary responses but has no suppressive effect on secondary responses (Rowley & Fitch 1964).

Lower vertebrates are quite incapable of giving secondary responses even though they can give adequate primary responses (Diener and Nossal 1966). This suggests that the cellular system responsible for memory induction evolved separately from that responsible for primary antibody production.

Memory Cells

The existence of a set of 'memory' cells has to be postulated in order to account for some of the differences between the primary and secondary responses summarised above. Whether the induction of the memory state is an event that is quite independent of the induction of antibody formation has not been definitely established. Nor is it really known whether the memory resides in a larger population of responsive 'X' cells or in a population of modified 'Y' cells.

When large doses of tritiated thymidine were given to primarily immunized rats 2 hours before a second injection of *s. adelaide*, it was found that almost all the antibody-producing plasma cells isolated from the regional lymph nodes were labelled. Identical results were obtained in rats stimulated either 4 or 40 weeks after primary antigenic challenge. This suggested that immunological memory was carried by a continually dividing line of large lymphoid cells established as a result of the primary stimulus and able to divide and differentiate into plasma cells in response to a second encounter with antigen (Nossal 1962). In contrast to those findings, other workers (Cohen & Talmage 1965) showed

that the precursors of antibody-forming cells in a secondary response in mice failed to incorporate tritiated thymidine just before secondary stimulation. This tends to support the idea that a non-dividing cell, presumably the small lymphocyte, may be the carrier of immunological memory. The fact that the small lymphocyte is a long-lived cell makes it an attractive candidate for the function of memory. Evidence to support this possibility comes from experiments in which X-irradiated rats gave a secondary type response to a first dose of bacteriophage φX_{174} after they had been injected with thoracic duct small lymphocytes from primarily immunized donors (Gowans & Uhr 1966). There is no inconsistency between this finding and the observation that secondary responses are insensitive to lymphocyte depletion by chronic thoracic duct drainage. Some of the memory cells may not be extractable by thoracic duct cannulation as they may be sessile or fixed in the lymphoid tissues either in the form of a dividing cell line or as a population of non-circulating small lymphocytes which are not part of the recirculating pool. The ability of rabbits to give a secondary response was much impaired by X-radiation when this was given not before but later than 1 month after primary immunization (Porter 1964). This suggests that a change in the cellular basis of immunological memory may occur with time, possibly from a non radiosensitive dividing cell line to a radiosensitive small lymphocyte. All the experimental evidence is thus not inconsistent with the possibility that two cellular mechanisms may be involved in immunological memory: a pool of replicating blasts and the production from these of long-lived small lymphocytes.

The Possible Significance of Germinal Centres

Germinal centres are absent in the lymphoid tissues of lower vertebrates and have not developed in very young or newly born laboratory rodents. These animals are incapable of producing IgG and of giving secondary immune responses. Hence, the development of germinal centres is associated both in phylogeny and ontogeny with the capacity to produce IgG and secondary immune responses (Diener & Nossal 1966; Williams & Nossal 1966).

Many investigators have demonstrated the presence of considerable amounts of immunoglobulins in the germinal centres and concluded that these structures are actively engaged in the synthesis of immunoglobulins. It has recently been shown that the immunoglobulins in the germinal centres are uniform mixtures of molecules of different class, type and subtype and bearing different genetic markers (Pernis 1967). The results of this work and of studies on antiserum transfusion are consistent with the notion that a part, at least, of the immunoglobulins of germinal centres are not synthesized locally, but are bound there secondarily—perhaps they are opsonins facilitating the uptake of antigen by the dendritic reticulum cells. A number of studies suggest that germinal centres formed during the course of the primary immune response (*vide supra*) 'prepare' the animal

in some way for the secondary immune response (e.g. Thorbecke *et al* 1962). After a secondary antigenic stimulus, cell division increases in germinal centres and new centres rapidly form. The centres may function as generative compartments where memory cells are produced and discharged into the medulla as antibody-producing cells after secondary antigenic stimulation. These are pure speculations: the origin, fate and functions of the cells in the germinal centres and the identity of the cell types alleged to respond to a second antigenic challenge have not been determined experimentally.

Immunological Tolerance

An animal in health does not produce immunological reactions against most of its own constituents. Acquired immunological tolerance has been suggested as a possible mechanism which prevents the immune system, through early and massive contact with autologous proteins, from producing a reaction against self. A failure of this mechanism would thus constitute one of the ways in which autoimmune reactions are produced. The development of autoimmunity is discussed in detail in Chapter 21.

Acquired immunological tolerance or paralysis is generally defined as a specific central failure of the mechanism of immunological response brought about by antigenic exposure. This definition insists upon the following essential characteristics:

1. There is a *central* failure of the mechanism of response. The population of responsive cells themselves are unable to respond: it is not the environment of the tolerant animal which masks or prevents the response.
2. Tolerance is immunologically *specific*: animals tolerant to one antigen can respond to an unrelated antigen. This distinguishes tolerance from general non-reactivity occurring in animals in which the immune system has not yet developed, has not properly developed as a result of neonatal thymectomy or has been damaged by irradiation or cytotoxic drugs.
3. Tolerance is induced by *antigen*, not by antibody. This distinguishes tolerance from the central depression of the immune response brought about by specific antibody (Rowley and Fitch 1964).

In order to evoke a response, an antigen must be recognized. Recognition is likely to occur at some critical site on or within the cell. A number of studies suggest that lymphocytes may bear 'recognition units' (Brent & Medawar 1963; Sell & Gell 1965; Mitchison 1967). The receptor may well be a 'sentinel antibody' located on the cell membrane, the antibody belonging to a fraction of the immunoglobulins that has the special properties of specificity.

A tremendous amount of work is being performed on the mechanism of induction of immunological tolerance in experimental animals. The reasons

for this are obvious: such a knowledge will open the doors to effective organ-grafts in clinical medicine.

Experimental Induction of Immunological Tolerance

Whether an antigen is capable of inducing tolerance or immunity depends on a multitude of factors: some are related to the host—its age, the capacity of its reticulo-endothelial system to handle antigen, the rate at which new immunological competent cells are produced, etc.; others are related to the antigen itself —its nature, its immunogenicity, the dose given and number of times it is given.

The age of the host has some importance in tolerance induction. As has been discussed in Section II, flagellar antigens are rapidly localized in adults, both in the lymphoid follicles and in the medullary macrophages. In newborn rats, there are no lymphoid follicles and, although there are macrophages, most of them are not yet capable of efficiently ingesting flagellar antigens. As a result, the antigen persists, scattered diffusely throughout the extracellular fluids and tissues for long periods. Inefficient phagocytosis and antigen sequestration resulting from immaturity of the reticulo-endothelial system thus appears to favour the induction of tolerance. By contrast, susceptibility to tolerance induction does not seem to be related to the stage of maturation of the immunologically competent cells. During the regenerative phase following irradiation or cytotoxic drugs it was thought that the proliferating blast cells would be more susceptible to tolerance induction than normal lymphoid cells. However, neither the age of the animal, nor procedures such as irradiation, cytotoxic drugs or thymectomy, affected the rate of induction of tolerance (Mitchison 1967).

Tolerance can be induced to cellular antigens as well as to protein antigens even when these are highly immunogenic proteins. Provided certain experimental conditions are fulfilled particulate antigens such as whole bacterial flagella can tolerize. Alum-precipitated material or antigens incorporated in adjuvants are never tolerogenic.

Recent work has revealed that there are two zones of antigen dosages capable of inducing tolerance or paralysis: sub-immunogenic microgram doses and much higher 'paralysing' doses. Intermediate dosage levels result in immunization rather than tolerance. Thus, using bovine serum albumin in adult mice, Mitchison (1967) has shown that very low doses (1–10 μg) given repeatedly induce low zone paralysis, higher doses, of the order of 100 μg to 1 mg, 'immunize' and higher doses still (5–50 mg) paralyse. The molar concentration of BSA in the extravascular fluid that resulted in low zone paralysis was found to be M^{-8} and for high zone paralysis M^{-5}. Other proteins such as diptheria toxoid, ovalbumin and lysozyme also showed two zones of dosages at which paralysis could be induced in adult mice. Monomeric flagellin could induce paralysis but only when given as a course of injection to *newborn* rats. Here too, a high

and low zone of antigen dosage was found to paralyse (Shellam & Nossal 1967). The concentration of flagellin in extravascular fluids was found to be M^{-14} for low zone paralysis and M^{-} for high zone paralysis. Concentrations of M^{-9} M^{-11} were associated with immunity rather than tolerance. Hence very low doses of antigen can induce tolerance so that it is not necessary to 'saturate' the lymphoid system as used to be thought originally.

The question of the immunogenicity of a protein is evident from Dresser's work (Dresser 1962). A component of bovine gammaglobulin does not 'immunize' mice provided that aggregated protein is removed by centrifugation. When this is done, the protein paralyses mice at exactly the same low zone range as demonstrated for bovine serum albumin. The immunogenic capacity of a protein may thus be abolished when material suited to phagocytosis is removed. It can thus be stated, as a general rule, that low doses of antigen will paralyse in circumstances when 'immunization' can be avoided, and that high doses are required where this cannot be arranged (Mitchison 1967). Inefficient phagocytosis or antigen sequestration thus provides a link between tolerance in adults by proteins such as bovine serum albumin, which are poorly immunogenic presumably because they are poorly phagocytosed thus becoming diffusely spread throughout the lymphoid system, and tolerance in newborn by highly immunogenic proteins such as flagella, which fail to be sequestered because of the immaturity of the reticulo-endothelial system of the newborn animal. The current hypothesis to account for tolerance induction (Nossal 1966; Mitchison 1967) is thus that encounter between an immunologically competent cell and antigen free in the extravascular fluid leads to tolerance, either by destruction of the cell (e.g. clonal deletion dependent upon lysis following antigen contact in which case the population is tolerant by virtue of the absence of cells capable of responding to that particular antigen) or by some alteration of the cell's genetic control mechanism (in which case tolerant cells are produced). 'Immunization' will result when the antigen is sequestered by macrophages or dendritic reticulum cells so that on the one hand, free access of antigen to circullating immunologically competent cells is not permitted, and on the other hand, the antigen is subjected to some type of processing by the macrophage. Macrophage-enclosed antigen was shown to be about one thousand times more immunogenic than free antigen (Mitchison 1967). Macrophages taken from paralysed donors processed antigen as effectively as macrophages from normal donors thus indicating that paralysis does not affect macrophage function even quantitatively.

Maintenance and Breakdown of Immunological Tolerance

The duration of the tolerant state depends on the size of the antigen dose inducing tolerance. In tolerance to cellular antigens, the tolerant animals are chimaeras

of haemopoietic or lymphoid tissues, the allogeneic cells having been introduced either in the newborn period or in the adult following total body irradiation. In the case of tolerance to proteins, a course of injections is necessary to ensure that a critical concentration of antigen persists for the critical period during which tolerance is induced. In some systems, the antigen levels in the circulation and in the lymphoid organs fell below one molecule per cell long before tolerance began to wane. Furthermore, there was no detectable antigen in the lymphocytes themselves (Nossal 1966) suggesting that if tolerant cells do indeed exist they are not held in a tolerant state by the presence within them of antigen.

Tolerance breaks presumably when new virgin immunologically competent cells are recruited and there is no antigen available to drive these new recruits towards tolerance. An increase in the number of potentially reactive cells occurs during the post-irradiation recovery phase: in the absence of a continuing concentration of antigen the new cells react normally to the tolerogen when it is injected and so the state of tolerance is broken (Nossal 1966). Tolerance can also be terminated by 'cross-immunization'. Rabbits paralysed with bovine serum albumin and then 'immunized' with human serum albumin were found to produce significant amounts of anti-bovine serum albumin antibody (Weigle 1962). Tolerance to a given protein could also be terminated by injecting a conjugate of the protein with haptens. It is possible that 'cross-immunization' leading to production of antibodies against otherwise tolerated antigens might cause autoimmune disease (Weigle 1962).

There is no clear evidence that the thymus facilitates or mediates in a specific manner the acquisition or maintenance of immunological tolerance. Some experimental findings have provided support for the hypothesis that interaction of antigen with lymphocyte precursors in the thymus may be the basis of tolerance in certain experimental systems (Smith *et al* 1966) and Burnet (1962) has suggested that the thymus exercises a 'censorship' function by eliminating cells potentially capable of reacting against self-antigens, a process which would thus result in self-tolerance. The dose-response kinetics data of Mitchison, however, suggest that cells do not have to be young or immature in order to be paralysed. The possibility remains that if there are in the thymus a few immunologically competent cells, they together with all the other cells in the body, must be modified in an appropriate manner for tolerance to be induced completely. Specific immunological tolerance to defined antigens has been induced in adult thymectomized animals indicating that the lymphoid system can be rendered tolerant independently of or in the absence of the thymus. Experimental evidence points to the likelihood that the waning of tolerance is mediated by a thymus-dependent mechanism which allows the development of new, uninhibited, immunologically competent cells: thymectomy of adult animals tolerant to a specific antigen has prevented the reappearance of reactivity to that antigen (Claman & Talmage 1963; Taylor 1964).

Summary and Conclusions

In order to immunize or effect antibody production, an antigen must be presented to the responsive cells by 'the proper diplomatic channels' (Medawar 1963b). Paralysis results from the direct access of antigen to lymphoid cells whereas antibody production occurs when the antigen has been the subject of prior processing by the macrophages. A dual response may thus be produced by the injection of antigen: perhaps most cells are driven towards tolerance whilst a few become set on antibody production. To produce tolerance at the organism level, conditions should be such that the antigen does not immunize. This can be achieved with antigens which are poor immunogens because they are not readily phagocytosed or with highly immunogenic proteins in newborn animals which have an immature reticulo-endothelial system and cannot efficiently handle the antigens. The role of immunological tolerance may be (1) to guard against autoimmunity, a subject discussed in Chapter 21 and (2) to provide a homeostatic mechanism to limit the number of responsive cells that can be driven towards immunity, as discussed in the following section.

REGULATION OF THE IMMUNE RESPONSE

What regulates the extent of the immune response? It is evident that some control must operate (*a*) to prevent unlimited proliferation of antibody-producing cells (*b*) to limit the recruitment of antigen-sensitive cells so that one particular antigen will not stimulate every uncommitted cell in the immune system and thus prevent a response to a second or third antigen, and (*c*) to replenish the pool of uncommitted cells when these are used up. The possibility that the precursors of antigen-sensitive cells are provided by the thymus has been discussed in Section III.

Regulation of the primary immune response is possible at different levels by various mechanisms—genetic, anatomical, physiological and immunological. The extent of the response depends upon factors which affect (*a*) the number of antigen-sensitive cells recruited and triggered off, (*b*) the rate of proliferation of antigen-sensitive cells and antibody-producing blasts and the total number of progeny produced, and (*c*) the antibody ouput of individual antibody-producing cells.

Genetic Factors Regulating the Immune Response

At the level of the X cell, clones of antigen-sensitive cells may be genetically restricted in their capacity to react to antigen. According to the clonal selection theory (Burnet 1959) the immune system contains multiple clones of cells, each of which is genetically capable of producing antibody specific for one, or only a very few, antigenic determinants. As a result, each antigenic determinant

can trigger off different sets of cells and there can be no competition between non-cross-reacting antigens.

At the level of the plasmablasts or of the Y cell, there may be a genetically determined limitation of the maximum number of divisions of which these cells are capable. Thus, even in the absence of an inhibitory feedback mechanism, the size of the progeny produced may be limited.

Anatomical Factors Regulating the Immune Response

The existence of sites relatively protected from antigen (e.g. the thymus) may be a factor in allowing any antigen-sensitive cells present there to escape the triggering action of antigen. The anatomical pathways through which the immunologically competent cells recirculate in spleen and lymph nodes may be such that only a fraction of the recirculating cells can make effective contact with the antigen or with the macrophages that have processed the antigen.

Physiological Factors Regulating the Immune Response

The process of lymphocyte recirculation provides a nice mechanism for recruiting a particular class of immunologically competent cells from the blood into the spleen or regionally stimulated lymph nodes. The mere passage of these cells through spleen and lymph nodes may not be sufficient for their differentiation into antibody-producing cells: the cells may have to make effective contact with macrophages. Recent evidence suggests that antigen processed by macrophage is effectively immunogenic for only a limited period of time (approximately 6 hr—McCulloch 1967). Hence, for antibody production to occur, an antigen-sensitive cell must not only make contact with the macrophage but it must do so within 6 hours of the macrophage having ingested antigen.

Although there is no evidence to support this, it is not inconceivable that the antigen-sensitive cell itself may undergo cyclic phases during some of which it is refractory to antigen.

Immunological Factors Regulating the Immune Response

Both the antigen, which triggers off the response, and the antibody produced may be factors regulating the extent of the response.

The amount, type and rate of antibody produced depends in part on the nature and dose of antigen injected. Large doses of antigen give a maximal response over a wide range of dosages. Doses of antigen less than a critical amount produce a response of which the maximal amplitude is related to the amount of antigen given. With low doses of antigen, it is likely that fewer antigen-sensitive cells are recruited. It is also possible that the progeny of

stimulated antigen-sensitive cells may reach their full proliferative potential only when stimulated repeatedly by antigen. Thus, for instance, one molecule of antigen may serve only to prime the antigen-sensitive cells; a second molecule may be required to cause proliferation of the primed cells.

Once antibody is produced two opposing feedback mechanisms come into play: on the one hand, the opsonin activity of the antibody produced facilitates the rapid phagocytosis of the antigen so that a more efficient immunogenic stimulus is available to trigger off more responsive cells; on the other hand, the antibody, by its combining action with the antigen and its inhibitory action on antigen-sensitive cells or their progeny (see below), limits the extent of the immune response.

Several studies have established that specific antiserum injected into animals together with the corresponding antigen leads to a partial or complete inhibition of the primary immune response but not of the secondary response (Uhr 1964; Rowley & Fitch 1964; Möller & Wigzell 1965). 'Late' immune antiserum, containing predominantly IgG antibodies, inhibited (*a*) IgM production, when given together with the antigen, (*b*) IgG production when given from 1–3 days (but not 5–7 days) after the antigen, and thus at a time when IgM production was increasing exponentially, and (*c*) the development of IgG memory. In contrast, 'early' antiserum, containing predominantly IgM antibody was much less effective in inhibiting antibody production; some inhibition was obtained when the antibody was given either before or very shortly after the administration of antigen.

The inhibition of the primary response by antibody may simply be due to the combining of antibody with antigen destroying the antigen or preventing it from reaching the appropriate antigen-sensitive cells. However, this explanation is rendered unlikely by further experiments. The primary response to sheep erythrocytes of heavily irradiated rats transplanted with spleen cells from normal unimmunized donors of the same inbred strain was inhibited by a short incubation *in vivo* or *in vitro* of the donor cells with the specific antiserum before the cells were washed and injected (Rowley & Fitch 1964). The inhibition was specific as the immune response to other antigens was not impaired. These findings indicate that antibody does not prevent further antibody synthesis in already committed cells nor the further development of memory cells, rather it inhibits either the further proliferation of recently committed cells or the commitment or irreversible differentiation of antigen-sensitive cells which were recruited relatively late by antigen. Whatever the case may be, it provides a nice feedback mechanism serving to limit the immune response or to maintain reserve capacity in the pool of cells potentially capable of responding, or both.

If immunological factors do indeed regulate the immune response as outlined above, it becomes unnecessary to assume that there are multiple pre-existing clones of lymphoid cells genetically capable of reacting to only one antigenic

determinant. The virgin immunologically competent cells (X cells) may well be multipotential, i.e. potentially capable of reacting to many antigens. The immunological factors (antigen and antibody) regulating the immune response would themselves automatically create the heterogeneity of the type postulated to exist by the clonal selection theory. The normal action of antigen may be to drive some X cells, perhaps even the majority, towards tolerance, and to commit others to only one pathway of differentiation and response, the cell or its progeny being then pre-empted from ever reacting to any other antigen. On the other hand, the antibody produced may prevent all the remaining X cells from responding, at least temporarily, to the corresponding antigen. An automatic mechanism would thus be available for maintaining a pool of antigen-sensitive cells incapable of responding to a particular antigen but capable of responding to many other antigens.

ACKNOWLEDGMENT

Thanks are due to Dr M. A. Hession, editor of *Hospital Medicine*, for permission to reproduce Figs. 11.2, 11.3 and 11.5.

REFERENCES

ASKONAS B.A. & RHODES J.M. (1965) Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. *Nature* **205,** 470.

BUCKTON K.E. & PIKE M.C. (1964) Chromosome investigations on lymphocytes from irradiated patients: Effect of time in culture. *Nature* **202,** 714.

BOYDEN S.V. (1964) Cytophilic antibody in guinea-pigs with delayed hypersensitivity. *Immunology* **7,** 474.

BRENT L. & MEDAWAR P.B. (1963) Tissue transplantation: a new approach to the 'typing' problem. *Brit. med. J.* **ii,** 269.

BUCHEM VA, F.L. (1962) Histologisch onderzoek van de plasma-cellulaire reactie en zijn plaats in de histophysiologie van de lymphklier. M.D. Thesis, Groningen.

BURNET F.M. (1959) *The Clonal Selection Theory of Acquired Immunity*. Cambridge University Press, Cambridge, England.

BURNET F.M. (1962) Role of the thymus and related organs in immunity. *Brit. med. J.* **ii,** 807.

BURNET F.M. (1965) Somatic mutation and chronic disease. *Brit. med. J.* **i,** 338.

CLAMAN H.N. & TALMAGE D.W. (1963) Thymectomy: prolongation of immunological tolerance in the adult mouse. *Science* **141,** 1193.

COHEN E.P. & PARKS J.J. (1964) Antibody production by noninmune spleen cells incubated with RNA from immunized mice. *Science* **144,** 1012.

COHEN E.P. & TALMAGE D.W. (1965) Onset and duration of DNA synthesis in antibody forming cells after antigen. *J. exp. Med.* **121,** 125.

COHEN I.R. & NORINS L.C. (1966) Natural Human Antibodies to Gram-negative bacteria: Immunoglobulins G, A and M. *Science* **152,** 1257.

DIENER E. & NOSSAL G.J.V. (1966) Phylogenetic studies on the immune response. 1. Localization of antigen and immune response in the toad *Bufo marinus*. *Immunology* **10,** 535.

DRESSER D.W. (1962) Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. *Immunology* **5,** 378.

FISCHER D.S. (1964) Theories of antibody formation: a review. *Yale J. Biol. Med.* **37,** 1.

FISHMAN M. & ADLER F.L. (1963) Antibody formation *in vitro*. In *Immunopathology*, p. 79. IIIrd International Symposium. Schwabe, Basel.

FOLLETT D.A., BATTISTO J.R. & BLOOM B.R. (1966) Tolerance to a defined chemical hapten produced in adult guinea-pigs after thymectomy. *Immunology* **11,** 73.

FORD C.E., HAMERTON J.L., BARNES D.W.H. & LOUTIT J.F. (1956) Cytological identification of radiation chimaeras. *Nature*, **177,** 452.

FORD C.E. & MICKLEM H.S. (1963) The thymus and lymph nodes in radiation chimaeras. *Lancet* **i,** 359.

FRIEDMAN H. (1964) Antibody plaque formation by normal mouse spleen cell cultures exposed *in vitro* to RNA from immune mice. *Science* **146,** 934.

GARVEY J.S. & CAMPBELL D.H. (1957) The retention of S^{35}-labelled bovine serum albumin in normal and immunized rabbit liver tissue. *J. exp. Med.* **105,** 361.

GOOD R.A. & PAPERMASTER B.W. (1964) Ontogeny and phylogeny of adaptive immunity. *Adv. Immunology* **4,** 1.

GORDON G.B., MILLER L.R. & BENSCH K.G. (1965) Studies on the intracellular digestive process in mammalian tissue culture cells. *J. Cell. Biol.* **25,** 41.

GORDON H.A. (1959) Morphological and physiological characterization of germfree life. *Ann. N.Y. Acad. Sci.* **78,** 208.

GOWANS J.L. & KNIGHT E.J. (1964) The route of re-circulation of lymphocytes in the rat. *Proc. roy. Soc. B.* **159,** 257.

GOWANS J.L. & MCGREGOR D.D. (1965) The immunological activities of lymphocytes. *Progr. Allergy*, **9,** 1.

GOWANS J.L. and UHR J.W. (1966) The carriage of immunological memory by small lymphocytes in the rat. *J. exp. Med.* **124,** 1017.

GRANGER G.A. & WEISER R.S. (1966) Homograft target cells: contact destruction *in vitro* by immune macrophages. *Science* **151,** 97.

HOWARD J.G., BOAK J.L. & CHRISTIE G.H. (1966) Further studies on the transfer of thoracic duct cells into liver macrophages. *Ann. N.Y. Acad. Sci.* **129,** 327.

ISAKOVIC K., SMITH S.B. & WAKSMAN B.H. (1965) Immunologic tolerance in thymectomized irradiated rats grafted with thymus from tolerant donors. *Science* **148,** 1333.

ISHIZAKA K., ISHIZAKA T. & HORNBROOK M. (1966) Allergen-binding activity of γE, γG and γA antibodies in sera from atopic patients: *in vitro* measurements of reaginic antibody. *J. Immunol.* **98,** 490.

JERNE N.K. & NORDIN A.A. (1963) Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405.

KENNEDY J.C., TILL J.E., SIMINOVITCH L. & MCCULLOCH E.A. (1966) The proliferative capacity of antigen-sensitive precursors of hemolytic plaque-forming cells. *J. Immunol.* **96,** 973.

KEUNING F.J., VAN DER MEER P., NIEUWENHUIS P. & OUDENDIJK P. (1963) The histophysiology of the antibody response. II. Antibody responses and splenic plasma cell reactions in sublethally X-irradiated rabbits. *Lab. Invest.* **12,** 156.

LITTLE J.R., BRECHER G., BRADLEY T.R. & ROSE S. (1962) Determination of lymphocyte turnover by continuous infusion of H^3 thymidine. *Blood* **19,** 236.

LANGEVOORT H.C. (1963) The histophysiology of the antibody response. I. Histogenesis of the plasma cell reaction in rabbit spleen. *Lab. Invest.* **12,** 106.

MAURER P. (1963) Antigenicity of polypeptides (Poly Alpha Amino Acids). X. Studies with Polymers of D Amino Acids. *Proc. Soc. exp. Biol. Med.* **113,** 553.

MAXIMOW A.A. (1909) Der Lymphozyt als gemeinsame Stamzelle der verschiedenen Blutelemente in der embryonalen Entwicklung und cu postfolaten Leben der Sangetiere. *Folia Haematol.* **8,** 125.

MCCLUSKEY R.T., BENACERRAF B. & MCCLUSKEY J.W. (1963) Studies on the specificity of the cellular infiltrate in delayed hypersensitivity reactions. *J. Immunol.* **90,** 466.

MCCULLOCH E.A. & TILL J.E. (1962) The sensitivity of cells from normal mouse bone marrow to gamma radiation *in vitro* and *in vivo*. *Radiat. Res.* **16,** 822.

MCCULLOCH P.J. (1967) Unpublished results.

MEDAWAR P.B. (1958) The homograft reaction. *Proc. roy. Soc. B.* **149,** 145.

MEDAWAR P.B. (1963a) Introduction: definition of the immunologically competent cell. In *The Immunologically Competent Cell: its Nature and Origin*, pp. 1–3, ed. WOLSTENHOLME G.E.W. & KNIGHT J. Ciba Foundation Study Group No. 16. London: Churchill.

MEDAWAR P.B. (1963b) Conclusion générales. In *La Tolerance Acquise et la Tolerance Naturelle a l'Egard de Substances Antigeniques Definies*, pp. 473–6. C.N.R.S. Colloque, Paris.

METCALF D. (1956) The thymic origin of the plasma lymphocytosis stimulating factor. *Brit. J. Cancer.* **10,** 442.

METCALF D. (1964) Functional interactions between the thymus and other lymphoid tissues. In *The Thymus*, p. 53, ed. DEFENDI V. & METCALF D. Wistar Inst. Symp. Monog. No. 2. Philadelphia: Wistar Inst. Press.

METCALF D. (1966) The nature and regulation of lymphopoiesis in the normal and neoplastic thymus. In *The Thymus: Experimental and Clinical Studies*, pp. 242–63, ed. WOLSTENHOLME G.E.W. & PORTER R. Ciba Foundation Symposium. London: Churchill.

MILLER J.F.A.P. (1966) Immunity in the foetus and the newborn. *Brit. med. Bull.* **22,** 21.

MILLER J.F.A.P. & OSOBA D. (1967) Current concepts of the functions of the thymus. *Physiol. Rev.* **47,** 437.

MILLER J.J. III (1964) An autoradiographic study of plasma cell and lymphocyte survival in rat popliteal lymph nodes. *J. Immunol.* **92,** 673.

MITCHELL G.F. & MILLER J.F.A.P. (1967) The thoracic duct lymphocyte output of unanaesthetized mice thymectomized at birth. *Nature.* **214,** 992.

MITCHISON N.A. (1967) Immunological paralysis as a dosage phenomenon. In *Regulation of the Antibody Response*, ed. CINADER B. & THOMAS C.C. Fort Lauderdale.

MOLLER G. & WIGZELL H. (1965) Antibody synthesis at the cellular level. Antibody-induced suppression of 19S and 7S antibody response. *J. exp. Med.* **121,** 969.

MOLLER G. & MOLLER E. (1966) Interaction between allogeneic cells in tissue transplantation. *Ann. N.Y. Acad. Sci.* **129,** 735.

MORRIS B. (1966) Lymphoid cells—their role in the establishment of systemic immunity. *Proceedings of the XIth Congress of the International Society of Haematology*, pp, 25–36. N.S.W. Government Press, Sydney, Australia.

NELSON D.S. & BOYDEN S.V. (1963) The loss of macrophages from peritoneal exudates following injection of antigens in guinea-pigs with delayed-type hypersensitivity. *Immunology* **6,** 264.

NOSSAL G.J.V. (1962) Genetic control of lymphopoiesis, plasma cell formation and antibody production. *Intern. Rev. Exp. Pathol.* **1,** 1.

NOSSAL G.J.V. (1965) Mechanism of action of antigen. *Austr. Ann. Med.* **14,** 321.

NOSSAL G.J.V. (1966) Principles of antibody production. *Proceedings of the XIth Congress of the International Society of Blood Transfusion*, pp. 212–19. N.S.W. Government Press, Sydney, Australia.

NOSSAL G.J.V. & ABBOT A. (1966) Lymphoid cells and antibody formation. *Proceedings of the XIth Congress of the International Society of Haematology*, pp. 5–18. N.S.W. Government Press, Sydney, Australia.

NOSSAL G.J.V., ADA G.L. & AUSTIN C.M. (1963) Behaviour of active bacterial antigens during the induction of the immune response. B. The cellular distribution of I^{131}-labelled flagella. *Nature* **199,** 1259.

NOSSAL G.J.V., AUSTIN C.M. & ADA G.L. (1965) Antigens in immunity. VII. Analysis of immunological memory. *Immunology* **9,** 333.

NOSSAL G.J.V. & MAKELA O. (1962) Elaboration of antibodies by single cells. *Am. Res. Microbiol.* **16,** 53.

NOSSAL G.J.V. & MITCHELL J. (1966) The thymus in relation to immunological tolerance. In *The Thymus: Experimental and Clinical Studies,* pp. 105–30, ed. WOLSTENHOLME G.E.W. & PORTER R. Ciba Foundation Symposium. London: Churchill.

NOSSAL G.J.V., SZENBERG A., ADA G.L. & AUSTIN C.M. (1964) Single cell studies on 19S antibody formation. *J. exp. Med.* **119,** 485.

OSOBA D. & MILLER J.F.A.P. (1963) Evidence for a humoral thymus factor responsible for the maturation of immunological faculty. *Nature,* **199,** 653.

PERNIS B. (1967) The immunoglobulins present in the germinal centres. In 'Germinal centres in immune responses' Springer-Verlaag, New York.

PLAYFAIR J.H.L., PAPERMASTER B.W. & COLE L. (1965) Focal antibody production by transferred spleen cells in irradiated mice. *Science* **149,** 998.

PORTER R.J. (1964) Temporal studies on suppression by X-ray of adaptation for the secondary antibody response. *J. Immunol.* **92,** 425.

POTTER M. (1962) Plasma Cell neoplasia in a single host: a mosaic of different protein-producing cell types. *J. exp. Med.* **115,** 339.

PUCK T.T. & MARCUS P.I. (1956) Action of X-rays on mammalian cells. *J. exp. Med.* **103,** 653.

ROWLEY D.A. & FITCH F.W. (1964) Homeostasis of antibody formation in the adult rat. *J. exp. Med.* **120,** 987.

SCHOENBERG M.D., MURNAW V.R., MOORE R.D. & WEISBERGER A.S. (1964) Cytoplasmic interaction between macrophages and lymphocytic cells in antibody synthesis. *Science* **143,** 964.

SCOTHORNE R.J. (1957) Studies on the response of the regional lymph node to skin homografts. *Ann. N.Y. Acad. Sci.* **64,** 1028.

SELL S. & GELL P.G.H. (1965) Studies on rabbit lymphocytes *in vitro*. I. Stimulation of blast transformation with an antiallotype serum. *J. exp. Med.* **122,** 423.

SHELLAM G. & NOSSAL G.J.V. (1967) Mechanism of induction of immunological tolerance IV. The effects of ultra low doses of flagellin. *Immunology.* In press.

SMITH S.B., ISAKOVIC K. & WAKSMAN B.H. (1966) Role of the thymus in tolerance. II. Transfer of specific unresponsiveness to BSA with thymus grafting. *Proc. Soc. exp. Biol. Med.* **121,** 1005.

STROBER S. & GOWANS J.L. (1965) The role of lymphocytes in the sensitization of rats to renal homografts. *J. exp. Med.* **122,** 347.

SYEKLOCHA D., SIMINOVITCH L., TILL J.E. & MCCULLOCH E.A. (1966) The proliferative state of antigen-sensitive precursors of hemolysin-producing cells, determined by the use of the inhibitor, vinblastine. *J. Immunol.* **96,** 472.

TALIAFERRO W.H., TALIAFERRO L.G. & JAROSLOW B.N. (1964) *Radiation and Immune Mechanisms.* An American Institute of Biological Sciences and United States Atomic Energy Commission Monograph. New York and London: Academic Press.

TAYLOR R.B. (1964) An effect of thymectomy in recovery from immunological paralysis. *Immunology* **7,** 595.

TILL J.E. & MCCULLOCH E.A. (1964) Repair processes in irradiated mouse hematopoietic tissue. *Ann. N.Y. Acad. Sci.* **114,** 115.

THORBECKE G.J., ASOFSKY R.M., HOCHWALD G.M. & SISKIND G.W. (1962) Gamma globulin and antibody formation *in vitro*. III. Induction of secondary response at different intervals after the primary; the role of secondary nodules in the preparation for the secondary response. *J. exp. Med.* **162,** 295.

THOMAS L. (1959) Reactions to homologous tissue antigens in relation to hypersensitivity, discussion of paper by P. B. Medawar. In *Cellular and Humoral Aspects of Hypersensitive States*, pp. 504–29, ed. LAWRENCE H.S. New York: Hoeber.

TURK J.L. & STONE S.H. (1963) Implications of the cellular changes in lymph nodes during the development and inhibition of delayed type hypersensitivity. In *Cell-bound Antibodies*, pp. 51–60, ed. AMOS B. & KOPROWSKI H. Philadelphia: Wistar Inst. Press.

UHR J.W. (1964) The heterogeneity of the immune response. *Science* **145,** 457.

URSO P. & MAKINODON T. (1963) The roles of cellular division and maturation in the formation of precipitating antibody. *J. Immunol.* **90,** 897.

VOLKMAN A. & GOWANS J.C. (1964) The origin of macrophages from bone marrow in the rat. *Brit. J. Exp. Pathol.* **46,** 62.

WEIGLE W.O. (1962) Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens. *J. exp. Med.* **116,** 913.

WEISS L (1964) The white pulp of the spleen. *Bull. J. Hopkins Hosp.* **115,** 99.

WEISS N S., MITCHELL G.F. & MILLER J.F.A.P. (1967) The proliferation and differentiation of antigen-sensitive cells in neonatally thymectomized mice. *Nature.* **214,** 995.

WILLIAMS G.M. & NOSSAL G.J.V. (1966) Ontogeny of the immune response. I. The development of the follicular antigen-trapping mechanism. *J. exp. Med.* **124,** 47.

YOFFEY J.M. (1962) The present status of the lymphocyte problem. *Lancet* **2,** 206.

YOFFEY J.M., HUDSON G. & OSMOND D.G. (1965) The lymphocyte in guinea-pig bone marrow. *J. Anat.* **99,**841.

CHAPTER 12

IMMUNOLOGICAL FUNCTIONS OF LYMPHO-RETICULAR TISSUES

R.G. WHITE

INTRODUCTION

The early history of immunology included several vigorous polemics one of which arose out of the sharp division of opinion between the 'cellular' and 'humoral' schools. Today, the contributions of cells and serum factors are both acknowledged to be essential for adequate functioning of the immunity process. Within the lympho-reticular tissues, the family of plasma cells is clearly a major source of specific antibodies. Possibly non-specific defence factors such as complement are made by similar cells. The specific antibodies and complement facilitate the phagocytic activities of the leucocytes and the cells of the reticulo-endothelial system. The reticulo-endothelial cells apparently play a major role in disposing of antigenic material, and by this activity they may initiate and control the synthesis of antibody. The relation of reticulo-endothelial cells, plasma cells and lymphocytes, with, on the one hand antigen, and on the other antibody, provides the main substance of this chapter.

PHAGOCYTOSIS AND THE RETICULO-ENDOTHELIAL SYSTEM

The function of phagocytosis has existed from the first unicellular animal, and throughout the evolutionary stages of increasing specialization of cellular function the multicellular animal has always apparently provided for the retention of a corps of phagocytic cells. Ontogenesis apparently repeats this process. In the early stages of embryonic development the cells of all three germ layers are capable of phagocytosis. Later this capacity is concentrated within the leucocytes and the reticulo-endothelial system.

Viewed from the standpoint of the host-parasite relationship, one role of the lympho-reticular tissues throughout the body is to provide a strategically disposed and suitably conditioned set of phagocytic cells. The concept that phagocytes are of importance in resistance against infections originated in the eighties of the last century. The uptake of bacteria by cells was certainly described by many pathologists writing around the middle of the nineteenth century, but the phagocytic cells appear to have been generally conceived of as scavengers. Ziegler, indeed, stressed the role of the mobile phagocytes in facilitating the spread of bacteria through the tissues. It was to Metchnikoff, a rumbustious Russian zoologist, that we owe the clear enunciation in 1882 of the crucial role of phagocytic cells as the major host mechanism of defence against invading micro-organisms.

His own words clearly reveal the personal impact of this discovery:

> One day, when the whole family had gone to a circus to see some extraordinary performing apes, I remained alone with my microscope, observing the life in the motile cells of a transparent starfish larva, when a new thought suddenly flashed across my brain. It struck me that similar cells might serve in the defence of the organism against intruders. Feeling that there was in this something of surpassing interest, I felt so excited that I began striding up and down the room and even went to the seashore in order to collect my thoughts.
>
> I said to myself that, if my supposition was true, a splinter introduced into the body of a starfish larva, devoid of blood vessels or a nervous system, should soon be surrounded by mobile cells as is to be observed in a man who runs a splinter into his finger. This was no sooner said than done.
>
> There was a small garden to our dwelling, in which we had a few days previously organized a 'Christmas tree' for the children on a little tangerine tree; I fetched from it a few rose thorns and introduced them at once under the skin of some beautiful starfish larvae as transparent as water.
>
> I was too excited to sleep that night in the expectation of the results of my experiment, and very early next morning I ascertained that it had fully succeeded.
>
> That experiment formed the basis of the phagocyte theory, to the development of which I devoted the next twenty-five years of my life (Metchnikoff 1921).

Metchnikoff, from his studies in comparative physiology, was able to indicate the basic importance of these processes throughout evolution. He was able to

trace the continuity of phagocytic and digestive activity from the amoeba, in which nutrition and destruction or elimination of potentially harmful micro-organisms are inseparable functions within a living cell, through the colonial protozoa (sponges) in which two cell layers can be distinguished, an outer provided with flagellae and providing a means of locomotion, and an inner core of amoebic cells with phagocytic activity. In more developed sponges two types of phagocytic cells are distinguishable. The inner food canal is lined by endodermal phagocytes which capture the food (including bacteria) and water flowing past them, and pass these on to mesodermal phagocytes, which either digest them or reject them to the exterior. With the development of the ability to secrete digestive enzymes, phagocytosis becomes no longer a necessary attribute of the gut-lining cells and these are no longer phagocytic in higher animals.

Most of the evidence for the role of the phagocytes in defence is admittedly circumstantial. Metchnikoff gave as a prototype the interaction between the water flea *Daphnia* and the spores of a primitive type of fungus *Monospora bicuspidata*. Daphnia is a crustacean which is small enough and transparent enough to be directly observed in the living state under the microscope. The spores are narrow, pointed bodies which can penetrate from the gut into the body cavity. A direct correlation exists between resistance and the success of phagocytosis, and when this fails the fungus flourishes and its host, the water flea, dies.

In man, it could be argued that the successful immunity from bacterial invasion by a host of different 'non-pathogenic' microbes depends upon the unerring success of phagocytosis of these particular parasites. Certainly a diminution of phagocytic function, as by chemical depletion by benzol of circulating polymorphs (Rich & McKee 1939), or by splenectomy or blockade of the reticulo-endothelial system can lead to a lowering of resistance. Probably the clearest example is provided by a pathogenic organism, the pneumococcus. The virulence of this organism for the mouse (and other animals) is directly dependent upon its possession of one of a variety of type-specific surface capsular polysaccharides. *Within a fluid medium* the encapsulated organism is taken up by the polymorphs with extreme difficulty and clearance of such encapsulated organisms from the blood of the rabbit by macrophages is also relatively ineffective (Enders, Shaffer & Wu 1936). The ability of the pneumococcus to cause disease in the mouse is therefore inversely proportional to the ability of this animal to mobilize its phagocytes. Again this animal is susceptible to a very few encapsulated organisms by the intraperitoneal route, to a much larger dose by the intramuscular route, and may survive 10,000 organisms when they are given intravenously (Dutton 1955).

Besides their role in defence against potential parasites the phagocytes act to eliminate effete red cells from the circulation. Apparently only dead or senile cells are thus dealt with; the phagocytes do not actively determine the survival

time of the red cells; and thus the role of the reticulo-endothelial system is no more than a cemetery for dead cells. Nevertheless, this poses an important problem in respect of the ability of the phagocyte to recognize an age change in the red cell. Loutit & Mollison (1943) observed that one-third of red cells preserved for 28 days and subsequently transfused into human subjects were eliminated in the first 24 hours; the remaining two-thirds were eliminated in accordance with a linear function like fresh blood.

Besides their function in eliminating old and useless cells, one might predict a major role for the phagocytes in preserving the antigenic integrity of the individual. In acquired haemolytic anaemia associated with presence of warm incomplete auto-antibodies, sections of the spleen show extensive erythrophagocytosis by sinus-lining macrophages. In severe cases of this disease the elimination of transfused red cells may follow an exponential curve (Mollison 1962) suggesting that the erythrocytes are recognized and treated by the phagocytes like a foreign particle. Presumably, in this case, the presence of the auto-antibody at the surface of the cell may function as the opsonin, and also determine the specificity of the reaction. Whether any specificity of phagocytic action ever resides in the phagocytic cell *per se* is an important problem which is too involved to embark on in this context (see Chapter 13). Even with inorganic particles, evidence exists that their uptake by phagocytes is dependent upon specific serum factors. Thus absorption of serum with several of such inorganic particulate substances results in a specific loss of opsonic effect from the serum (Boyden 1962). Attempts to demonstrate a recognition mechanism in the phagocytes for protein molecules have proved inconclusive. Thus Weigle & Dixon (1958) were unable to show consistently that the blood clearance of a heterologous serum protein was more rapid in the early days after its injection into another animal species than that of a similar autologous or homologous serum protein. The phase of accelerated clearance of a heterologous serum globulin occurs at 6 to 8 days or thereabouts after a primary intravenous injection and considerable evidence supports the view that this phase results from the rise in production of circulating antibody.

The term macrophage refers to the fully activated free and mobile cell. The inactive form in the tissues is usually referred to as a histiocyte. For the purposes of this discussion we can regard the phagocytic cells as organized on three levels: the local inflammatory response (the area of primary lodgement), the lymph node and lymphatic chain (lymphatic lodgement), and the bloodstream lodgement. On the basis of the injection of certain particles and dyes, the histiocytes have been thought to constitute a *system*, the 'reticulo-endothelial system'. A large number of these cells are in contact with the blood; these are principally the Kupffer cells of the liver, the sinus-lining and reticular cells of the spleen and the sinus and the reticular cells of the bone marrow. However, a picture of the full extent of the reticulo-endothelial system cannot be obtained

merely by the injection of colloidal particles or dyes into the blood. By introduction of the same materials via the lymphatics a further system of sinus-lining and reticular cells can be visualized in lymph nodes and finally the direct introduction of these materials into any tissue results in their uptake by local tissue histiocytes.

The injected dye or metal is taken up by a vital reactive process which bears no relation to staining, and the cells in the neighbourhood are not coloured. If very weak solutions of the dye or metal are used the cells have the property of concentrating the dye within themselves. The term *reticulo-endothelial cell* and *system* should properly be applied to those elements which are revealed by the injection of colloidal dyes or suitable carbon particles. To extend this term, as is done in some quarters to include other cells of the reticular tissue, is to deprive it of its original *raison d'être*.

Understanding of the origin of such cells and the concept of them as a special corps of differentiated cells has derived from use of silver and gold impregnation techniques previously developed in neuropathology. By the use of an impregnation with silver carbonate, Hortega revealed one of the elements of the neuroglia as a thick rod-like cell body with numerous peripheral twig-like cytoplasmic processes. He called them the *microglia*. Hortega also clearly followed the transformation of such cells in foci of cerebral inflammation into rounded actively phagocytic amoeboid forms: the *compound granular* corpuscles.

The resemblance of such cells to macrophages elsewhere in the body prompted Hortega & de Asúa (1921) to study tissues outside the central nervous system by the same silver impregnation methods. By the use of this method, Hortega & de Asúa (1924) revealed the presence of a beautifully organized reticular system of cells in the spleen pulp. It is beyond the scope of this account to include a full description of the distribution and morphology of such 'metalophilic cells'. It is clear that they correspond in many situations with cells which are included in the reticulo-endothelial system of Aschoff, and by metallic impregnation of the tissues of vitally stained animals it can be shown that the impregnated cells are the same as those which contain the dye. Nevertheless, in the spleen and lymph nodes many metalophilic cells in the medulla of the gland and pulp of the spleen show little evidence of vital staining. The most likely explanation of these facts is that the dendritic metalophil cells which fail to stain intravitally with dye represent less avid precursors of the phagocytic forms. Alternatively in some situations it may be that circulatory factors prevent easy access to injected dyes.

Plate 12.1* shows a drawing of the arrangement of metalophil cells in a section of rabbit spleen. Four distinct types of cells can be distinguished. The first comprises the *amoeboid macrophages* which lie free in the lumen of the sinuses. The second includes fixed cells which extend as flattened spindle forms on the walls of the sinuses. The third type makes up the bulk of the red pulp. Many of

*Plates in this chapter follow p. 352.

these appear to be joined to form syncytial connections with their neighbours through numerous coarse and irregularly branching cytoplasmic processes. The fourth type is distributed around the periphery of the Malpighian bodies and appears as separated cells with much finer and more branched cytoplasmic processes than the previous type. They resemble in outline the microglia of the brain. Knowledge concerning the arrangement and differentiation of all these cells is of some importance. Thus the cells of the third and fourth types take up much lower doses of particulate dyes. Other evidence also exists of functional distinctions among various reticulo-endothelial cells. Lison & Smulders (1948) found that two forms occur in the liver: type G is distributed in the perilobular region and takes up particles with a diameter greater than 80 Å; type F in the walls of the sinusoids normally takes up particles of size less than 80 Å. Inactive stellate cells were also found in the centrilobular regions to change into active amoeboid cells after injection of large doses of dyes or stimulants such as choline or histamine.

Two results which are of great potential immunological significance derive from the finding that intravenously injected dyes fail to reach the substance of the brain or thymus gland. Even repeated injection of dyes leaves these two tissues uncoloured except for the choroid plexuses of the third and fourth ventricles, the capsule and trabeculae of the thymus. On the basis of the finding by silver impregnation techniques that both these tissues contain numerous microglia-like metalophil cells and that after a local injury (such as a cautery burn) the intravenously injected dye can penetrate to, and become taken up by such cells, 'blood-brain' and 'blood-thymus' barriers have been postulated (Marshall & White 1961).

Repeated injection of dyes reveals that the cells of this sytem have considerable powers of rapid multiplication in that if the system is presented with the task of disposing of a heavy load of circulating particulate material, there will be a relatively rapid increase in size of those tissues in which phagocytic cells are present in the greatest concentration. Splenomegaly, together with enlargement of the lymph nodes and liver, and proliferation of the cells of the bone marrow is therefore a systemic defence reaction common to a large variety of diseases, in which systematized reticulo-endothelial hyperplasia becomes a striking feature.

Measurement of Reticulo-Endothelial Function

Various attempts have been made to quantitate the functional activity of parts of the reticulo-endothelial system. There is, of course, no way to reach all of the widely scattered cells of this system, but fortunately a large number of these cells are in contact with the blood, and the phagocytic activity of this group can be studied and measured with the aid of intravenously injected colloidal suspensions which do not cross the capillary wall. The kinetics of this process

are studied by measuring the concentration of the particles in the blood at specific times after the intravenous injection. Particles which have been used include:

1. Carbon particles measuring about 250 Å obtained from 'Indian ink' (Günther Wagner, Hanover). The commercially available ink is 'stabilized' with fish glue and an essential preliminary to the use of these particles is the elimination of stabilizers by washing and centrifugation (Biozzi, Benacerraf & Halpern 1953).
2. Chromium phosphate particles marked with radioactive ^{32}P suspended in 5% dextrose.
3. Saccharated iron oxide particles suspended in a solution of saccharose and gelatin measuring about 25 Å (British Schering) (Benacerraf, Stiffel, Biozzi & Halpern 1954).
4. Heat-aggregated serum protein labelled with radioactive iodine ^{131}I (Benacerraf *et al* 1955).
5. Particles of a methyl methacrylate polymer marked with a fluorescent dye (H.W. 185) (Juhlin 1956).

In such a system the clearance of carbon or saccharated iron oxide particles can be expressed by an exponential function

$$C_t = C_o\, 10^{-kt}$$

when C_t is the concentration of carbon (iron oxide) at time t, C_o is the starting concentration of carbon (iron oxide) at time o.

The constant K is a measure of the clearance of particles and thus of the phagocytic activity of this group of cells under the conditions of the particular test. K varies inversely with the dose of carbon injected. In the case of saccharated iron oxide, for example, in the range of doses from 0.5 mg to 5 mg Fe per 100 g, the product of $K \times$ dose is remarkably constant. Benacerraf and his colleagues have verified that the relationship $K \times$ dose = a constant applies in various animal species (rabbit, rat, mouse and guinea-pig). Also, for doses in this range, the liver and spleen appear to be responsible for about 90% of this phagocytic activity. With larger doses of particles, and especially if unsatisfactory carbon particle suspensions (e.g. with added shellac) are used, appreciable amounts are also found in the lungs. The low efficiency of the phagocytes of the lung relative to those of the liver in monitoring the blood stream presumably derives from the presence of the organization of sinuses in the liver and their absence in the lungs. The efficiency of the hepatic mechanism is correlated with the extent of the portal blood flow. Removal of all the intestines of a rat greatly reduced the value of K showing that a reduction of portal blood flow is accompanied by a considerable diminution in the granulopectic activity, although the weight of the liver remains unchanged.

Rats and mice of different strains were found to show considerable differences in K values for the same dose of carbon. These differences were associated with differences in the relative size of the liver and spleen, and a *corrected phagocytic index* α could be derived as a measure of activity per unit of liver and spleen tissues.

In order to investigate the efficiency of phagocytosis, the above methods are satisfactory provided a large enough dose of colloid is used so that $K \times$ dose = constant.

The Functional Activity of the Reticulo-Endothelial System

Influence of Age and Environment

In comparing the effect of various drugs and procedures on phagocytic activity by these quantitative methods, several environmental factors need to be taken into account. The influence of cold is very striking. Rats which had a drop of body temperature down to 22–26° were shown to have low K values. Sewell (1962) has described a seasonal variation of phagocytic activity in mice with K values varying widely from winter to summer. In rats, Benacerraf, Biozzi, Halpern & Stiffel (1955) found that the phagocytic index K increases slowly from birth up to 3 weeks of age, and then decreases in the adult and in the older animal.

Effect of Physical State of Particles

Besides many colloidal or finely particulate materials, the cells of the reticulo-endothelial system are responsible for removing from the blood stream partially denatured proteins. They show extremely fine discrimination in detecting minor alteration in serum proteins which cause the molecules to aggregate, even though they stay in solution (Benacerraf, Biozzi, Halpern & Stiffel 1955).

Effects of Overloading

By overloading the cells with relatively large amounts of colloidal suspension, such as carbon in the form of Indian ink (16–48 mg per 100 g) or thorium dioxide, it is possible temporarily to depress their phagocytic activity towards themselves or other colloids. The animals, however, recover their normal phagocytic activity very rapidly, since within 3 days (rats) K has returned to normal. This recovery is associated with and apparently depends on an increase in the size of the liver and spleen. It is interesting that cortisone and nitrogen mustard, presumably by their action in inhibiting cell proliferation, maintain the phagocytic activity at a low level after a blocking dose of carbon, although the same dosage of cortisone or nitrogen mustard has no effect upon the phagocytic activity in normal rats (Benacerraf *et al* 1954).

Influence of Bacterial Endotoxin

Bacteria contain components which markedly alter phagocytic function. Biozzi *et al* (1955) found that from 4–10 hr after 5×10^9 *Salmonella typhi* heat-killed organisms were injected intravenously, the phagocytic activity of the reticulo-endothelial system of the mouse was greatly depressed. Within 24 hours the phagocytic activity returns to normal and then climbs to increased levels over 5–8 days. In the early years of this century Almroth Wright in his studies of typhoid vaccination observed a negative phase of resistance which ensued within a few hours of vaccine injection and preceded the development of demonstrable immunity. The same changes could be produced by endotoxin derived from a wide variety of Gram-negative bacteria. Also, if the same dose of killed *Salm. typhi* or endotoxin is repeated after 24 hours it no longer leads to a depression of the phagocytic index. In the presence of a hyper-reactive reticulo-endothelial system there is therefore an increased tolerance to endotoxin. Further evidence has been presented by Good & Thomas (1952) which relates the reticulo-endothelial system with the defence of the organism against the toxins of Gram-negative bacteria, for it is shown that a previous blocking injection of thorotrast to rabbits results in an enormous increase in susceptibility to endotoxin. A single injection of this toxin 6 hours after the administration of the blocking colloidal suspension results in the development of bilateral cortical necrosis of the kidney (generalized Schwartzman reaction) and death of most of the rabbits.

Also, as might have been expected from the well-known histological changes produced after infection with tubercle bacilli, this organism causes a marked stimulation of phagocytic activity. After injection of living BCG vaccine into the mouse the phagocytic index stays at a high level for at least 3 weeks. All bacterial infections do not produce the same effect, and large doses of dead *Staphylococcus aureus* produced no change in phagocytic activity as measured by these methods.

Hormone Influences

The influence of hormones on reticulo-endothelial function has been much studied. Even a cursory review of the literature of the past decade will yield abundant clinical evidence that infections constitute one of the important hazards of the prolonged use of corticosteroids clinically. The evidence relating to the effect of cortisone varies according to dose and species. In the rat, no effect on reticulo-endothelial blood clearance is apparent from the use of even large doses. In the mouse, moderate doses (5 mg per kilo) produce slight depression and higher doses (20 mg per kilo) severe depression. The rabbit on a dose of cortisone at 10 mg per kilo shows a striking deficiency in its ability to clear encapsulated pneumococci from the blood (White & Marshall 1951). However, it is apparent in mice that both hypo-adrenal as well as hyper-adrenal states confer increased susceptibility to infection (Kass 1960). Thus from the thera-

peutic point of view, cortisone dosage in a hypo-adrenal state needs to be carefully judged and too little or too much can be equally harmful. The ability of some steroid hormones to stimulate the reticulo-endothelial system has been explored by Nicol & Bilbey (1960) who found the highest activity in oestradiol benzoate.

The severe depressive effect of cortisone on antibody production (Berglund, 1956a and b) in a 'cortisone-sensitive' species like the rat could conceivably result from an effect on the activity of macrophages. However, cortisone causes a striking depopulation of lymphocytes from all the lymphoid tissues, and could therefore act by elimination of the precursors of antibody-forming cells.

The evidence of Weissman and Thomas (1962) that cortisone can stabilize the lysosomal membranes of phagocytic cells may be highly relevant and cortisone could impair the activity of phagocytes in antibody production by decreasing the hydrolytic breakdown by lysosomal enzymes of antigens within the macrophage.

Stimulation by Simple Substances

Recently Stuart, *et al* (1960) have found that several relatively simple organic substances such as olive oil and glyceryl trioleate cause pronounced stimulation of phagocytic function.

Morphological Differentiation of the Activated Macrophage

A homogeneous population of mononuclear phagocytes from the normal peritoneal cavity of the mouse will if maintained in tissue culture undergo a progressive increase in the size of individual cells without significant cell division. Phase-dense granules, mitochondria and lipid droplets accumulate in the cytoplasm. The phase-dense granules are lysosomes which react strongly for acid phosphatase. At the same time the culture shows marked increases in specific activity of other hydrolytic enzymes such as cathepsin and β-glucuronidase. Peritoneal macrophages from mice stimulated *in vivo* with bacterial lipopolysaccharide undergo a similar series of morphological and biochemical events (Cohn & Benson 1965). Contrariwise, the activation which is secured by triolein is unaccompanied by increase in lysosomal enzymes (Lee & Cooper 1964), and this substance is postulated to increase phagocytosis by a purely surface effect.

CELLULAR ASPECTS OF THE IMMUNOLOGICAL (ALLERGIC) RESPONSE

The introduction of foreign cells or macromolecules into the tissues of the host will be followed by certain cellular and humoral events, which are regarded as a

response to the 'antigenicity' of these. The terms *antigen* and *antigenicity* have often in the past been defined in terms of antibody production, but by current concepts the response to an antigen is fundamentally a series of cellular events and antibody production is to be regarded as an easily identifiable and conveniently measurable indicator of these. However, antigen-induced specific cellular changes may not necessarily be directed to an inevitable production of antibody, and cells may result which do not have as their immediate function the synthesis of antibody. Such cells which may bear the imprint of specificity and react to the presence of the specific antigen are referred to as *immunologically competent cells*. It also has to be accepted that one possible result of antigenic stimulation is to induce specific immunological 'tolerance' or antigenic 'paralysis'.

The Chemical Characteristics of Antibody

The reader will derive some idea of the complexities of the cellular apparatus which is necessary for antibody synthesis from a brief consideration of some properties of this class of proteins. After Tiselius had shown that plasma proteins could be separated on the basis of their mobilities in an electric field, it was soon observed that antibodies occurred in the γ-globulins, or more rarely, the β-globulins. But all subsequent efforts to classify antibodies rigidly in terms of electrophoretic mobility met with frustration and it is nowadays apparent that the class of proteins to which antibodies belong differs from other plasma proteins in not being homogeneous, but rather consisting of a family of a wide variety of closely related proteins. The clearest illustration of this is that in the technique of immunoelectrophoresis (see Chapters 1 and 13) the line of precipitation against anti-γ-globulin extends forwards through the β-globulins and even into the α-globulins.

It is clear that the definition of γ-globulin solely in terms of electrophoretic mobility leads to terminological difficulties. For this reason the term immunoglobulins has been proposed to describe the whole family of related globulins. In the rabbit, when the antigenic stimulus is intense, the antibody which is formed to any specific antigen appears to cover the whole range of electrophoretic mobilities of the immunoglobulins in this species. In other species (e.g. the horse and the guinea-pig) it is common to find that the antibody covers only a very limited part of the whole spectrum. The subject of the division of immunoglobulins into their main classes IgM (or γM), IgA (or γA), IgG (or γG), IgD (or γD), and IgE (or γE), etc. is dealt with in Chapter 13.

The reason for this complexity of antibody molecules is little understood but may involve the adaptability which these macromolecules must manifest under conditions of parasitic defence or for the maintenance of the antigenic integrity of the individual. It is also now apparent that different immunoglobulins are strictly compartmentalized in the multicellular organism. Thus the maintenance of immunity in the foetus depends upon selective permeation of

immunoglobulin γG molecules from the mother, while the milk and other secretions contain principally γA immunoglobulins.

These facts imply that the cells which make the different immunoglobulins are not the same but come from a number of distinct cell lines (or clones). This concept gains definite support from the disease state multiple myeloma, which has all the appearances of a neoplasm of immunoglobulin-secreting plasma cells. In mice such tumours can occur as peritoneal nodules following intraperitoneal injection of paraffin oil (technique of Potter). It is found that different tumours can produce electrophoretically different globulins. Moreover, different nodules taken from the same animal can produce quite different proteins when propagated separately in new hosts.

Maturation of the Immune Response

The human infant will usually start its life with a level of immunoglobulin in the blood which is derived from its mother, and is approximately equal to the normal adult concentration. In animals with a type of placental barrier which does not permit the transfer of antibodies, the serum at birth may be almost totally devoid of immunoglobulin. Thus piglets which are removed by caesarian section and prevented from obtaining maternal colostrum have only 40 μg per ml. of γ-globulin (Sterzl *et al* 1965). This small quantity was apparently made by the foetus since incorporation of methionine into this γ-globulin of the newborn could be shown by the use of Methionine-S^{35}. However, it could not be shown to have any detectable antibody activity. In the human newborn the level of immunoglobulin declines during the first 12–14 weeks of life since in this period synthetic activity is unable to maintain the level. At some time between the end of the 1st month and the 4th month of life, varying from child to child, the decline in γ-globulin gradually ceases and, as production develops, immunoglobulins rapidly accumulate in the serum.

Although the newborn of man and other species respond poorly, if at all, to many immunizing procedures it is not now accepted that the mammalian foetus is totally unable to respond to an antigenic stimulus up to the time of birth. Thus many reports have appeared recently on the ability of the foetus of many species to form circulating antibody. These include the foetal calf, lamb, opossum as well as the human foetus (Eichenwald & Shinefeld 1963). Presumably the inability to find plasma cells in a normal foetus merely reflects the protection afforded by the normal placenta against extraneous antigens. When this barrier is breached by the *Treponema pallidum* or *Toxoplasma gondii*, the human foetus responds by a precocious development of lymphoid tissues and impressive collections of plasma cells at the sites of infection (Silverstein & Lukes 1962). Toxoplasmic newborn infants may also show non-maternal macroglobulin antibodies in their cord blood (Eichenwald & Shinefeld 1963). Silverstein (1962) has speculated that the appearance of the lesions of congenital syphilis only

after the 5th or 6th month of gestation may indicate the time of onset of immunological competence in man. The fact that younger foetuses can be infected with treponemes but do not develop the lesions of syphilis has been interpreted as evidence that in the absence of immunological competence, the pathogenicity of the treponemes is not manifest. In the lamb an immunological response to bacteriophage with antibody production can be mounted by the 35-day foetus—just about the time that thymocytes first appear in the primitive thymus and before the appearance of functional lymph nodes and spleen (Silverstein & Kraner 1965). Also, according to the latter authors, the data in the lamb suggests that ability to produce antibody to different antigens appears at markedly different stages of gestation. Antibodies to the protein ferritin were not formed until the 65th day of gestation and antibodies to ovalbumin were not formed until the 125th day. The foetus appeared unable to respond throughout gestation to other antigens: diphtheria toxoid, *Salmonella typhi* or BCG. Competence to reject skin homografts developed only at about 80 days' gestation.

The initial antibody response by foetus, premature or full-term infant consists almost entirely of macroglobulin. Smith (1960) immunized human premature infants with typhoid vaccine. The antibody first formed to the H-agglutinin was macroglobulin (19S), in contrast to older children and adults in whom the major production was 7S:γG immunoglobulin. It is also claimed that when antigen in Freund-type adjuvant was injected into the foetal calf, large amounts of γ-globulin were produced while antibody only just reached detectable levels (Silverstein & Kraner 1965).

The most important source of early antigenic stimulation in nature is usually the bacteria of the gut. In so-called germ-free animals which are deprived of living (but not dead) bacteria in their food and environment generally, the lymphoid tissue in relation to the gut is considerably less developed than in a normal animal. This deficiency affects the development of germinal centres in the lymphoid nodules of Peyer's patches and there is a relative deficiency of plasma cells. Germ-free animals possess lower levels of γ-globulin in their serum than normal animals. In the 2–3 week old rabbit the production of γ-globulin, as shown by *in vitro* culture of isolated tissues, is confined almost entirely to the appendix. As will be discussed below, in the adult many other tissues are active in γ-globulin production (e.g. bone marrow, spleen and peripheral lymph nodes).

Sites of Antibody Production in Adult Animals

Antibody production is, in general, the concern of lymphoid tissues (other than the thymus in mammals) such as the spleen, lymph nodes and bone marrow. Thus in the rare 'Swiss type' of hypogammaglobulinaemia (see Chapter 19) in which a rudimentary development of the thymus is associated with a failure of development of all the lymphoid organs of the body, all immunological

responses including antibody production, graft rejection and delayed-type hypersensitivity are lacking (Hitzig & Willi 1961). Similarly selective damage to the lymphoid tissues by X-rays, anti-lymphocyte sera, nitrogen mustard and other radio-mimetic drugs, result in diminished immunological responses of all the above categories.

Broadly the situation seems to be that if antigen is injected repeatedly intravenously, antibody is produced mainly in the spleen, lung and bone marrow. When antigens are distributed by lymphatics from a local focus of infection or following injection of antigen, antibody is made predominantly in the group of regional draining lymph nodes. Under certain circumstances, and especially when the antigen is mixed with the so-called adjuvants, subcutaneous injection is followed by local granuloma formation and often antibody formation takes place locally in such granulomata.

The cells involved in the immunological responses leading to antibody production differ from those in delayed-hypersensitivity and graft rejection responses, and in recent years convincing evidence has accrued to support the view that these differing immunological responses are subserved by separate populations of lymphoid cells. Thus Warner, Szenberg and Burnet (1962), by injection of testosterone proprionate *in ovo*, prevented the development of lymphoid tissue in the bursa of Fabricius while allowing, in some birds, a normal thymus to develop. Such birds were unable to produce antibody following antigen injection, but could reject skin homografts in a normal manner. Other birds which developed a complete atrophy of the thymic cortex as well as bursal lymphoid tissue, had in addition impaired rejection of skin homografts. Furthermore, it is now evident that thymectomy, even in the newborn mouse, exerts a more powerful suppression of homograft rejection mechanisms and delayed-type hypersensitivity than of antibody production. On the basis of these facts, the question must arise as to whether any organ which is functionally equivalent to the bursa of Fabricius exists in the mammal; possibly among such entodermally related lymphoid organs as the tonsils, appendix and Peyer's patches.

In so far as both thymus and bursa of Fabricius are not themselves capable of producing antibody (see below) but either provide the cells for seeding other productive lymphoid tissues, or control by the production of hormones the development of these, it is possible (Miller, Marshall & White 1962) to envisage the existence of two levels of organization of the lymphoid tissues. Substantial support is given for this concept by the finding of Parrott, de Sousa & East (1966) that removal of the thymus from newborn mice results in depletion of lymphocytes from select areas of the cortex of lymph nodes which abut on the medulla and which have been termed 'thymus dependent areas', and which correspond topographically with the areas (termed tertiary cortical follicles by Scothorne & McGregor, 1955) which develop the large pyroninophilic lymphoid cells in

homograft reactions and the 'paracortical areas' of Oort and Turk (1965), which develop similar cells in reactions to chemicals sensitizing delayed hypersensitivity.

Antibody Formation by the Spleen

Evidence for the spleen as a site of antibody production comes from studies of the effects of splenectomy. The splenectomized rat (Rowley 1950a) is found to make little or no antibody when small amounts of particulate antigens are injected into the blood stream. If antigen is injected intraperitoneally or intradermally the splenectomized rat equals the intact animal. In humans also the production of haemolysins and haemagglutinins following the injection of small doses of foreign red cells intravenously was far less in splenectomized patients than in controls (Rowley 1950b).

These results would appear to stress the role of the spleen in forming antibody to antigens which are particulate and distributed via the blood stream. Therefore, in human haemolytic disease, when antibodies occur against antigenic components of erythrocytes it would be logical to expect that the spleen plays a foremost role in their production.

Antibody Formation Within Lymph Nodes

The major evidence for lymph nodes as producers of antibody was derived from the classic approach of McMaster & Hudack (1935). They controlled the possibility that the antibody which was extractable from lymph nodes was the result of seepage into an inflamed node of antibody which had been formed elsewhere, by injecting two different antigens of killed bacilli, one into each ear of a mouse. The injections would, of course, cause the regional nodes of both sides to become more permeable than normal, but the antibodies of the two kinds would have an equal chance to concentrate in the inflamed area. In point of fact it was found that agglutinins to the antigen injected in the right ear appeared earlier and in high concentration in the lymph nodes draining that ear, but not in the lymph nodes draining the other ear. Conversely, in the cervical nodes of the left side antibodies appeared first to the antigen injected in the left ear, and not to that injected on the right side.

Further evidence for the fact that the main production of antibody occurs in the grouping of lymph nodes in the region draining the lymph from the site of injection will be considered later, when the individual cells involved are considered. But it should be stressed that the method and route of administration of antigens determines which tissues predominate in antibody production. Presumably this pattern results from the fact that the antigens (or at least that part which is effective in antibody production) are rapidly fixed in the lymph tissues which first receive the drainage of fluid containing these. It is surprising to find that this holds not only for the particulate antigens, like bacilli or erythrocytes, but also for soluble protein antigens.

Local Production of Antibody

The foregoing discussion concerning the spleen and lymph nodes has stressed that the preliminary fixation of the antigen is an important factor in determining whether a tissue manufactures antibody. It is possible to summarize the vast amount of work which has investigated the possibility of local production of antibody by the general statement that, providing methods are used which secure the local fixation of the antigen, almost any tissue is found capable of accumulating antibody-producing cells.

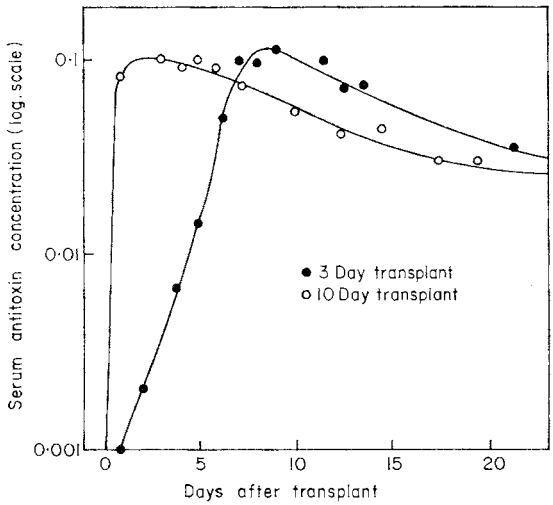


Fig. 12.1. Local production of antibody by a fat transplant. Serum antitoxin rises in two rabbits, which received a 3-day, and a 10-day transplant into the omentum. Each donor was injected primarily with 60 Lf of diphtheria APT (alum-precipitated toxoid), 28 days afterwards a secondary injection of 12 Lf of diphtheria toxoid was made into the interscapular fat, before transplantation.

From Oakley, Warrack & Batty (1954) *J. Path. Bact.* **67**, 485.

The problem can be approached by the same technique which was used to show antibody production in lymph nodes, i.e. by extraction of the local tissue, and finding that the antibody titre of the extract is greater than in the plasma. The same objection occurs, as with the lymph node studies, that antibody has merely become concentrated rather than synthetized in the local tissues, but the same device of injecting two antigens, one at the site of study and one elsewhere, can control this concentration effect.

A second approach was to transplant tissues from immunized animals to non-immunized recipients and to show that antibody appeared in the recipient at about the time that it would have appeared in the donor. Oakley, Batty &

Warrack (1951) transplanted fatty tissue, after local injection of alum-precipitated diphtheria toxoid, into the omentum of rabbits, with results as shown in Fig. 12.1. It will be noted that whereas the 10-day transplant produced antibody from the beginning, the 3-day transplant required a day or two to get going—behaving as it would have done in the donor. It may be objected that the antigen persisting in the tissue transplanted could induce the recipient to form antibody anyhow. But such primary formation would not start until a much longer time had elapsed following the transfer. It should be realized that the antibody in this case is apparently not formed by the fat cells but by the cells of the granulomata which develop about the alum-precipitated toxoid antigens.

An important aspect of the local production of antibody is whether this is possible within the organs which house antigen-containing fluids such as the bladder, uterus and mammary glands. After the introduction of *Trichomonas* and *Brucella abortus* into the uterus and vagina, it is possible to extract antibody from the walls of these organs. There is also evidence that when *Br. abortus* is introduced into the 'milk cistern' of one-quarter of a cow's udder agglutinins to this antigen appear in the milk from that quarter well in advance of agglutinins from other quarters, suggesting very strongly that antibody can be formed in a local sector of the udder (Giltner, Cooledge & Huddleson 1916). The problem of such local antibody production has recently assumed new interest and complexity from the demonstration by Chodirker & Tomasi (1963) that γA globulin is the predominant immunoglobulin type in human parotid saliva, colostrum and lacrimal secretions. The γG/γA ratio in this group of fluids is less than one, whereas in normal human serum this ratio is approximately six. Moreover, Tomasi *et al* (1965) found that γA of colostrum and saliva differs from serum γA since: first, it is more highly polymerized and consists principally of a component with a sedimentation coefficient of 11S; secondly, it is not disrupted by disulphide bond reduction in the absence of urea; and thirdly, saliva and colostrum γA has an additional antigenic determinant. Evidence from radio-immunoelectrophoresis (Hochwald, Jacobson & Thorbecke 1964) has revealed that both salivary and mammary tissue are able to incorporate ^{14}C-labelled amino acids into newly synthesized γA *in vitro*, although this technique is unable to indicate the fraction of the total γA which is locally produced. Nevertheless, even if most of the production derives from circulating plasma immunoglobulin, the results of Tomasi and his colleagues must indicate that an additional 'transport piece' of the γA molecule is added during the selective transport of this minor polymeric form of γA by the glandular epithelium.

Antibody Production in the Liver

It seems that several of the plasma proteins are formed in the liver, which would appear to be the only site of formation of the plasma albumin, β-globulin and fibrinogen. Miller & Bale (1954) showed for instance that the

isolated rat's lever, when perfused with ^{14}C-lysine, vigorously incorporated this radioactive amino acid into these three fractions of the plasma proteins, but showed little or no incorporation into γ-globulin. Contrariwise, the perfusion of the eviscerated carcase of the rat produced only plasma proteins with the mobilities of γ-globulins. Since antibodies are regarded as ordinarily associated with the γ-globulin fraction of serum, this evidence argues strongly against the role of the liver in antibody production.

Another way to study the proportional activity of the different tissues in the synthesis of antibody, is to measure the incorporation of radioactive amino acids into the antibody formed by various tissues taken from antigen-injected

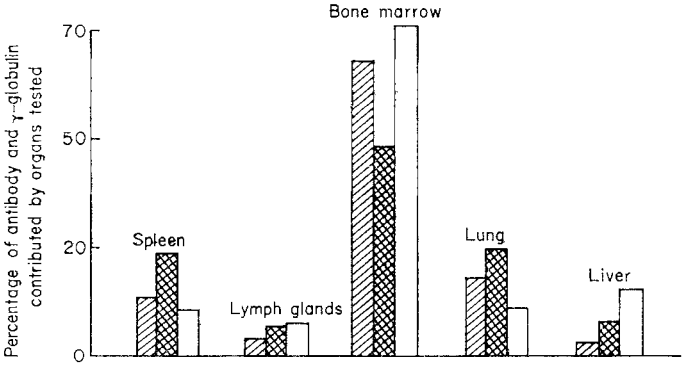


FIG. 12.2. Synthesis of specific antibody to ovalbumin and to pneumococcus type III capsular polysaccharide and non-specific γ-globulin by tissues of a rabbit maintained a few hours *in vitro*. Percentages are shown of antibody and γ-globulin contributed by different tissues to the overall synthesis, in a rabbit immunized by intravenous injection of alum-precipitated ovalbumin and pneumococcus type III.

▨ anti-pneumococcus polysaccharide III
▩ anti-ovalbumin
□ non-specific γ-globulin

From Askonas & Humphrey (1958) *Biochem. J.* **68**, 252.

animals and maintained in tissue culture. The technique consists in the incubation of tissue slices in a nutrient medium over a period of 4–5 hr, in the presence of ^{14}C-labelled amino acids. At the end of the incubation period the antibody produced is isolated by specific precipitation and estimated quantitatively by its count of emitted radiation. Although such a method does not, without numerous assumptions, give a true estimate of the absolute rate of synthesis, the technique is valid for comparing one tissue with another and one method of immunization with another. By allowing for the total weight of the tissue in the animal, the relative contributions of the different tissues can be easily calculated.

The results of this method are shown in Fig. 12.2 for the different organs of a

rabbit hyper-immunized with intravenous injections of killed pneumococcus type III and alum-precipitated ovalbumin. It will be seen that these results confirm the above evidence of a relatively insignificant production of antibody by the liver. Moreover, this method of administration of antigen (by intravenous injection of a particulate antigen) would be expected to favour the role of the liver, since under these circumstances the phagocytes of the sinuses trap a very high proportion of the antigenic material. As is seen in Figs. 12.3 and 12.4 when the antigen is injected intra-muscularly into a rabbit or subcutaneously into a guinea-pig, the liver plays an even smaller role.

As will be seen later, antibody production within the liver can usually be

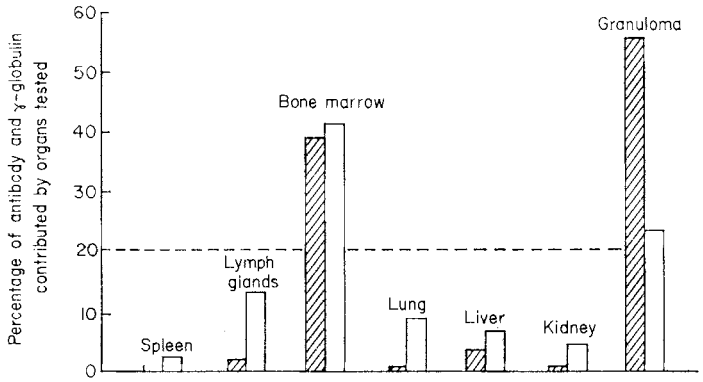


FIG. 12.3. Synthesis of specific antibody to ovalbumin and non-specific γ-globulin. The individual tissues were maintained during a period of a few hours *in vitro*. Percentages are shown of the contributions made by the different tissues to the overall synthesis, in a rabbit immunized by intramuscular injection of ovalbumin in Freund's adjuvant mixture.

▨ anti-ovalbumin
□ non specific γ-globulin

From Askonas & Humphrey (1958) *Biochem. J.* **68**, 252.

correlated with the presence of invading cells forming scattered periportal granulomata. The true hepatic parenchymal cell does not seem capable of forming antibody.

ANTIBODY FORMATION IN THE BONE MARROW

The scattered nature of the bone marrow has resulted in this tissue receiving less attention from the ablation and isolation techniques of the experimental pathologist. In the experiments of Miller & Bale, quoted above, the significant contribution of the eviscerated carcase of the rat to the synthesis of γ-globulin was interpreted as depending upon the activity of the bone marrow chiefly.

In the tissue culture experiments, as illustrated in Figs. 12.2, 12.3 and 12.4, the

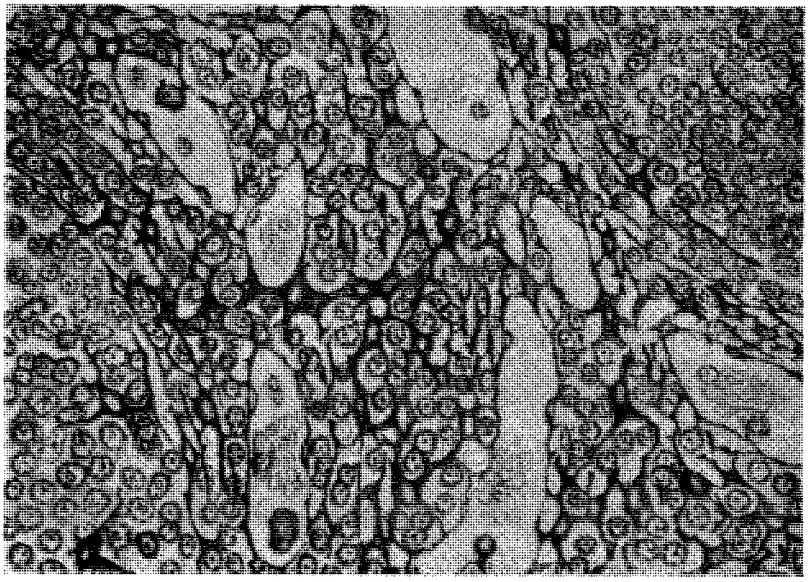


PLATE 12.1. Drawing in pencil of the pulp strand of a normal rabbit spleen, made by the author from a preparation of Dr A. H. E. Marshall treated by Weil-Davenport method to demonstrate argyrophil cells (Marshall & White 1950). *Note:* the four morphological types of reticulo-endothelial cell:

(i) spheroidal macrophages lying free in the lumina of the sinuses;
(ii) sinus-lining cells;
(iii) coarsely branching reticular cells lying in the stroma of the red pulp, whose processes appear to join with or overlap those of neighbouring cells;
(iv) finely branching reticular cells occurring at the periphery of the Malpighian bodies which resemble microglia in the form of their cyto-plasmic branches.

facing p. 352

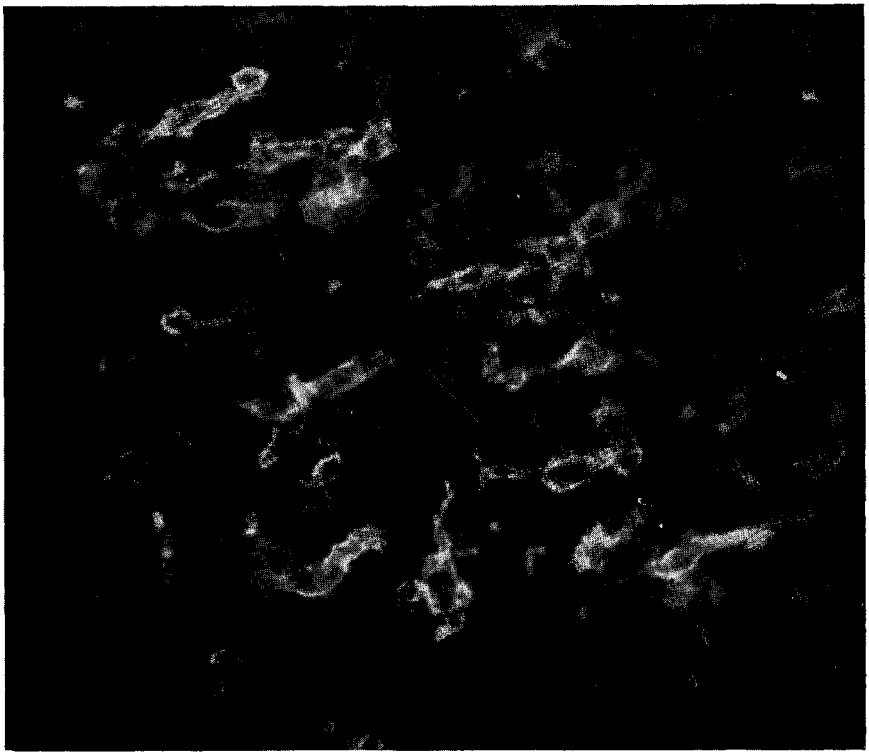


PLATE 12.2. Fluorescence micrograph. Single layer technique using fluorescein isothiocyanate-labelled rabbit anti-human serum albumin to demonstrate antigen (HSA) denoted by surface fluorescence following the dendritic outline of scattered cells within a germinal centre of the chicken spleen at 6 days after injection of 1 mg of HSA intravenously. × 1100.

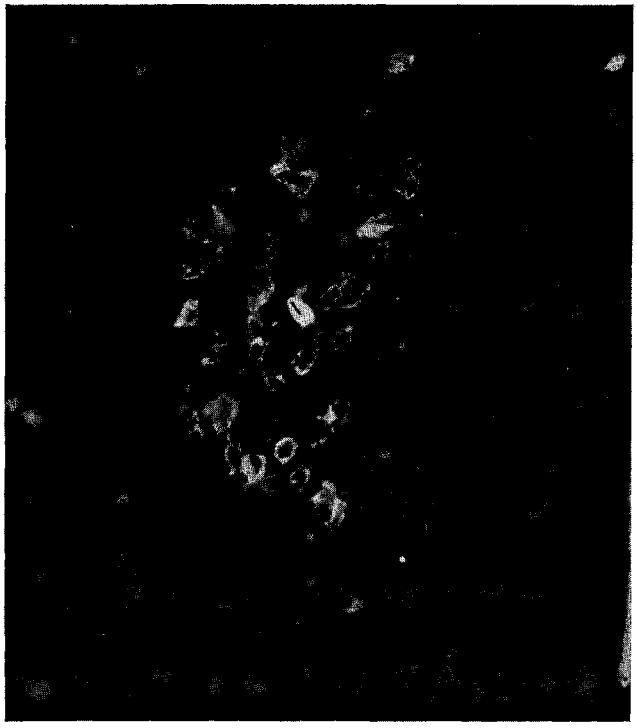


PLATE 12.3. Fluorescence micrograph. Single layer technique using fluorescein isothiocyanate-labelled rabbit anti-human serum albumin to demonstrate antigen (HSA) localized to 'dendritic macrophages' within a small, early germinal centre of the chicken spleen at 4 days after injection of HSA intravenously. ×400.

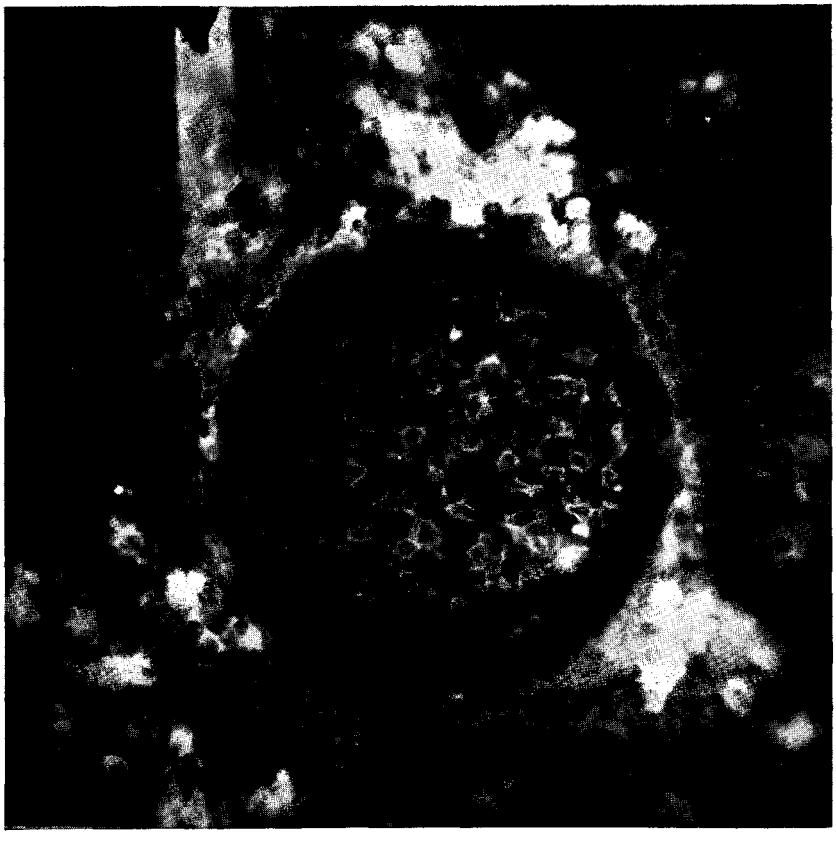


PLATE 12.4. Fluorescence micrograph. Single layer technique using fluorescein isothiocyanate-labelled anti-chick γ-globulin to demonstrate the sites of chicken antibody. It is seen that a pattern of outlined dendritic cell bodies is produced similarly to the pattern of antigen (Plates 9.3 and 9.4) showing the association of antibody with antigen in and on dendritic macrophages within a germinal centre of the chicken spleen. $\times$ 400.

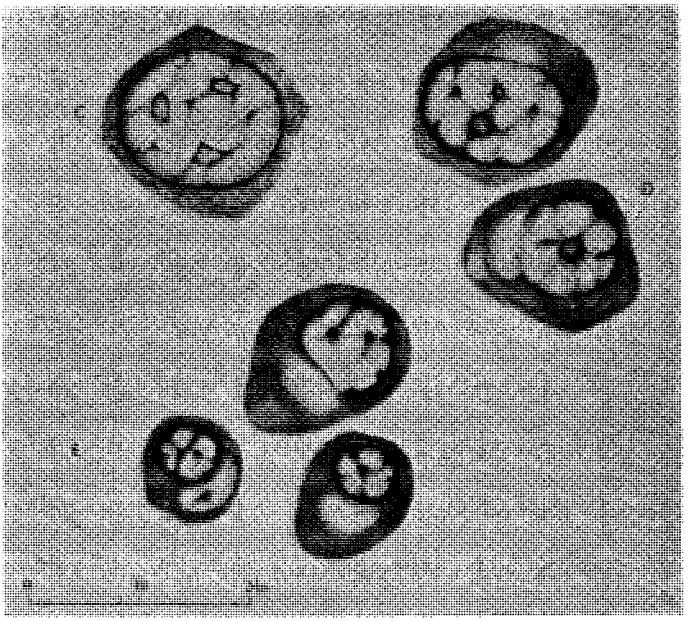


PLATE 12.5. Drawing of typical members of the plasma cell series stained by haematoxylin and pyronin as they appear in formalin-fixed, paraffin-embedded tissues.

C. haemocytoblast (plasmablast, transitional cell);
D. immature plasma cells;
E. mature plasma cells.

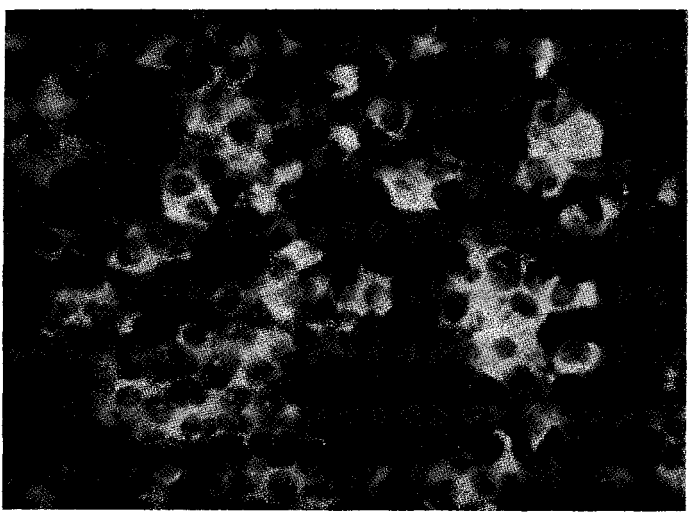


PLATE 12.6. Fluorescence micrograph. Sandwich technique to demonstrate anti-ovalbumin in a frozen section of the regional lymph node of a rabbit 4 days after a recall injection in a hyperimmunized animal. Antibody appears as a bright fluorescence throughout the cytoplasm and as irregular areas within the nuclei of some cells. The cells which show the presence of antibody are mainly immature plasma cells, with a few mature plasma cells, in the medullary cord of the node (magnification × 600).

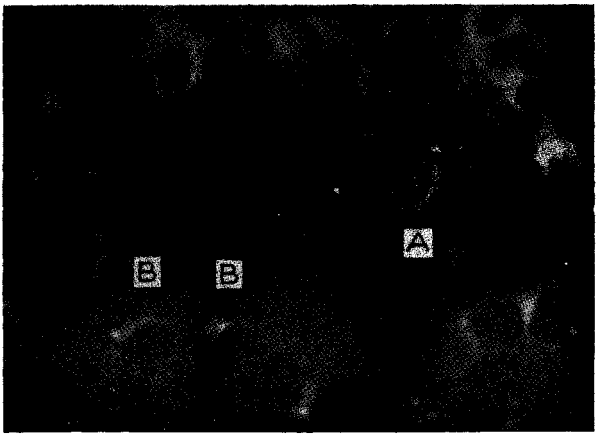


PLATE 12.7. Fluorescence micrograph. Sandwich technique used to demonstrate the presence of diphtheria antitoxin in the regional lymph node 2 days after a secondary stimulus. *Note* the haemocytoblast A, and the two immature plasma cells B, B. In the latter the greatest concentration of antibody can be seen to be in and around the region of the Golgi element. *Note also* the irregular ring of antibody within the nuclear shadow of the haemocytoblast (magnification × 800).

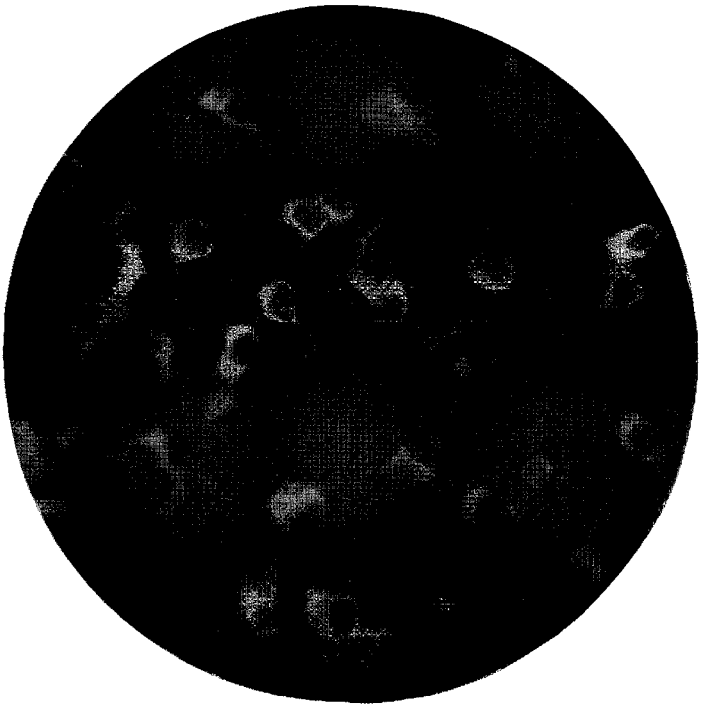


PLATE 12.8. Fluorescence micrograph. Sandwich technique used to demonstrate diphtheria antitoxin in the regional lymph node of a rabbit 4 days after a secondary stimulus. *Note* the mature and immature plasma cells within a medullary cord (magnification × 600).

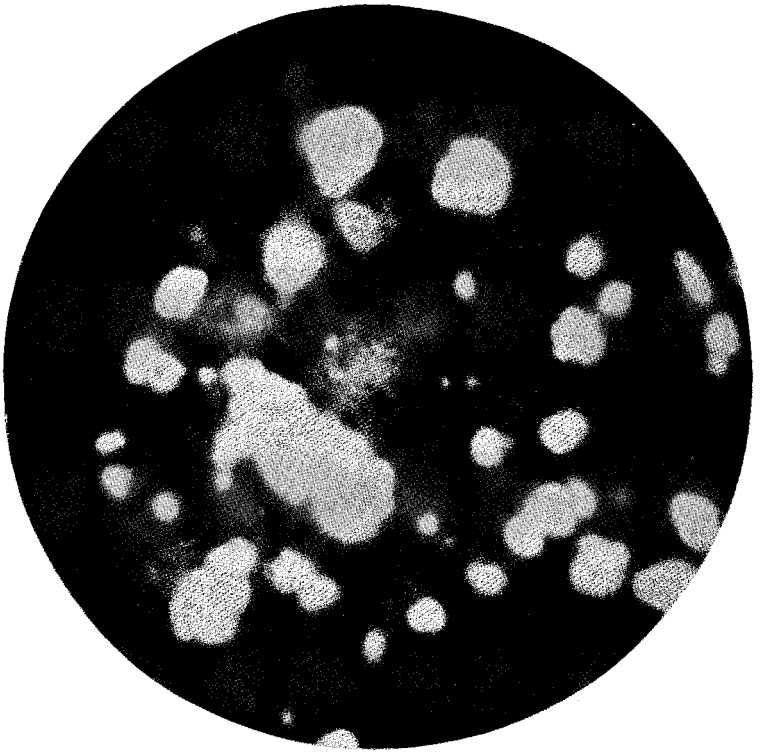


PLATE 12.9. Fluorescence micrograph. Russell bodies appearing as clusters of brilliantly fluorescent round objects within the cytoplasm of plasma cells. From a lymph node 6 days after a secondary stimulus of ovalbumin. In addition to the 'solid' spheres, there are a few with a 'hollow' appearance (magnification × 1200).

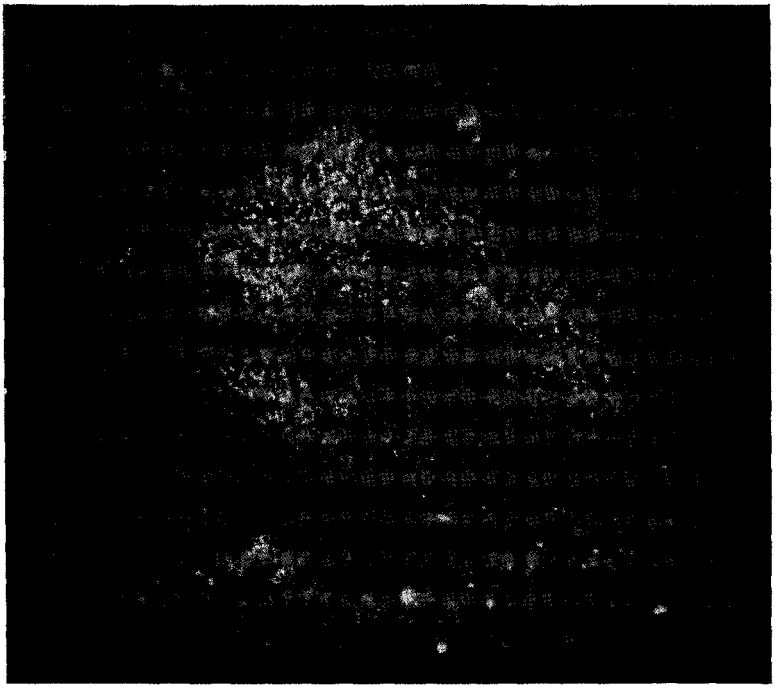


PLATE 12.10. Fluorescence micrograph. Cortical lymphoid module of a regional lymph node, showing antibody within and between the cells of the germinal centre (magnification × 200).

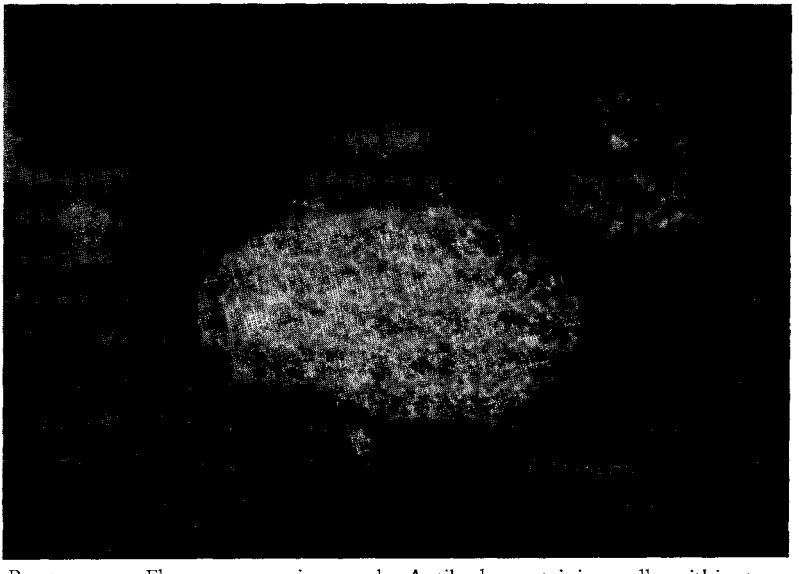


PLATE 12.11. Fluorescence micrograph. Antibody-containing cells within two germinal centres of a chicken at 14 days after a primary injection of 1 mg of human serum albumin. Sandwich technique for antibody to human serum albumin. *Note* the almost total absence of medullary antibody-containing cells, and the presence of antibody in the majority of the cells within the larger germinal centre. × 200.

bone marrow, by all three methods of antigen administration, made a major contribution to the overall calculated antibody synthesis. The activity per unit mass of this tissue was never strikingly high, but the total bulk of the bone marrow so far outweighs that of the other lympho-reticular tissues that its importance for antibody production ranks relatively high. The immunoglobulins involved in these experiments were principally 7S γ_1 and γ_2, since the tissues were excised from guinea-pigs at 3 weeks. Bone marrow taken at 8–10 days after injection of bacteriophage appeared to produce almost all the macroglobulin 19S antibody of the animal (Fleming, Wilkinson & White 1967).

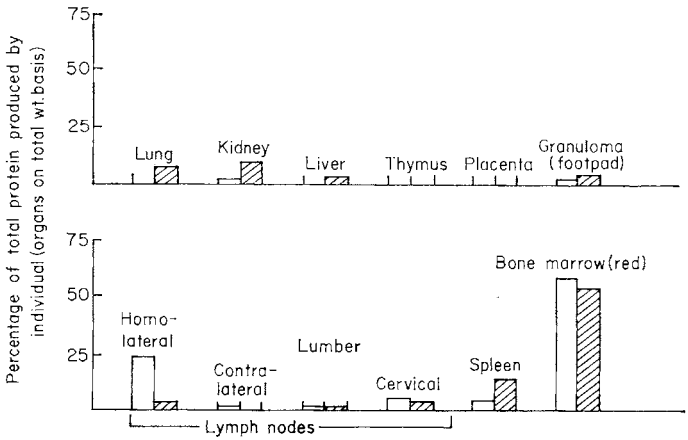


FIG. 12.4. Percentages of antibody and non-specific γ-globulin contributed by the different tissues to the overall synthesis in a guinea-pig, which was immunized by injections of an oily emulsion of ovalbumin with added Wax D of *Mycobacterium tuberculosis* into the footpad.

□ anti-ovalbumin.

▨ non-specific γ-globulin

From Askonas & White (1956) *Brit. J. exper. Path.* **37**, 61.

ANTIBODY FORMATION IN THE THYMUS

Most workers seem agreed that little antibody is made in the thymus (see Fig. 12.4), although by sensitive methods such as radio-immunoelectrophoretic analysis of cultured glands, some synthesis of immunoglobulin can be detected. But even when particulate or other antigens are repeatedly injected in a vein, extraction of the thymus fails to reveal any more antibody than similar extractions of muscle, and there is no production of plasma cells or germinal centres. Such a behaviour could result if antigen failed to reach the thymic cells, and Marshall & White (1961) postulated the lack of a receptor system for antigens. Clark (1963) has shown that the thymic cells are separated from connective

tissue and blood vessels by a basement membrane and a continuous layer of epithelial-reticular cells. However, this epithelial layer did not offer an absolute barrier to soluble antigens since ferritin and albumin were found to pass into the gland (Clark 1964). When antigens are injected directly into the thymus large numbers of antibody containing cells result. It could be objected that this cellular reaction might result from imported cells from the blood just as it might happen in a tissue-like muscle or adipose tissue. However, lymphoid nodules with germinal centres also resulted and clearly suggested that the reaction involved thymic cells. Finally, the lack of reactivity could depend on incompetence of the thymic lymphocytes, although several claims to have demonstrated the competence of transplant populations of such cells in antibody production and graft-versus-host reactions have been made.

Antibody Production in the Central Nervous System

Somewhat similar considerations apply in the case of the central nervous system, which also shows no uptake of intravenously injected vital dyes or carbon particles, due presumably to the existence of a hypothetical 'blood-brain' barrier. In point of fact, potential antigenic components appear to exist within the normal brain which when injected into the extra-neural parts of the body give rise to antibody production. This argues that the brain must lack the power of responding under normal conditions to antigens within its substance. This may be associated with the lack of a system of lymphatics in the brain draining into lymph nodes. However, when once an immune response is established to the antigens of the brain by repeated injection into the animal of brain tissue, this immune response can react upon the antigens already present in the brain and cause damage (disseminate allergic encephalomyelitis).

Homografts are often well tolerated when grafted into the normal brain, presumably since no immune response is forthcoming. However, animals who have made a successful response resulting in the rejection of a graft from a given donor which was in this case implanted on the skin of the animal, can also react against skin grafts from the same source which are implanted into its brain.

One might argue from this that if the mechanism for the immune response were set up by antigen absorption outside the central nervous system the presence of antigen within the system could then cause an immune response and possibly antibody production.

Certainly, in neurosyphilis the Wassermann reaction is occasionally positive in the cerebrospinal fluid and negative in the blood stream. Morgan, Schlesinger & Olitsky (1942) injected monkeys intracerebrally with poliomyelitis virus and demonstrated the highest antibody titres in the body within the anterior horns of the spinal cord and little or no antibody in the blood. In such inflammatory conditions the cells which are associated with antibody formation

elsewhere in the body—macrophages, lymphocytes and plasma cells may accumulate in the lesions of the nervous tissue and would provide the most probable cellular site of the formation of such antibody.

ROLE OF THE MACROPHAGE SYSTEM IN THE INITIAL MANIPULATION OF ANTIGEN

Following the work of Metchnikoff, Aschoff and others, the uptake of bacteria and all kinds of foreign particulate matter by macrophages led to the easy assumption that these cells must process the antigen to form antibody, and the fact that blockade with an overdose of inert particles can reduce antibody production was at least consistent with this view. At present the role of the macrophage is far from clear. No one has yet followed antigen into a cell and succeeded in showing a subsequent production of antibody *by the same cell.* This argues at least that this simple role for the macrophage is highly unlikely and the blockade evidence would be consistent with the concept that the macrophage is essential merely for the initial reception or manipulation of the antigen.

However, one point at least is clear: there is an obvious discrepancy between the ability of a tissue to take up antigen and its ability to synthetize antibody. This is clearly so for the liver, which as discussed previously, will take up 90% of an intravenous dose of particulate antigen, and yet contributes only a small fraction of the subsequently synthesized antibody. Indeed Campbell and Garvey (1961) showed that less than 2% of injected antigen goes to the major lymphoid organs of the antibody-forming system. In other situations too, it is possible to show that macrophages neither contain nor synthesize antibody. Askonas & White (1956) studied the granuloma which develops after the injection of antigen in Freund-type adjuvant mixture into the footpad. This tissue, although packed with hypertrophic macrophages, some of epithelioid type, proved to be a relatively poor antibody producer when maintained *in vitro.*

Another difficulty arises from the fact that the dose of an antigen necessary to provoke antibody formation is far less than that used in histological investigations on the fate of the same antigen. Thus in the classical studies of Coons & Kaplan (1950) with pneumococcus polysaccharides the amounts injected ranged from 0.5 to 10 mg per mouse. According to Felton (1949) the dose necessary to produce immunity is half a microgram—a thousandfold lower dose. In point of fact, with this antigen in the mouse, doses of 1 mg and upwards produce immunological paralysis rather than immunity. Nossal, Ada & Austin (1964) have shown that remarkably small doses of protein antigens, namely, 100 ng (0.1 μg) and 10 ng (0.10 μg) of flagellin caused peak antibody titres (7S). Doses as small as 100 pg (0.0001 μg) occasionally caused antibody production.

The foregoing kind of evidence has led to the alternative view that the macrophage merely acts to catabolize antigens and by this function acts to protect the

antibody producing cell system from exposure to excessive antigen which would result in paralysis. Thus, Crampton, Frankel & Rodeheaver (1960) have argued that any cell which takes in a large dose of antigen would be precluded from antibody production.

In tissue culture, it has not generally proved possible to induce antibody production by simple exposure of a 'pure' population of lymphoid cells such as blood or thoracic duct small or large lymphocytes to antigen, although there are claims to have achieved this in both primary and secondary responses (Holub & Riha 1960; Elves, Taylor & Israëls 1963). However, the work of Fishman (1959) and his colleagues (reviewed in Fishman, van Rood & Adler 1965) has shown that specific antibody production can be initiated in normal rats or cultures of lymph node cells from such animals by ribonucleic acid-containing extracts of peritoneal macrophages which had been incubated with antigen *in vitro*.

The concept that the basic cellular unit for antibody production consists of an antigen-processing macrophage working in concert with the precursors of the actual antibody-producing cell (plasma cell) has received histological support recently from two independent series of investigations. Ada, Nossal & Pye (1964) and Nossal, Ada & Austin (1964) studied the localization of antigens of various kinds trace-labelled with ^{131}I or ^{125}I injected in small amounts into rats.

Antigens in doses as small as 16 ng, which is near to the lowest immunizing dose, localized in the macrophages of the medullary sinuses of lymph nodes and in the germinal centres of lymphoid nodules. The pattern of labelling over the follicles suggested that the antigen was attached to macrophages and not to the main cells—the medium and large lymphocytes (haemocytoblasts). Ada, Nossal & Austin (1964) studied a range of soluble and particulate antigenic substances, e.g. flagellin, bovine serum albumin, horse ferritin, diphtheria toxoid, fowl erythrocytes, and found that all consistently localized to macrophages in germinal centres. Homologous, presumably non-antigenic rat serum albumin, rat haemoglobulin and rat erythrocytes did not localize to the same site. On the basis of these results, it was postulated that a 'recognition mechanism' capable of distinguishing between self and non-self body components existed in respect of antigen localized at these sites. A possible exception was rat gamma-globulin which was found to localize to the germinal centres of the lymphoid nodules in spite of its 'self' origin. The authors concluded that this exception could have significance in indicating that antibody might act as 'opsonin' in allowing the specialized macrophages of the germinal centres to capture the antigen.

The localization of antigen had been previously studied by the fluorescent antibody technique by Coons, Leduc & Kaplan (1951). White (1963) had shown that proteins such as human serum albumin, and diphtheria toxoid when injected

intravenously into chickens, localized to 'dendritic macrophages' (Plate 12.2) of the white pulp. This localization could not be detected until about 48 hours after injection and then was seen to be associated with elongated spindle-shaped cells, which were scattered throughout the white pulp, around the central arteries. At this time no antigen could be seen within the germinal centres of the spleen. Later (at 3 and 4 days) antigen was localized to dendritic cells (Plate 12.3) within small germinal centres, as well as outside in the white pulp. At 6 days all the detectable fluorescence denoting antigen was within germinal centres appearing as a pattern of surface fluorescence outlining the cell body and processes of about fifteen to thirty scattered dendritic cells (Plate 12.2). Antigen clearly persisted in and on these cells for at least 15 days. This fact alone would indicate a special significance for this small fraction of the total injected antigen.

Further work has shown that antibody is associated with antigen at the surface of the dendritic cells, and may be essential for the localization process (White, French & Stark 1967; White 1967). Thus sections treated with fluorescein-labelled anti-chick γG gave a similar pattern to that of the localized antigens within the germinal centres. Further it was found that no localization of antigen would occur to the dendritic cells in chickens which were rendered tolerant by repeated injections of the homologous antigen (human serum albumin) from the time of hatching, presumably since the tolerant birds lacked circulating homologous antibody. These results are at variance with those of Ada, Nossal & Pye (1965) who found that localization, as detected by autoradiography, occurred normally in specifically tolerant rats.

The dendritic macrophages possibly correspond with the metalophilic cells resembling microglia, which have been discussed in a previous section and illustrated in Plate 12.1. They are regarded as a specialized form of the spleen or lymph node macrophages and to differ functionally from the amoeboid sinus macrophages or the reticulum cells of the medulla. Indeed, the long persistence of antigen on these dendritic germinal centre cells argues a functional difference. Possibly the reason for the apparently specific localization of antigen to these cells is that antigen initially enters reticulo-endothelial cells of all kinds but persists only in the dendritic cells owing to a defect in the intracellular hydrolytic activity of these cells.

The close structural and functional relationship between antigen-loaded cells and lymphocytes in the germinal centre has obvious similarities to the concept of Bessis & Breton-Gorius (1962) for the erythroblastic island in bone marrow. In this case reticular 'nurse' cells at the centre maintain contact with several layers of erythroblasts by extensive pseudopodia and it is claimed that transfer of ferritin across this surface can be visualized by the electron microscope. The relation of antigen localization in dendritic cells to the formation of germinal centres will be discussed below.

PLASMA CELL REACTIONS

If a bacillary vaccine or foreign protein, such as horse serum, is repeatedly injected intravenously into, say, a rabbit, after a week or so there appears an impressive increase in the size of the spleen, the pulp strands of which become massively infiltrated with pyroninophilic plasma cells. Such cells accumulate not only in the spleen, but in many other situations, such as the lungs—which become converted eventually into what is practically a lymphoid organ; the fatty tissue around the renal pelvis, and in smaller collections in the periportal zones of the liver (Fagraeus 1948).

The hypothesis that these pyroninophilic cells were engaged in the synthesis of antibody globulin was stimulated by the work of Caspersson and his Scandinavian colleagues on the association between cytoplasmic ribonucleoprotein and protein synthesis in protein-secreting glands such as the pancreas and the salivary glands. In the plasma cell, the accumulation of nucleotides has produced one of the cell's most characteristic features—its marked basophilia. While other cells of the reticular tissue show basophilia when young, presumably since they are vigorously synthesizing protein for growth, such cells as the granulocytes, lymphocytes and erythrocytes all lose their cytoplasmic ribonucleoprotein with the process of differentiation. The plasma cell, on the contrary, maintains basophilia as a mature cell.

Fagraeus (1948) used the methyl-green-pyronin technique to describe the developmental stages of the families of cells found in reticular tissues after antigen injections. First in the series was the cell termed by Fagraeus the transitional cell, which by the methyl-green-pyronin staining method is indistinguishable from the haemocytoblast (see Plate 12.5C) the precursor cell of the erythrocyte and leucocyte series of cells. The next stage was termed the immature plasma cell (Plate 12.5D). The leptochromatic large nucleus of the haemocytoblast stage has become smaller, its chromatin strands were condensed. The basophil cytoplasm has become somewhat more extensive on one side of the nucleus and a definite clear area can be made out against the nucleus. In the final stage, the mature plasma cell (Plate 12.5E), the process of condensation of nuclear chromatin has gone further to produce a small, round and very eccentric nucleus. The nuclear membrane is thick with lumps of chromatin arranged peripherally at its inner surface so that the well-known 'clock face' nucleus is produced at least in formalin-fixed, paraffin-embedded material. The cytoplasm is deeply basophil except for a prominent bean-shaped area overlapping the nucleus, an area corresponding with the Golgi element. The Golgi element is a prominent feature of all protein-secreting glandular cells, e.g. the pancreas, thyroid and salivary glands.

Further evidence for the plasma cell as a unicellular gland comes from recent studies with the electron microscope which show that the area of cytoplasm

corresponding with the basophilia is occupied by a complex system of parallel membranes: the endoplasmic reticulum or ergastoplasmic membranes. This is shown in Fig. 12.5. Such membraneous systems are present in the cytoplasm of various known protein secreting cells. By way of contrast, the small lymphocyte

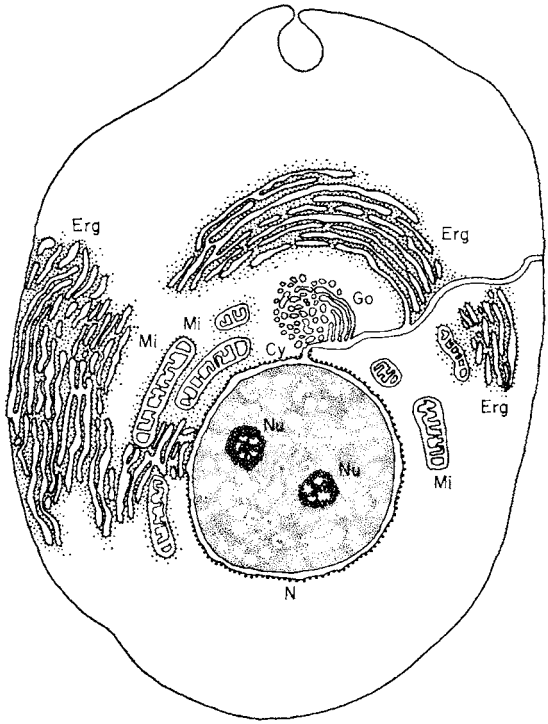


FIG. 12.5. Diagrammatic representation of the ultra-structure of a typical plasma cell or other protein-secreting cell.

N—nucleus with nucleolus (Nu).
Go.—Golgi complex.
Mi.—mitochondria.
Erg.—membranes of the endoplasmic reticulum or ergastoplasm with attached ribonucleoprotein granules (ribosomes)—RN.
Cy.—cytoplasmic matrix containing free ribosomal granules.

has a cytoplasm which is almost devoid of endoplasmic reticulum. Also its Golgi element is quite tiny.

The essential unit of the endoplasmic reticulum as seen in sectioned cells is a pair of parallel membranes seen in profile. These are regarded as making up a system of communicating sacs or *cisternae*, the lumina of which can be empty or occupied sometimes by the proteinaceous secretion of the cell. The membranes

are fairly uniformly around 70 Å thick and on the outer surface show regularly spaced granules or projections 150 Å diameter. Beyond the membranes and between adjacent similar structures is the cytoplasmic matrix, which includes particles which are of a similar size and probably are identical with the attached granules and which appear to be scattered freely between the membranes. These particles consist in large part of ribonucleic acid and they are termed ribosomes. The Golgi element consists of a collection of smooth-surfaced sacs which lack granules.

By analogy with the intracellular mechanisms which have been postulated for enzyme synthesis by pancreatic cells, the antibody molecules may be formed on the surface of the ribosomes. The endoplasmic reticulum may carry out the secondary segregation and intracellular transfer of such secreting products. The specificity of the antibody product is regarded as a property of the molecular configuration of the RNA occupying the ribosomal particle. The ultimate template for specific synthesis is the nuclear DNA; by the transfer of RNA of specific configuration (messenger RNA) to the cytoplasmic ribosome, the nucleus remotely controls the synthetic process.

The actual presence of antibody within the cytoplasm and nuclei of the members of the family of plasma cells was shown by Coons, Leduc & Connolly (1955) using an extension of the basic fluorescent antibody method (Coons & Kaplan 1950) which has been aptly termed the 'sandwich' technique. Antibody in an area of a frozen section exposed to a solution of specific antigen will fix some of the latter, and antigen thus fixed can be detected, after washing away the unfixed portion, by exposure to a solution of fluorescent antibody. The sites of deposition of the latter can be localized under a fluorescence microscope. A control slide without the intermediate layer of antigen will distinguish between antigen in the section and antigen added *in vitro*.

The results of such a technique applied to various situations in the tissues of an immunized rabbit are shown in Plates 12.3, 12.4 and 12.5. Plate 12.3 shows the appearance of antibody-containing cells in the spleen pulp strands of a hyperimmunized rabbit. In such masses of cells it is difficult to discern the individual cell-types. This can be achieved more easily in the cellular responses following a single secondary-type stimulus. Early in such a response, at 48 hr, isolated haemocytoblast forms occur. They are later accompanied by increasing numbers of immature plasma cell-types (Plate 12.7). At the 4th day the cells include a fair proportion of mature plasma cells. The fluorescence extends throughout the cytoplasm and appears to correspond with the area of basophilia or the area of endoplasmic reticulum in an electron micrograph. Like the basophilia in the mature plasma cell it appears to spare a juxta-nuclear area which corresponds with the Golgi element. In the immature plasma cells, on the other hand, the highest concentration appears to be found in the neighbourhood of the Golgi element (Plate 12.7) which would agree with the concept of Dalton and Felix

that the latter acts as an energy source to withdraw water from the secretory product of the microsomes, prior to the admission of this to the *cisternae* of the endoplasmic reticulum.

Antibody in the nucleus appears as irregular streaks or sometimes as an irregular ring around an oval or circular dark area (Plate 12.5). Such an appearance raises the possibility that a part of the antibody is actually synthesized within the nucleus, possibly in the region just outside the nucleolus.

Some plasma cells contain globular masses of antibody, and at times such objects, brilliantly fluorescent, are the most conspicuous objects in the section. They correspond to certain intracellular acidophil hyaline bodies which have been termed Russell bodies, after William Russell of Edinburgh, who in 1890 described their occurrence in the granuloma at the margin of malignant growths. Some years ago (White 1954) it was shown possible to induce the formation of plasma cells containing Russell bodies in the spleen following the intravenous injection of a wide variety of antigens. The Russell bodies appeared in greatest numbers with the mature plasma cells at the end of a secondary-type response. By the Periodic-Acid Schiff technique Russell bodies appear a bright purple; a reaction which suggests a high carbohydrate content. Material with the same staining properties can appear in adjacent plasma cells as lozenge-shaped crystals. In the case of the experimentally produced Russell bodies White (1954) showed by the sandwich fluorescent antibody that they gave an intensely bright reaction for specific antibody (Plate 12.9).

THE LYMPHOCYTES

Unfortunately for the accuracy of histological description the lymphocyte is a difficult cell to characterize. The typical small lymphocyte has a nucleus which is round, fills most of the cell, stains densely with basic dyes such as haematoxylin and lacks obvious features such as nucleoli. The cytoplasm is 5–8 μ in diameter and possesses only a few mitochondria close to the nucleus and a tiny Golgi element. However, while a high proportion of cells in the lymph (as for example in the thoracic duct of a rat) has this appearance, 5–10% are larger than 8 μ diameter and gain the title of 'medium and large lymphocytes'. More important than their size, the intensity of the basophilic staining of the cytoplasm is variable and may be very intensive in some of the medium-sized cells. Usually this takes the form, in the electron microscope, of cytoplasm packed with large numbers of free ribosomes but in some cells Braunsteiner and Pakesch (1960) and others have observed an extensively developed endoplasmic reticulum (see below). In other words, cells previously referred to as lymphocytes may in reality be immature plasma cells. Gowans, Gesner & McGregor (1961) found that small lymphocytes of thoracic duct lymph which have been labelled *in vitro* with thymidine can pass out of the blood and appear in the cortical tissue of

lymph nodes as large pyroninophilic cells. The cells used were homologous parental cells which were transfused into F_1 hybrid recipients and presumably the acquisition of a larger size and a pyroninophilia (which has been shown in the electron microscope to be due to accumulation of free ribosomes) represents a reaction of the parental cells against the additional antigens containined in F_1 hybrid host cells. This reaction might therefore correspond to the observed transformation of lymphocytes in the tertiary nodules of a lymph node regional to a homograft (Scothorne & McGregor 1955) but does not represent a conversion of lymphocytes into plasma cells for the purposes of antibody formation. Indeed in the work of Gowans and his colleagues, the pyroninophilic large lymphocyte apparently divided and again assumed the morphology of small lymphocytes.

The small lymphocyte of the blood is a cell which recirculates from the blood out into the lymphatic tissue of lymph nodes (and possibly spleen) and back through the lymphatic channels and thoracic duct to the blood (Gowans & Knight 1963). Continuous drainage of the circulating pool lymphocytes from a rat by a thoracic duct fistula over 6 days so impairs the immunological capacity of the animal that it cannot undertake a primary response to sheep erythrocytes (McGregor & Gowans 1963). In other words, a pool of cells which are essential for the immunological competence of the whole animal are in the circulating blood, and the fact that these are the 'small lymphocytes' is shown by the fact that the animal can be restored to competence by transfusing with a purified suspension of these cells separated from the withdrawn mixture from the thoracic duct lymph.

It would seem, therefore, that the initial problem of antigenicity is to arrange a liaison between the appropriate members of this population of recirculating cells and the antigen as it enters this pathway. Presumably this occurs in the areas of organized lymphoid tissue in most instances but the organization is such that in, say, the special circumstances of graft rejection, the encounter can be by cell to cell contact of virgin lymphocyte and xenotypic cell within the graft (Strober & Gowans 1965). Relatively little more is presently known about the situation as it relates to graft rejection except that a major part of the subsequent development of small lymphocyte to large pyroninophilic cells (large lymphocytes) takes place in the regional lymph node (Scothorne & McGregor 1955) and that transplantation immunity can be transferred to other normal animals by cells obtained from such regional (but not from more remote) lymph nodes (Billingham, Brent & Medawar 1954).

A histological reaction which has repeatedly been observed to follow the injection of antigens into experimental animals is the formation of germinal centres (or lymphocytopoietic centres) in the lymph nodes and spleen (Sjövall & Sjövall 1930; Conway 1937; Marshall & White 1950). The course of this response as it is seen in the spleen of the rabbit following a course of injections of

horse serum or TAB vaccine intravenously is as follows. Starting at 3–4 days at sites within the white pulp, foci of large stellate cells with somewhat basophil cytoplasm appear. These are in relation to the sheath of the cortical blood vessel, have been regarded as arising from adventitial primitive reticular cells, and referred to in their basophilic form as activated reticular cells. Mingled with these are also typical haemocytoblasts (lymphoblasts, large lymphocytes). Mitotic figures are obvious and later these foci of dividing cells appear to enlarge to round or oval masses of medium and later of small lymphocytes. Judged by the proportion of the total number of cells which are in mitosis, cellular multiplication in such a centre reaches a peak at 8–10 days. The synthesis of so much nuclear material should reflect a high turnover of phosphorus and Gyllensten, Ringertz & Ringertz (1955) have shown very high rates of ^{32}P uptake in such centres (in their experiments in lymph nodes) after antigenic stimulation. The centre appears to grow larger for perhaps another 8 or 10 days, but gradually loses its clear contrast against the surrounding dark cortex.

It has been made clear by several authors (e.g. Sjövall & Sjövall 1930) that the germinal centre response after subcutaneous injection of antigen is a localized change, or at least develops far more in regional than in remote lymph nodes. After the intravenous injection of antigens the formation of germinal centres is largely confined to the spleen although the lymph nodes of the lung may also react. How long after the antigen stimulus active cell division continues is unknown, and also the fate of the newly formed cells is obscure.

Germinal centres can be formed as a local response to antigen. A dramatic illustration of this is provided by their presence in lymphadenoid goitre (Hashimoto's disease of the thyroid) where they probably result from a chronic leak of thyroglobulin and other segregated thyroid antigens (White 1957). Germinal centres are also seen in the thymus after direct injection of antigens (Marshall & White 1961).

In hyper-immunized animals it is not uncommon to see antibody in the germinal centres of the spleen and lymph nodes (Coons, Leduc & Connolly 1955). This phenomenon has been analysed in the rabbit (White 1960) and chicken (White 1963). In the rabbit it was found that antibody regularly appears in the cells of the germinal centre at 3–6 days after a secondary stimulus, providing that both primary and secondary injections were made into the same region of lymphatic drainage. Control animals which received a single, or primary, injection only did not show antibody in the regional centres of the regional nodes. Often the resulting pattern of fluorescence outlined the cytoplasm of a proportion of the lymphoid cells (haemocytoblasts and medium lymphocytes) in the centre. At other times the fluorescence assumed a reticular pattern (Plate 12.10). Evidence that the majority of the lymphoid cells produced by a germinal centre can be immunologically competent is contained in Plate 12.10. In the chicken, an injection of 1 mg of human serum albumin intravenously will lead

to the appearance of a population of antibody-containing plasma cell derivatives in the splenic red pulp at 6–8 days later. This plasma cell reaction in the red pulp has disappeared almost totally by 12 days. Then, from 13 days onwards antibody-containing cells made their appearance in the germinal centres of the white pulp (Plate 12.10). These cells have the appearance of medium basophilic lymphocytes or immature plasma cells. Mature plasma cells only rarely appear in this situation.

The evidence of these experiments in the chicken and rabbit is compatible with the primary antigenic stimulus causing the *de novo* formation of germinal centres. In the chicken the initial localization of antigen to dendritic macrophages throughout the white pulp (after 24 hours) followed by the progressive concentration of dendritic cells within germinal centres (from 4 days onwards) suggests that the antigen-bearing dendritic cells become aggregated in association with other non-antigen bearing cells (small lymphocytes) into spherical collections which, when they have reached a critical size and when the individual cells have reacted by enlargement and division can be discerned against the surrounding lymphoid tissue as an early germinal centre. This formation could occur by a kind of mixed agglutination reaction between dendritic cells bearing antigen at their surface and lymphoid cells bearing complementary marker antibody at their surface. On this view, the antigen-antibody complex at the surface of the white pulp dendritic cells would act as a *lymphocyte-netting mechanism* for filtering off and gathering together into germinal centres a selected population of cells able to react with specific antigen from the recirculating pool of small lymphocytes. By providing the stimulus for division of the gathered cells into larger numbers of similarly reactive cells the germinal centre could provide the basis for immunological memory—the enhanced reactivity of the secondary type response (White 1960).

REFERENCES

Ada G.L., Nossal G.J.V. & Pye J. (1964) Antigens in Immunity. III. Distribution of iodinated antigens following injection into rats *via* the hind footpads. *Aust. J. exp. Biol. Med. Sci.* **42**, 295–310.

Ada G.L., Nossal G.J.V. & Austin C.M. (1964) Antigens in Immunity. V. The ability of cells in lymphoid follicles to recognize foreignness. *Aust. J. exp. Biol. Med. Sci.* **42**, 331–46.

Askonas B.A. & White R.G. (1956) Sites of antibody production in the guinea-pig. The relation between *in vitro* synthesis of anti-ovalbumin and γ-globulin and distribution of antibody-containing plasma cells. *Brit. J. exp. Path.* **37**, 61.

Askonas B.A. & Humphrey J.H. (1958) Formation of specific antibodies and gamma-globulin *in vitro*. A study of the synthetic ability of various tissues from rabbits immunized by different methods. *Biochem. J.* **68**, 252.

Benacerraf B., Halpern B.N., Biozzi G. & Benos S.A. (1954) Quantitative study of the granulopectic activity of the reticulo-endothelial system. *Brit. J. exp. Path.* **35**, 98.

BENACERRAF B., STIFFEL C., BIOZZI G. & HALPERN B.N. (1954) Mesure de l'activité granulopexique du système réticulo-endothélial à l'aide du saccharate d'oxyde de fer chez le rat. *C.R. Soc. Biol. (Paris)*, **148,** 486.

BENACERRAF B., HALPERN B.N., STIFFEL C., CRUCHAUD S. & BIOZZI G. (1955) Phagocytose d'une fraction du sérum chauffé et iodé par le système réticuloendothélial et comportement consécutif de ses cellules à l'égard d'autres colloids. *Ann. Inst. Pasteur* **89,** 601.

BENACERRAF B., BIOZZI G., HALPERN B.N. & STIFFEL C. (1955) The physiology of phagocytosis of particles by the reticulo-endothelial system. 1st Symposium on *The Physiopathology of the Reticulo Endothelial System*, p. 52. Oxford: Blackwell Scientific Publications.

BERGLUND K. (1956a) Studies on factors which condition the effect of cortisone on antibody production. 1. The significance of time of hormone administration in primary haemolysin response. *Acta path. microbiol. scand.* **38,** 311.

BERGLUND K. (1956b) Studies on factors which condition the effect of cortisone on antibody production. 2. The significance of the dose of antigen in primary haemolysin response. *Acta path. microbiol. scand.* **38,** 329.

BESSIS M.C. & BRETON-GORIUS J. (1961) Iron metabolism in the bone-marrow as seen by electron microscopy: a critical review. *Blood* **19,** 635.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1954) Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc. Roy. Soc. B.* **143,** 58.

BIOZZI G., BENACERRAF B. & HALPERN B.N. (1953) Quantitative study of the granulopectic activity of the reticulo-endothelial system. *Brit. J. exp. Path.* **34,** 441.

BIOZZI G., BENACERRAF B. & HALPERN B.N. (1955) The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticulo-endothelial system in mice. *Brit. J. exp. Path.* **36,** 226.

BOYDEN S.V. (1962) Personal communication.

BRAUNSTEINER H. & PAKESCH F. (1960) Electronenmikdroskopische Untersuchungen zur Frage der Blutplasmazellen. *Wein Z. inn. Med.* **41,** 58.

CAMPBELL D.H. & GARVEY J.S. (1961) The fate of foreign antigen and speculation as to its role in immune mechanisms. *Lab. invest.* **10,** 1126.

CHODIRKER W.B. & TOMASI T.B. (1963) Gamma-globulins: quantitative relationships in human serum and non-vascular fluids. *Science* **142,** 1080.

CLARK S.L. (Jr) (1963) Electronmicroscopy of the thymus in mice of strain 129/J. *Amer. J. Anat.* **112,** 1.

CLARK S.L. (Jr) (1964) In *The Thymus*, p. 85. Ed. DEFENDI V. & METCALF D. Wistar Institute Symposium Monograph. Philadelphia: Wistar Press.

COHN Z.A. & BENSON B. (1965) The differentiation of mononuclear phagocytes. Morphology, cytochemistry and biochemistry. *J. exp. Med.* **121,** 153.

CONWAY E.A. (1937) Cyclic changes in lymphatic nodules. *Anat. Rec.* **69,** 487.

COONS A.H. & KAPLAN M.H. (1950) Localization of antigen in tissue cells. II. Improvement in method for detection of antigen by means of fluorescent antibody. *J. exp. Med.* **91,** 1.

COONS A.H., LEDUC E.H. & KAPLAN M.H. (1951) Localization of antigen in tissue cells. VI. Fate of injected foreign proteins in the mouse. *J. exp. Med.* **102,** 49.

COONS A.H., LEDUC E.H. & CONNELLY J.M. (1955) Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. exp. Med.* **102,** 49.

CRAMPTON C.F., FRANKEL F.R. & RODEHEAVER J.L. (1960) Mechanism of immunological unresponsiveness. *Nature* **184,** 873.

DUTTON A.A.C. (1955) The influence of the route of injection on lethal infections in mice. *Brit. J. exp. Path.* **36,** 128.

EICHENWALD H.F. & SHINEFELD H.R. (1963) Antibody production by the human foetus. *J. Pediat.* **63,** 870.

ELVES M.W., TAYLOR G. & ISRAËLS M.C.G. (1963) The *in vitro* production of antibody lymphocytes. *Lancet* **i,** 1292.

ENDERS J.F., SHAFFER M.F. & WU C.J. (1936) Studies on natural immunity to pneumococcus type III. III. Correlation of the behaviour *in vivo* of pneumococci type III varying in their virulence for rabbits with certain differences observed *in vitro*. *J. exp. Med.* **64,** 307.

FAGRAEUS A. (1948) Antibody production in relation to the development of plasma cells. *Acta med. scand.* suppl. 204.

FELTON L.D. (1949) The significance of antigen in animal tissues. *J. Immunol.* **61,** 107.

FISHMAN M. (1959) Antibody formation in tissue culture. *Nature (Lond.)* **183,** 1200.

FISHMAN M., VAN ROOD J.J. & ADLER F.L. (1965) The initiation of antibody formation by ribonucleic acid from specifically stimulated macrophages. In *Molecular and Cellular Basis of Antibody Formation*, p. 491, ed. STERZL J. Proceedings of a Symposium, Prague 1964. Czechoslovak Academy of Sciences, Prague.

FLEMING W.A., WILKINSON P.C. & WHITE R.G. (1967) Sites of biosynthesis of immunoglobulins in guinea-pigs immunized with bacteriophage Φx174. *Immunology*, **13,** 613.

GILTNER W., COOLEDGE L.H. & HUDDLESON I.F. (1916) *J. Am. Vet. Med. Assoc.* **50,** 157.

GOOD R.A. & THOMAS L. (1952) Studies on the generalized Shwartzman Reaction. II. The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial toxin in rabbits previously treated with thorotrast or Trypan Blue. *J. exp. Med.* **96,** 625.

GOWANS J.L., GESNER B.M. & MCGREGOR (1961) The immunological activity of lymphocytes. In *Biological Activity of the Leucocyte*, p. 32, ed. WOLSTENHOLME G.E.W. & O'CONNOR M. Ciba Foundation Study Group No. 10. London: Churchill.

GOWANS J.L. & KNIGHT F.J. (1964) The route of recirculation of lymphocytes in the rat. *Proc. Roy. Soc. B.* **159,** 257.

GYLLENSTEN L., RINGERTZ N. & RINGERTZ W.R. (1955) Uptake of labelled phosphate in lymph nodes during experimental lymphadenitis. *Acta path. microbiol. scand.* **38,** 81.

HEILMEYER L. (1955) Physiologische Beziehungen zwischen Milz und Knochenmark. *Bibl. haemat.* **3,** 21.

HITZIG W.H. & WILLI H. (1961) *Schweiz. med. Wschr.* **91,** 1625.

HOCHWALD G.M., JACOBSON E.B. & THORBECKE G.J. (1964) C^{14}-amino acid incorporation into transferrin and β_2A globulin by ectodermal glands *in vitro*. *Fed. Proc.* **23,** 557.

HOLUB N. & ŘÍHA I. (1960) Morphological changes in lymphocytes cultivated in diffusion chambers during primary antibody response to a protein antigen. In *Mechanisms of Antibody Formation*, p. 30. Ed. HOLUB M. & JAROŠKOVA L. Proceedings of a Symposium, Prague 1959. Czechoslovak Academyof Sciences, Prague.

HORTEGA DEL RIO P. & DE ASUA F. (1921) *Arch. cardiol. y haematol.* **2,** 161.

HORTEGA DEL RIO P. & DE ASUA F. (1924) *Bol. de la Soc. Espan de Hist. Nat. An.* **11,** 7.

HOWARD J.G. & MICHIE D. (1962) Induction of transplantation immunity in the new born mouse. *Transplantation Bulletin* **29,** 1.

JUHLIN L. (1956) The spreading of spherical particles in dermal connective tissue. *Acta dermat.-venereol.* **36,** 131.

KASS E.H. (1960) Hormones and host-resistance to infection. *Bact. Rev.* **24,** 177.

LEE A. & COOPER G.N. (1964) Effect of simple lipids on the phagocytic properties of peritoneal macrophages. III. Microelectrophoresis and biochemical studies on triolein-treated cells. *Aust. J. exp. Biol. med. Sci.* **42,** 725.

LISON L. & SMULDERS J. (1948) Discriminating 'athrocytes' in the reticulo-endothelial system. *Nature (Lond.)* **162,** 65.

LOUTIT J.F. & MOLLISON P.L. (1943) Advantages of a disodium-citrate glucose mixture as a blood preservative. *Brit. med. J.* **2,** 744.

MARSHALL A.H.E. & WHITE R.G. (1950) Reactions of reticular tissues to antigens. *Brit. J. exp. Path.* **31,** 157.

MARSHALL A.H.E. & WHITE R.G. (1961) Experimental thymic lesions resembling those of myasthenia gravis. *Brit. J. exp. Path.* **42,** 379.

MCGREGOR D.D. & GOWANS J.L. (1963) The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct. *J. exp. Med.* **117,** 303.

MCMASTER P.D. & HUDACK S.S. (1935) The formation of agglutinins within lymph nodes. *J. exp. Med.* **61,** 783.

METCHNIKOFF O. (1921) *Life of Elie Metchnikoff,* pp. 116–17. Boston and New York: Houghton Mifflin Co.

MILLER J.F.A.P., MARSHALL A.H.E. & WHITE R.G. (1962) The immunological significance of the thymus. *Advances in Immunology* **2,** 111. New York: Academic Press.

MILLER L.L. & BALE W.F. (1954) Synthesis of all plasma protein fractions except gammaglobulin by the liver. *J. exp. Med.* **99,** 125.

MOLLISON P. L. (1962) *Blood Transfusion in Clinical Medicine,* 3rd ed. Oxford: Blackwell.

MORGAN L.M., SCHLESINGER R.W. & OLITSKY P.K. (1942) Induced resistance of the central nervous system to experimental infection with equine encephalomyelitis virus. I. Neutralizing antibody in the central nervous system in relation to cerebral resistance. *J. exp. Med.* **76,** 357.

NICOL T. & BILBEY D.L.J. (1960) In *Reticuloendothelial Structure and Function,* p. 301, ed. HELLER J.H. New York: Ronald Press.

NOSSAL G.J.V., ADA G.L. & AUSTIN C.M. (1964) Antigens in Immunity. IV. Cellular localization of ^{125}I- and ^{131}I-labelled flagella in lymph nodes. *Aust. J. exp. Biol. Med. Sci.* **42,** 311–30.

OAKLEY C.L., BATTY I. & WARRACK G.H. (1951) Local production of antibodies. *J. Pathol. Bacteriol.* **63,** 33.

OORT J. & TURK J.L. (1965) A histological and autoradiographic study of lymph nodes during the development of contact sensitivity in the guinea-pig. *Brit. J. exp. Path.* **46,** 147.

PARROTT D.M.V., DE SOUSA K.A.B. & EAST J. (1966) Thymus dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. exp. Med.* **123,** 191.

RICH A.R. & MCKEE C.M. (1939) The pathogenicity of avirulent pneumococci for animals deprived of leucocytes. *Bull. Johns Hopkins Hosp.* **64,** 434.

ROWLEY D.A. (1950a) The effect of splenectomy on the formation of circulating antibody in the adult male albino rat. *J. Immunol.* **64,** 289.

ROWLEY D.A. (1950b) The formation of circulating antibody in the splenectomized human being following intravenous injection of heterologous erythrocytes. *J. Immunol.* **65,** 515.

SCOTHORNE R.J. & MCGREGOR I.A. (1955) Cellular changes in lymph nodes and spleen following skin homografting in the rabbit. *J. Anat.* **89,** 283.

SEWELL T.A. (1962) The effect of anti-bacterial agents on the reticulo-endothelial system. Ph.D. Thesis, London University.

SILVERSTEIN A.M. (1962) Congenital syphilis and the timing of immunogenesis in the human foetus. *Nature* **194,** 196.

SILVERSTEIN A.M. & KRANER K.L. (1965) Studies on the ontogenesis of the immune response. In *Molecular and Cellular Basis of Antibody Formation,* p. 341. Ed. STERZL J. Czeckoslovak Academy of Sciences, Prague.

SILVERSTEIN A.M. & LUKES R.V. (1962) Fetal response to antigenic stimulus. I. Plasmacellular and lymphoid reactions in the human foetus to intrauterine infection. *Lab. Inv.* **11,** 918.

SJÖVALL A. & SJÖVALL H. (1930) Experimentelle Studien über die Sekundhärknötchen in der Kniekehlenlymphknoten des Kaninchens idei Bacillus pyocyaneusinjektion. *Virch. Arch.* **278,** 258.

SMITH R.T. (1960) Response to active immunization of human infants during the neonatal period. In *Cellular Aspects of Immunity*, p. 348. Ciba Foundation Symposium. London: J. & A. Churchill.

ŠTERZL J., MANDEL L., MILER I. & ŘÍHA I. (1965) Development of immune reactions in the absence or presence of an antigenic stimulus . In *Molecular and Cellular Basis of Antibody Formation*, p. 351. Ed. ŠTERZL, J. Proceedings of a symposium, Prague 1964, Czechoslovak Academy of Sciences, Prague.

STROBER S. & GOWANS J.L. (1965) The role of lymphocytes in the sensitization of rats to renal homografts. *J. exp. Med.* **122,** 347.

STUART A.E., BIOZZI G., STIFFEL C., HALPERN B.N. & MOUTON D. (1960) The stimulation and depression of reticulo-endothelial phagocytic function by simple lipids. *Brit. J. exp. Path.* **41,** 599.

TOMASI T.B., TAN E.M., SOLOMON A. & PRENDERGAST R.A. (1965) Characteristics of an immune system common to certain external secretions. *J. exp. Med.* **121,** 101.

WARNER N.L., SZENBERG A. & BURNET F.M. (1962) The immunological role of different lymphoid organs in the chicken. I. Dissociation of immunological unresponsiveness. *Aust. J. exp. Biol.* **40,** 373–88.

WEIGLE W.O. & DIXON F.J. (1958) Relationship of circulating antigen-antibody complexes, antigen elimination, and complement fixation in serum sickness. *Proc. Soc. exp. Biol. Med.* **99,** 226.

WEISSMAN G. & THOMAS L. (1962) Studies on Lysosomes. I. The effects of endotoxin, endotoxin tolerance, and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *J. exp. Med.* **116,** 433.

WHITE R.G. & MARSHALL A.H.E. (1951) Effect of Cortisone on pneumococcal bacteraemia. *Lancet* **i,** 891.

WHITE R.G. (1954) Observations on the formation and nature of Russell bodies. *Brit. J. exp. Path.* **35,** 365.

WHITE R.G. (1957) An immunological investigation of Hashimoto's disease. *Proc. roy. Soc. Med.* **50,** 953.

WHITE R.G. (1958) Antibody production by single cells. *Nature (Lond.)* **182,** 1383.

WHITE R.G. (1960) The relation of the cellular responses in germinal or lymphocytopoietic centres of lymph nodes to the production of antibody. Symposium of the Czechoslovak Academy of Science, Prague 1960. Czecholovak Academy of Sciences, Prague.

WHITE R.G. (1963) Functional recognition of immunologically competent cells by means of the fluorescent antibody technique. In *The Immunologically Competent Cell: Its Nature and Origin*, p. 6. Eds. WOLSTENHOLME G.E.W. & KNIGHT J. Ciba Foundation Study Group No. 16. London: Churchill.

WHITE R.G. (1967) Presidential address Royal Society of Medicine, Antigen and Adjuvant. *Proc. Roy. Soc. Med.* In press.

WHITE R.G., FRENCH V. & STARK J.M. (1967) Germinal Centre formation and antigen localization in Malpighian bodies of the chicken spleen. In *Germinal Centers in Immune Responses*, ed. COTTIER H. & ODARTCHENKO N. Berlin: Springer.

CHAPTER 13

IMMUNOGLOBULINS

D.S.Rowe

INTRODUCTION

Immunoglobulins constitute a functionally and structurally diverse group of proteins. They are distinguished by their properties as antibodies, by a characteristic polypeptide chain structure, and by synthesis in the lympho-reticular tissues.

Since the demonstration by Tiselius in 1937 that antibodies are to be found in the γ-globulin electrophoretic region numerous investigators have emphasized the heterogeneity both of antibodies and of γ-globulins, a heterogeneity which continues to be explored (e.g. Fahey 1962; Holborow 1963; Fudenberg 1965). Different categories of proteins have been defined on the basis of properties such as antigenic characteristics, rates of sedimentation in the ultracentrifuge, and chemical composition. Although for a number of years proteins in these categories were included within the term γ-globulins, this electrophoretic designation ultimately became an embarrassment when describing molecules whose mobility ranged from the α- to the γ-globulin regions. The use of different

names for the same protein was an additional source of confusion. These names were based on different methods of identification, e.g. 19S γ-globulin as defined by electrophoresis and ultracentrifugation was identified as γ_{1M} or β_{2M} globulin by immunoelectrophoretic analysis. This confusion was largely resolved by a *Nomenclature for Human Immunoglobulins* (1964) published by the World Health Organization. Table 13.1 includes this nomenclature, which is now in general use. Unfortunately it was not possible to recommend a single name for each protein. The Ig (immunoglobulin) nomenclature will be used in this account.

IMMUNOGLOBULIN STRUCTURE

Four major classes of immunoglobulins are now recognized in human serum: IgG, IgA, IgM and IgD (Rowe and Fahey 1965). Their appearance on immuno-

TABLE 13.1

Nomenclature and molecular characteristics of immunoglobulins

Present name (class)	Previous names	Polypeptide chain structure		Molecular weight	Sedimentation constant	Carbohydrate (%)
		Type K	Type L			
IgG or γG	γ, 7Sγ, 6.6Sγ, γ_2, γSS	$\gamma_2\kappa_2$	$\gamma_2\lambda_2$	160,000	6.6	3.1
IgA or γA	β_{2A}, γ_{1A}	$(\alpha_2\kappa_2)$n	$(\alpha_2\lambda_2)$n	(160,000)n	6.6, 10, 13	10.7
IgM or γM	β_{2m}, γ_{1m}, 19Sγ γ-macroglobulin	$(\mu_2\kappa_2)5$	$(\mu_2\lambda_2)5$	900,000	19, 26, 32	10.4
IgD or γD	γ_{1J}	$\delta_2\kappa_2$	$\delta_2\lambda_2$	—	7	—

electrophoresis is shown in Plate 13.1, facing p. 372. In serum, IgG is predominant in concentration, and most serum antibodies fall within this class. The different classes possess both common and distinctive features in their molecular structure. Porter and his co-workers have shown that molecules of each class are comprised of units containing four polypeptide chains (see Fig. 13.1), two identical heavy chains and two identical light chains (Fleischman *et al* 1963). A similar four-chain unit is found in the immunoglobulins of many other species. The molecular weight of the heavy chains is about 50,000, that of the light chains about 20,000 (Pain 1963). Heavy chains are distinctive for each class, i.e. γ chains are characteristic of IgG, α chains of IgA, μ chains of IgM and δ chains of IgD. The antigenic distinctiveness of heavy chains (Cohen 1963) permits the preparation of antisera which can be used as reagents for the detection and quantitation of the different immunoglobulin classes.

In contrast, the light chains of the immunoglobulin units are common to all classes. Two varieties of light chains have been recognized on the basis of antigenic distinctiveness (Mannik & Kunkel 1963a; Fahey 1963). These are now termed Type K (κ chains), and Type L (λ chains). A single immunoglobulin unit may either be Type K, having two κ light chains, or Type L, having two λ light chains, but hybrids containing both κ and λ light chains are not found. The antigenic differences between κ and λ chains reflect differences of primary structure, which will be discussed below.

The immunoglobulin unit of four heavy chains is held together by inter-chain disulphide and by non-covalent bonds. The ability to form such units is inherent in the polypeptide chains, since light and heavy chains of IgG, when mixed *in vitro*, readily associate in this way (Olins & Edelman 1964). The number

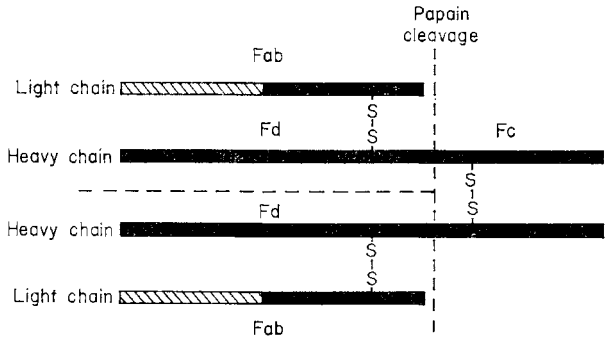


FIG. 13.1. A schematic diagram of the arrangement of polypeptide chains of IgG, similar to that proposed by Porter. N-terminal ends of the chains are to the left. The variable composition of the N-terminal half of the light chains is indicated by cross-hatching. Papain splits the molecule to give one Fc and two Fab fragments. Each Fabs include one light chain, and the Fd part of one heavy chain. The antigen combining site is located in Fd.

of units differ in the molecules of each class. IgG and IgD are comprised of single units (monomers). IgM is found predominantly in the form of a five-unit polymer, (Miller & Metzger 1965), accounting for its high molecular weight. IgA includes monomer and a variable proportion of polymers, recognized as proteins sedimenting at 7, 9, 11 and 13S (Vaerman *et al* 1964). Immunoglobulins also contain carbohydrate, linked to their heavy chains; IgA and IgM contain about 10%, and IgG about 2% of carbohydrate. In addition to these four-chain units, serum also contains a small amount of free light chains not associated with heavy chains (Berggard 1961). These are more easily found in urine, presumably on account of their ability to cross the glomerular membrane. Free heavy chains have not been found, so that it appears that there may normally be a slight excess of light chain synthesis.

Proteolytic enzymes split immunoglobulin molecules into components other than their constituent polypeptide chains. The protein most studied in this respect is IgG. One region of the γ heavy chains of IgG of a number of species appears to be particularly susceptible to cleavage (Fig. 13.1). Papain with cysteine splits IgG into three main fragments, two identical Fab fragments, and one Fc fragment (Porter 1959). Fab comprises one light chain and a part of one heavy chain termed Fd. Fc comprises the remainder of the two heavy chains. Pepsin splits IgG at a similar but not identical site. Fc is further digested, and the two Fab fragments do not separate from each other (Nisonoff *et al* 1960).

The capacity of antibody chains and fragments to combine with antigen has been studied with the object of locating the combining site in the intact molecules. Antigen binding by isolated polypeptide chains is much reduced as compared with the parent molecule, but study of *in vitro* recombinants of chains from antibody and non-antibody IgG indicates that the specific site is on the heavy chains (Franek & Neslin 1963). Antigen binding by papain fragments resides in Fab (Fragment antigen-binding). Activity of Fab and of heavy chains together implicate Fd as containing the antigen binding site. At the present time it seems likely that specificity for antigen is determined by a specific aminoacid sequence in Fd, but the way by which antigen induces synthesis of appropriately sequenced antibody molecules remains a central problem in immunology. Although there is little evidence that isolated antibody light chains can combine with antigen, heavy chains or Fd fragments from a specific antibody combine preferentially with light chains from the same antibody rather than with 'non-specific' light chains. These recombinants are highly active as antibody (Roholt *et al* 1966). It appears that some specificity of structure of both heavy and light chains is a feature of antibody molecules. Fc is not involved in antigen binding, but it possesses other important biological properties which will be discussed below. It also contains those antigenic determinants which are characteristic of γ heavy chains.

M PROTEINS, DISCRETE COMPONENTS OF IMMUNOGLOBULINS

Elucidation of the heterogeneity of immunoglobulins has been greatly assisted by studies on the discrete components of immunoglobulins (M proteins) which may appear in the plasma in various plasmacytic and lymphocytic disorders amongst which multiple myeloma and Waldenström's macroglobulinaemia are predominant. Individual M proteins belong to a particular immunoglobulin class, i.e. IgG, IgA, IgM or IgD. When associated with the appropriate disease they are designated G-, A-, or D-myeloma proteins, or M-macroglobulins (Waldenström). M-proteins show greater homogeneity than the normal proteins of the same class. For example, normal IgG extends from the β to the

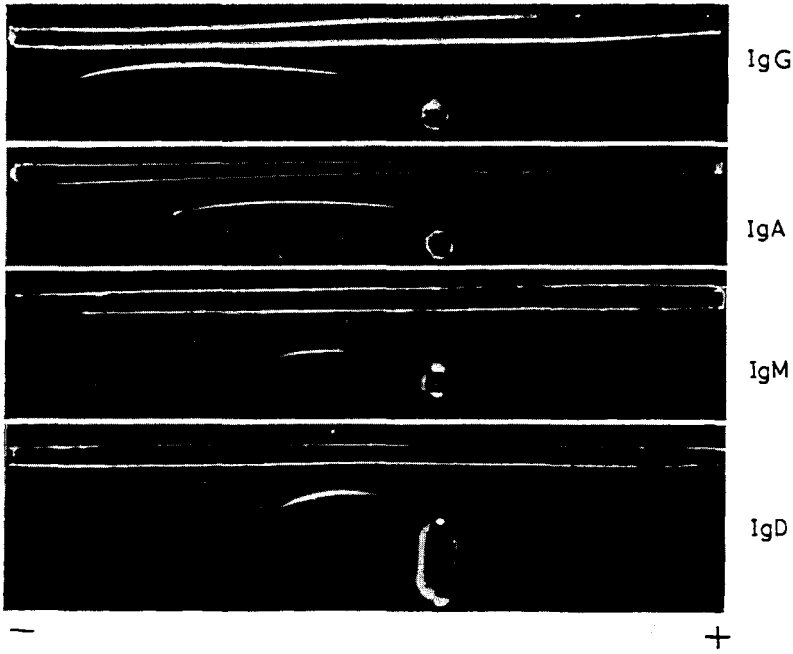


PLATE 13.1. Immunoelectrophoretic analysis of human serum to show four classes of immunoglobulins. Specific antisera in the troughs produce characteristic precipitin lines of IgG, IgA, IgM and IgD.

facing p. 372

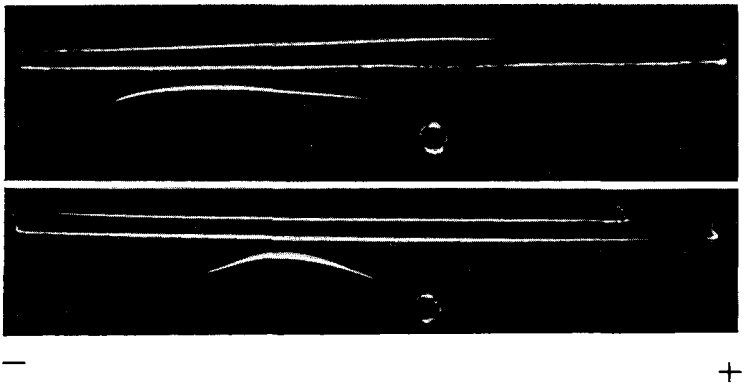


PLATE 13.2. Immunoelectrophoretic demonstration of the restriction of electrophoretic mobility of a G-myeloma protein. Specific anti-IgG antiserum was used. Normal human serum in the upper analysis shows the range of electrophoretic mobilities of normal IgG. Serum from a case of multiple myeloma in the lower analysis shows a myeloma protein arc of restricted mobility within the IgG range. The amount of 'normal' IgG is reduced in this serum.

slow γ region on immunoelectrophoretic analysis, but G-myeloma proteins are of restricted mobility within this range (Plate 13.2). This restriction of electrophoretic mobility applies to M proteins of all classes, and enables them to be recognized by abnormal sharp bands produced in the α_2 to γ regions on electrophoretic analysis. Differentiation between classes is not possible by electrophoresis alone, but can be made by immunoelectrophoresis using specific antisera. In addition the ultracentrifuge can distinguish M-macroglobulins of Waldenström sedimenting at 19S and above, and certain A-myeloma proteins sedimenting between 7 and 19S, but does not distinguish the remaining A-, G-, and D-myeloma proteins which sediment close to 7S.

Another important aspect of the homogeneity of M-proteins is their restriction to a single light chain type only. Thus M-proteins are either Type K or Type L. (Mannik & Kunkel 1962). A different aspect of the homogeneity of these light chains is shown by starch gel electrophoresis at acid pH in eight molar urea. Light chains from normal IgG show many bands, indicating heterogeneity of charge; light chains from M-proteins show one or two bands only (Cohen 1963).

Bence Jones proteins are another variety of M-proteins which appear in serum and urine. They occur commonly in myeloma and macroglobulinaemia, and may be the only protein abnormality found in some cases of multiple myeloma (Drivsholm 1964). Although the association of Bence Jones proteins with this disease has been recognized since 1846 (Bence Jones 1847), they remained a biochemical curiosity for over 100 years. It has now been shown that light chains isolated from a myeloma protein possess the same thermal properties, are of similar size and amino acid composition, and are of the same antigenic type and electrophoretic mobility as the Bence Jones protein present in the same individual. (Edelman & Gally 1962). Bence Jones proteins thus form the pathological counterpart of the normal free light chains of serum and urine.

A nother and extremely rare variety of M-protein is that found in cases of Franklin's disease, or so-called 'Heavy chain disease' (Franklin *et al* 1964). The fe examples so far described have occurred in association with unusual rapidly progressive lymphomatous tumours (Osserman & Takatsuki 1964). A M-protein is found in the plasma possessing the antigenic characteristics of γ vy chains, but not of light chains. These proteins appear to consist of only the c portions of γ heavy chains. The occurrence of this fragment suggests the possibility that Fc represents a synthetic unit of immunoglobulins.

Two explanations have been proposed for the relationship between M-proteins and the normal immunoglobulins. Either these are abnormal proteins, not previously present in the body; or they arise from excessive synthesis of a homogeneous protein which normally forms only a small proportion of the range of protein molecules which constitute the normal class. In either case the cellular basis for the homogeneity of M-proteins can be regarded as increased synthesis by a single group or clone of lymphoid cells. The abnormal protein

hypothesis is favoured by the absence of unequivocal evidence of antibody activity of M-proteins and by the presence of antigenic determinants which may appear to be specific to an individual M-protein (Korngold 1963). The normal protein hypothesis is favoured by the demonstration that very many of the features of M-proteins can be demonstrated in the normal class (Deutsch *et al* 1956). Moreover certain antibodies of IgG class resemble myeloma proteins in showing a range of electrophoretic and antigenic heterogeneity which is restricted as compared with the class as a whole (Mannik & Kunkel 1963b; Kunkel, Mannik & Williams 1963).

The homogeneity of M-proteins, whatever the explanation, has rendered them most useful models for the elucidation of immunoglobulin heterogeneity. Appreciation of the distinctiveness of the four immunoglobulin classes and of the two types of light chains, has, in large measure, been dependent upon their study, which has also helped to define sub-classes within the main classes.

The Diagnostic Significance of M-Proteins

The demonstration of M-macroglobulin in the plasma is a prerequisite for the diagnosis of Waldenström's macroglobulinaemia, where certain features of the disease are consequent upon the increased plasma viscosity due to this protein (Fahey *et al* 1965). In multiple myeloma the demonstration of myeloma or Bence Jones proteins is also a valuable aid to diagnosis. However M-proteins are not specific to macroglobulinaemia and myeloma. They also occur, although rarely, in association with leukaemias, reticuloses and carcinoma (Waldenström 1961a).

Population surveys have shown an incidence of M-proteins increasing in frequency with age, to an incidence of 3% in individuals over the age of 70 (Hallen 1963). Many of the people in whom these proteins were found had no demonstrable disease and remained healthy for a number of years. These M-proteins have not been structurally distinguished from those associated with pathological states. However the serum concentration is usually 3 g/100 ml or less, and remains constant; Bence Jones protein is usually but not always absent, and anaemia is not prominent. This condition has been termed 'benign monoclonal gammopathy' (Waldenström 1961b). Further study of its natural history is important since the condition can now be readily recognized. Some cases have been observed to progress after many years to multiple myeloma (Kyle & Bayrd 1966).

HETEROGENEITY OF IMMUNOGLOBULINS WITHIN THE MAJOR CLASSES

As already noted the four immunoglobulin classes show heterogeneity with respect to electrophoretic mobility and light chain types. Another aspect of the

heterogeneity of IgG can be shown by the use of antisera raised in rhesus monkeys (Terry & Fahey 1964), or by antisera to individual G-myeloma proteins raised in rabbits (Grey & Kunkle 1964). Such antisera demonstrate four sub-classes of IgG which have been termed γ_{2a} (or Na), γ_{2b} (or We), γ_{2c} (or Vi), and γ_{2d} (or Ge). The distinctive determinants of these sub-classes are possessed by their heavy chains and, if they survive papain digestion, by their Fc fragments. Sub-classes of IgM and IgA have also been described.

A related heterogeneity of IgG can be recognized in a different way by the use of the antibody-like reagents to IgG which occur among rheumatoid factors and in rare normal sera. These reagents are demonstrated by their ability to agglutinate Rh +ve red cells coated with certain incomplete anti-D antibodies of IgG class. The IgG of different human sera can be categorized by its ability to inhibit agglutination by these reagents. Using such systems two main groups of inhibitors can be distinguished, Gm and InV. (For further details see Chapter 31 on Rheumatoid Arthritis). Gm and InV have been shown to be separate genetic loci controlling synthesis of a series of characteristics or factors present in some but not all human sera (Steinberg 1962). Gm factors (Gma, Gmb, etc.) are only found in IgG, and are exclusive properties of γ heavy chains. InV factors (InVa, InVb, etc.) are properties of light chains, and so may be held by immunoglobulins of all classes (Harboe *et al* 1962). These factors are useful genetic markers in population studies. Such genetically determined differences within a species (allotypes) are not confined to humans; allotypes have also been recognized in the immunoglobulins of rabbits, mice and guinea-pigs.

There are correlations between Gm and IgG sub-classes and InV allotypes and light chain types. Studies on myeloma proteins have shown that currently recognized Gm factors are restricted to γ_{2b} and γ_{2c} sub-classes (Terry *et al* 1965). Gm factors associated with γ_{2b}, (a, x, b^2 and f), are distinct from those associated with γ_{2c} (b^1, b^3, b^4 and c). InV factors are associated with light chains of antigenic Type K only.

The study of the heterogeneity of immunoglobulins is, at the time of writing, being extended to the level of primary structure by the massive task of the determination of the amino acid sequences of their constituent polypeptide chains. For this purpose, Bence Jones proteins provide a convenient point of attack on account of their homogeneity and relatively small size. Studies on three Type K Bence Jones proteins show they are composed of two halves, one of similar composition in all the three proteins, the other half of variable composition in the different proteins (Hilschmann & Craig 1965, Titani *et al* 1965; Milstein 1966). The C terminal halves (see Fig. 13.1), are identical in a sequence of 106 amino acid residues, with the exception of a single substitution in three places. Of these the substitution of leucine by valine is associated with the allotypic change from InVa to InVb. In contrast to the similarity of the C terminal halves the N terminal halves show considerable variation. The

significance of this variability in relation to antibody activity has been discussed by Burnet (1966) and Brenner & Milstein (1966). These findings also indicate a structural basis for the serological heterogeneity of light chains. It is likely that the antigenic Type K or L is determined by a prolonged amino acid sequence. Allotypic (Inv) differences may represent single amino acid substitutions within this sequence. Antigenic determinants specific to individual Bence Jones proteins may be determined by sequences in the variable half of the chain.

Much less is known at present of the primary structure of heavy chains. There is evidence that Fc is fairly homogeneous and that Gma+ and Gmb+ differ in a single peptide (see Fudenberg 1965). This difference is, however, due to substitution of more than one amino acid. It may be anticipated that a prolonged amino acid sequence within Fc defines the antigenic characteristics distinguishing γ chains from other heavy chains. Variation within this sequence defines IgG sub-classes, and variation within the sub-class sequence defines Gm specificity.

BIOLOGICAL ACTIVITIES OF IMMUNOGLOBULINS

A counterpart to the structural heterogeneity of immunoglobulins is found in their diversity of biological function. Two broad categories of biological activities may be distinguished; firstly the definitive property of antibody function; and secondly properties such as complement activation, adherence to cell surfaces, placental transfer, etc. which may largely determine the *in vivo* effects of antigen-antibody combination. These properties must be due to structural features of different immunoglobulin molecules. Important differences in both types of activities occur between the different immunoglobulin classes. Antibody activity has been shown in all classes of immunoglobulin except IgD and in the three sub-classes of IgG (Terry & Fahey 1964). Following exposure to antigen there is a typical sequence of the appearance of antibody. Usually the first antibody to be detected is in IgM, and subsequently IgG antibodies appear and become predominant. A second exposure to the same antigen results in a more rapid response, often predominantly in IgG. Less is known of IgA antibodies in this sequence but their production has been shown to be an early and continued response to certain antigens. (Turner & Rowe 1964). Certain antibodies do not progress in this sequence, but remain at least predominantly confined to IgM. These include 'natural' α and β isoagglutinins, Wassermann antibodies and some rheumatoid factors. The usual criteria by which IgM and IgG antibodies are distinguished are size-separation by ultracentrifugation or gel filtration into 19S and 7S antibodies, and the capacity of 2-Mercaptoethanol to inactivate IgM but not IgG antibodies. Such tests must however be interpreted with caution; IgA antibodies for example may fall into both categories.

Some biological functions of immunoglobulins other than antibody activity are listed in Table 13.2. In the case of IgG these properties can be attributed to the Fc parts of the molecules. Although a particular activity has been shown to occur within a class, not all the proteins of the class may possess this property. For example, of the IgG sub-classes γ_{2b}, γ_{2c}, and γ_{2d} are active in sensitizing guinea-pig skin for reverse passive cutaneous anaphylaxis, whereas γ_{2a} is not. (Terry 1964).

The definition of the immunoglobulin class to which human tissue-sensitizing (reaginic) antibodies belongs has been rendered difficult by the extremely low concentration of reagins in human serum and the practical limitations of the Prausnitz-Küstner test. Earlier claims that reaginic activity occurred in IgA

TABLE 13.2

Some biological properties of the immunoglobulins

Class	Antibody Activity	Complement dependent haemolysis	Complement fixation by complexes and aggregates	Placental transfer	Secretion in colostrum and saliva	Combination with rheumatoid factors	Reaginic antibody	Fixation to guinea pig skin (PCA)
IgG	+	+	−	+	+	+	−	+
IgA	+	−[1]	?	−	++	−	−	−
IgM	+[2]	++	+	−	+	−	−	−
IgD	−	?	?	−	−	?	−	?

1. IgA antibodies may be bacteriolytic in the presence of complement and lysozyme (Adinolfi *et al* 1966).
2. IgM is frequently the first detectable antibody.

have not been confirmed by the recent painstaking work of Ishizaka (e.g. Ishizaka *et al* 1966). This group have given the name IgE to a protein which is present in reagin-rich fractions of human serum, which is capable of binding allergen, and which is precipitated by antisera not reactive with IgG, IgA, IgM or IgD. Reaginic activity of human serum is greatly diminished by precipitation of IgE by a rabbit specific anti-IgE globulin. IgE migrates as a γ_1-globulin, but its other physico-chemical properties are at present unknown. By analogy with reaginic antibodies it may be expected to sediment at approximately 8S, and to contain light chains. Present evidence strongly suggests that reaginic activity resides in IgE, but it still remains possible that reaginic antibodies are unrecognized contaminants of IgE preparations.

IMMUNOGLOBULIN CONCENTRATIONS IN THE SERUM

The most generally available measurement of serum immunoglobulin concentration is the electrophoretic measurement of γ-globulins. However not all immunoglobulins are included in the γ-globulin region, and electrophoresis cannot distinguish between the different immunoglobulin classes. Measurements which are specific for the individual classes can at present only be made by using specific antisera as quantitative reagents. Gel diffusion methods are suitable for such estimations, and a particularly convenient single diffusion method using antiserum incorporated into agar gel has recently been developed (Mancini *et al* 1965; Fahey & McKelvey 1965). In these methods the precipitate developed

TABLE 13.3

Median values of some metabolic characteristics of immunoglobulins

Class	Serum concentration in g/100 ml	Half life in days	% of body content catabolized/day	Synthetic rate mg/Kg/day	% intravascular
gG	1.2	23	3	36	40
IgA	0.4	6	12	30	40
IgM	0.12	5	14	6.9	80
IgD	0.003	2.8	33	0.4	75

Data from Solomon *et al* (1963); Solomon & Tomasi (1964); Barth *et al* (1964); Rogentine *et al* (1966); Fahey (1965).

by the serum under test is compared with that given by a standard immunoglobulin preparation. At the present time (in 1967), standards are prepared by individual laboratories so that results obtained by different laboratories may not be directly comparable. It is hoped that standards for general use may become available in the near future.

In serum IgG is found in highest concentration, followed by IgA, IgM and IgD (see Table 13.3). All classes, and especially IgD, show a considerable range of concentrations. This range must reflect the variability both of environmental factors, such as exposure to antigens, and of genetic factors. The importance of the antigenic stimulation provided by bacteria and viruses is emphasized by the gross reduction of immunoglobulin synthesis and serum levels seen in animals reared in a germ-free environment (Sell & Fahey 1964). The significance of genetic factors is suggested by a closer correlation of levels of some immunoglobulin classes in monozygotic, as compared with dizygotic human twin pairs

(Rowe & Boyle 1968), and by the observation of familial trends of some immunoglobulin classes in an African community (Rowe & McGregor 1968).

At birth the predominant immunoglobulin is maternal IgG, selectively transferred across the placenta (Brambell 1958). The low level of immunoglobulin synthesis by the foetus is attributable to freedom from antigenic stimulation in the intra-uterine environment rather than to absence of synthetic potential. The immunological competence of the animal foetus in antibody and immunoglobulin synthesis has been shown by direct antigenic stimulation (Silverstein *et al* 1963). The competence of the human foetus has been inferred from the presence of plasma cells in children born with congenital syphilis (Silverstein 1962).

Levels of IgG fall during the first few months of life as the maternal protein is catabolized, and then rise due to increasing synthesis by the child. Adult levels may be reached by the 5th year (West *et al* 1962). IgM, present only in trace amounts at birth, rises more rapidly to achieve adult values within the 1st year, and the antibody response of infants is predominantly in this class (Smith & Eitzman 1964). IgA levels rise more slowly, to reach adult levels by the age of 10–15 years. IgD is not detectable in cord blood but usually appears during the first few months of life. (For further details of immunoglobulin levels in children the reader should refer to the Chapter 19 on the Antibody Deficiency Syndrome.)

IMMUNOGLOBULIN METABOLISM

The metabolic characteristics of the four classes of immunoglobulins have been studied chiefly by the use of isolated proteins which have been trace-labelled with radioactive iodine isotopes. Measurements of serum and whole body radioactivity following injection of labelled immunoglobulins have provided the information shown in Table 13.3.

Immunoglobulins show certain metabolic features in common with other plasma proteins; for example they are catabolized more rapidly in pyrexial states. Each class of immunoglobulins is however metabolically distinct, with different synthetic rates and fractional catabolic rates. IgG has the lowest, and IgD the highest fractional catabolic rate. Differences in fractional catabolic rates between immunoglobulin classes make serum levels unreliable guides to synthetic rates. IgA has a similar synthetic rate to IgG, although the mean serum concentration of IgA is only about one-quarter that of IgG. Similarly IgM and IgD synthesis relative to IgG is greater than is suggested by a comparison of relative serum levels. The relationship between catabolism and serum concentration also differs between the classes. The fractional catabolic rate of IgG rises at high serum levels and falls at low serum levels, and so tends towards the maintenance of a constant concentration of this protein (Fahey & Robinson 1963). For IgM and IgA the fractional catabolic rate appears to be independent of serum

concentration, and for IgD the rate may be inversely related to serum concentration (Rogentine *et al* 1966).

Immunoglobulins also shows differences in their distribution between different fluid compartments. More than half the total body IgG and IgA is extravascular, whereas IgM and IgD are predominantly intravascular. These differences cannot be directly ascribed to differences of molecular size of the molecules, since IgD is of similar size to IgG and IgA. These findings suggest that IgG and IgA are the immunoglobulins chiefly involved in antigen-antibody combination outside the vascular compartment, at least in regions when normal capillary permeability is maintained.

IMMUNOGLOBULINS IN BODY FLUIDS OTHER THAN SERUM

Immunoglobulins and antibodies are probably present in most body fluids. The fluids chiefly studied are colostrum, saliva and urine. The predominant immunoglobulin of colostrum is IgA, which may be present in higher concentrations than in the serum. IgM is also present, sometimes in excess of serum levels, but there are only traces of IgG and no detectable IgD. IgA is also predominant in saliva, although in much lower concentrations. In both colostrum and saliva, IgA is distinguished by the presence of an antigenically distinct component which is not demonstrable in serum IgA (Tomasi *et al* 1965). The failure of trace-labelled serum IgA to appear in these secretions indicates that the protein is synthesized in the secretory gland. IgA synthesis may be a general feature of the membranes lining the respiratory and alimentary tracts. Immunofluorescent staining of intestinal mucosa shows large numbers of IgA synthesizing plasma cells (Crabbé & Carbonara 1965).

Normal urine contains IgA and IgG, free light chains and the Fc′ fragment of γ heavy chains (Turner & Rowe 1966). Fc′ is a fragment of the papain Fc fragment; it is not the normal counterpart of protein of Franklin's disease which is more closely related to Fc. Normal urine contains antibodies of which at least the majority are associated with IgA and IgG (Turner & Rowe 1967), although claims have been made for antibody activity in fragments of much lower molecular weight (Merler 1966).

Immunoglobulins have an interest and significance that extends beyond any one discipline of biological research. As the most accessible component of the allergic or immune system they are of prime interest to the immunologist. Changes of immunoglobulins in hypogammaglobulinaemia, in multiple myeloma and macroglobulinaemia, and in infections and auto-allergic diseases are of interest to the clinician and the pathologist. The structure and synthesis of immunoglobulin molecules, and the characteristics of the antigen-binding and other biologically active sites, present challenges to the biochemist. Genetic

variants of immunoglobulins concern the population geneticist and the molecular biologist. Finally it is reasonable to expect that the principles of the regulation of immunoglobulin structure and synthesis will be broadly applicable to other proteins. Studies of antibodies and immunoglobulins as model systems are relevant to the general problem of biochemical individuality.

REFERENCES

ADINOLFI M., GLYNN A.A., LINDSAY M. & MILNE C.M. (1966) Serological properties of γA antibodies to *E. coli* present in human colostrum. *Immunology* **10,** 517.

BARTH W.F., WOCHNER R.D., WALDMANN T.A. & FAHEY J.L. (1964) Metabolism of human gamma macroglobulin. *J. clin. Invest.* **43,** 1036.

BENCE JONES H. (1847) Papers on Chemical Pathology read at the Royal College of Physicians, 1846. Lecture 3. *Lancet* **ii,** 88.

BERGGARD I. (1961) On a γ-globulin of low molecular weight in normal human plasma and urine. *Clin. Chim. Acta.* **6,** 545.

BRAMBELL F.W.R. (1958) Passive immunity of young animals. *Biol. Rev.* **33,** 488.

BRENNER S. & MILSTEIN C. (1966) Origin of antibody varieties. *Nature* **211,** 242.

BURNET F.M. (1966) A possible genetic basis for specific pattern in antibody. *Nature* **210,** 1308.

COHEN S. (1963) Properties of the separated chains of human γ-globulin. *Nature* **197,** 253.

CRABBÉ P.A. & CARBONARA A.O. (1965) The normal human intestinal mucosa as a major source of plasma cells containing γA immunoglobulin. *Lab. Invest.* **14,** 235.

DEUTSCH H.F., MORTON J.I. & KRATOCHVIL C.H. (1956) Antigenic identity of hypergammaglobulinaemia serum components with proteins of normal serum. *J. Biol. Chem.* **222,** 39.

DRIVSHOLM A. (1964) Myelomatosis, A clinical and biochemical study of 105 cases. *Acta. Med. Scand.* **176,** 509.

EDELMAN G.M. & GALLY J.A. (1962) Nature of Bence Jones proteins. Chemical similarities to polypeptide chains of myeloma proteins and normal γ-globulins. *J. exp. Med.* **116,** 207.

FAHEY J.L. (1962) Heterogeneity of γ-globulins. *Advances in Immunology.* **2,** 41.

FAHEY J.L. (1963) Structural basis of difference between Type I and Type II human γ-globulin molecules. *J. Immunol.* **91,** 448.

FAHEY J.L. & ROBINSON A.G. (1963) Factors controlling serum γ-globulin concentration. *J. exp. Med.* **118,** 845.

FAHEY J.L. & MCKELVEY E.M. (1965) Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* **94,** 84.

FAHEY J.L. (1965) Antibodies and Immunoglobulins, II. Normal development and changes in disease. *J.A.M.A.* **194,** 255.

FAHEY J.L., BARTH W.F. & SOLOMON A. (1965) Serum hyperviscosity syndrome. *J.A.M.A.* **192,** 464.

FLEISCHMAN J.B., PORTER R.R. & PRESS E.M. (1963) Arrangement of peptide chains in γ-globulin. *Biochem. J.* **88,** 220.

FRANEK F. & NESLIN R.S. (1963) Recovery of antibody combining activity by interaction of different peptide chains isolated from purified horse antitoxin. *Folia Microbiol.* **8,** 128.

FRANKLIN E.C., LOWENSTEIN J., BIGELOW B. & MELTZER, M. (1964) Heavy chain disease, a new disorder of serum γ-globulin. *Amer. J. Med.* **37,** 332.

FUDENBERG H.H. (1965) The immune globulins. *Ann. Rev. Microbiol.* **19,** 301.

GREY H.M. & KUNKEL H.G. (1964) H chain subgroups of myeloma proteins and normal 7S γ-globulins. *J. exp. Med.* **120,** 253.

HÄLLEN J. (1963) Frequency of abnormal serum globulins (M components) in the aged. *Acta. Med. Scand.* **173,** 737.

HARBOE M., OSTERLAND C.K. & KUNKEL H.G. (1962) Localization of two genetic factors to different area of the γ-globulin molecule. *Science* **136,** 979.

HILSCHMANN N. & CRAIG L.C. (1965) Amino acid sequence studies with Bence Jones proteins. *Proc. Nat. Acad. Sci.* **53,** 1403.

HOLBOROW E.J. (ed.) (1963). Antibodies. *Brit. med. Bull.* **19,** 169.

ISHIZAKA K., ISHIZAKA T. & HORNBROOK M.M. (1966) Physiochemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *J. Immunol.* **97,** 75.

KORNGOLD L. (1963) Antigenic specificity of γ_2 myeloma globulins. *J. Natl. Cancer Inst.* **30,** 553.

KUNKEL H.G., MANNIK M. & WILLIAMS R.C. (1963) Individual antigenic specificity of isolated antibodies. *Science* **140,** 1218.

KYLE R.A. & BAYRD A. (1966) Benign monoclonal gammopathy. *Amer. J. Med.* **40,** 426.

MANCINI G., CARBONARA A.O. & HEREMANS J.F. (1965) Immunochemical quantitation of antigens by single radial diffusion. *Immunochemistry* **2,** 235.

MANNIK M. & KUNKEL H.G. (1962) Classification of myeloma proteins, Bence Jones proteins and macroglobulins into two groups on the basis of common antigenic characteristics. *J. exp. Med.* **116,** 859.

MANNIK M. & KUNKEL H.G. (1963a) Two major types of 7S γ-globulins. *J. Exp. Med.* **117,** 213.

MANNIK M. & KUNKEL H.G. (1963b) Localization of antibodies in Group I and Group II γ-globulins. *J. exp. Med.* **118,** 817.

MERLER E. (1966) The properties of isolated serum and urinary antibodies to a single antigen. *Immunology* **10,** 249.

MILLER F. & METZGER H. (1965) Characterization of a human macroglobulin. I. The molecular weight of its subunit. *J. Biol. Chem.* **240,** 3325.

MILSTEIN C. (1966) Variations in aminoacid sequence near the disulphide bridges of Bence Jones proteins. *Nature,* **209,** 370.

NISONOFF A. WISSLER F.C., LIPMAN L.N. & WOERNLEY D.L. (1960) Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulphide bonds. *Arch. Biochem. Biophys.* **89,** 230.

Nomenclature for human immunoglobuline (1964) *Bull. Wld. Hlth. Org.* **30,** 447.

OLINS D.E. & EDELMAN G.M. (1964) Reconstitution of 7S molecules from L and H polypeptide chains of antibodies and γ-globulins. *J. exp. Med.* **119,** 789.

OSSERMAN E. & TAKATSUKI K. (1964) Clinical and immunochemical studies of four cases of H γ_2 chain disease. *Amer. J. Med.* **37,** 351.

PAIN R.H. (1963) The molecular weights of the peptide chains of γ-globulin. *Biochem. J.* **88,** 234.

PORTER R.R. (1959) The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem. J.* **73,** 119.

ROGENTINE G.N., ROWE D.S., BRADLEY J., WALDMAN T. & FAHEY J.L. (1966) Metabolism of human immunoglobulin D. *J. Clin. Invest.* **45,** 1467.

ROHOLT O.A., RADZIMSKI J. & PRESSMAN D. (1966) Specificity in the combination of Fd fragments with L chains to form haptene-binding sites. *J. exp. Med.* **123,** 921.

ROWE D.S. & FAHEY J.L. (1965) A new class of human immunoglobulins II. Normal serum IgD. *J. exp. Med.* **121,** 185.

ROWE D.S., BOYLE J.A. and BUCHANAN W.W. (1968) Plasma immunoglobulin concentrations in towns. *J. clin. exp. Med.* In press.

ROWE D.S., MCGREGOR I.A., SMITH S.J., HALL P. and WILLIAMS K. (1968) Immunoglobulins concentrations in a West African (Gambian) community and in a group of healthy British adults. *J. clin. exp. Immunol.* **3,** 63.

SELL S. & FAHEY J.L. (1964) Relationship between γ-globulin metabolism and low serum γ-globulin in germ-free mice. *J. Immunol.* **93,** 81.

SILVERSTEIN A.M. (1962) Congenital syphilis and timing of immunogenesis in the human foetus. *Nature* **194,** 196.

SILVERSTEIN A.M., UHR J.W., KRAMER K.L. & LUKES R.J. (1963) Fetal response to antigenic stimuli. II. Antibody production by the fetal lamb. *J. exp. Med.* **117,** 799.

SMITH R.T. & EITZMAN D.V. (1964) Development of the immune response. Characterization of response of human infants and adults to immunization with Salmonella vaccines. *Pediatrics* **33,** 163.

SOLOMON A., WALDMANN T.A. & FAHEY J.L. (1963) Metabolism of normal 6.6S γ-globulin in normal subjects and in patients with macroglobulinaemia and multiple myeloma. *J. Lab. Clin. Med.* **62,** 1.

SOLOMON A. & TOMASI T.B. (1964) Metabolism of β_{2A}-globulin. *Clin. Res.* **12,** 452.

STEINBERG A. (1962) Progress in the study of genetically determined human γ-globulin types. *Progress in Medical Genetics* **2,** 1.

TERRY W.D. (1964) Subclasses of Human IgG molecules differing in heterologous skin sensitizing properties. *Proc. Soc. Exptl. Biol. Med.* **117,** 901.

TERRY W.D. & FAHEY J.L. (1964) Subclasses of human γ_2-globulins based on differences of heavy polypeptide chains. *Science* **146,** 400.

TERRY W.D., FAHEY J.L. & STEINBERG A. (1965) Gm and InV factors in subclasses of IgG. *J. Exp. Med.* **122,** 1087.

TISELIUS A. (1937) Electrophoresis of serum globulins. II. Electrophoretic analysis of normal and immune sera. *Biochem. J.* **31,** 1464.

TITANI K., WHITELEY E., AVOGARDO L. & PUTNAM F.W. (1965) Immunoglobulin structure. Partial amino acid sequence of a Bence Jones protein. *Science* **149,** 1090.

TOMASI T.B., TAN E.M., SOLOMON A. & PRENDERGAST R.A. (1965) Characteristics of an immune system common to certain external secretions. *J. exp. Med.* **121,** 101.

TURNER M.W. & ROWE D.S. (1964) Characterization of human antibodies to Salmonella typhi by gel filtration and antigenic analysis. *Immunology* **7,** 639.

TURNER M.W. & ROWE D.S. (1966) A naturally occurring fragment related to the heavy chains of immunoglobulin G in normal human urine. *Nature* **210,** 130.

TURNER M.W. & ROWE D.S. (1966b) Antibody of IgG and IgA class in normal human urine. *Immunology.* **12,** 689.

VAERMAN J.P., FUDENBERG H.H., JOHNSON L.B. & MANDY W.J. (1964) Size heterogeneity of normal and pathological γ_{1A}-globulins. *Fed. Proc.* **23,** 558.

WALDENSTRÖM J. (1961a) Clinical diagnosis and biochemical findings in a material of 296 sera with M type narrow γ-globulins. *Acta. Med. Scand. Suppl.* **367,** 110.

WALDENSTRÖM J. (1961b) Disturbed γ-globulin formation. *Harvey Lec.* **56,** 211.

WEST C.D., HONG R. & HOLLAND N.H. (1962) Immunoglobulin levels from the newborn period to adulthood and in the immunoglobulin deficiency states. *J. clin. Invest.* **41,** 2054.

CHAPTER 14

COMPLEMENT

P.J.LACHMANN

INTRODUCTION

Many of the biological consequences of antigen-antibody interaction are brought about not by antibody alone but require in addition the participation of the whole system of serum factors known collectively as complement. A certain economy can be discerned in this arrangement in that some division of function between antibody—to recognize and bind on to the antigen, and complement—

to mediate a variety of effector mechanisms that follow antigen-antibody interaction, is apparent. Thus antibody shows exquisite specificity towards the chemical nature of the antigen while the specificity of complement is directed primarily to the immune complex as such. Again, antibody is a single reactant and circulates in an active form, whereas complement is found to be made up of multiple reactants, including inhibitors, circulating in inactive forms and requiring sequential activation.

This complexity should not be a matter for surprise. As is the case with blood clotting, complement activation at the wrong time or place could be most damaging; even more than is the case with blood clotting the system is found to be multiple, complex and subject to a multitude of checks and balances. Indeed the very complexity of the complement system, discouraging though it may sometimes be, can be looked on as a hallmark of its biological potency.

An understanding of complement action is a necessary, if not itself a sufficient, prerequisite for understanding, and perhaps controlling, the biological consequences of many types of allergic reaction. Far from being the wholly esoteric pursuit it is not uncommonly made out to be, the study of complement has *inter alia*, very practical aims.

THE HISTORY OF COMPLEMENT

Complement was first recognized at the end of the nineteenth century as a heat-labile factor occurring in normal (i.e. non-immune serum) which was required in addition to antibody for the immune lysis of red blood cells and of certain bacteria. It was established that complement levels were roughly constant in adult members of a given species and were unaffected by immunization; that complement activity was destroyed by heating at 56° for 30 min, and that complement would react with neither antigen nor antibody alone but only with the complexes formed by their interaction. In these ways complement was unequivocally distinguished from antibody. However, it was the description of the 'complement fixation test' by Bordet & Gengou (1901) that established complement as a genuine immunological entity by demonstrating that it was quantitatively involved in antigen-antibody reactions generally rather than being merely an accessory factor required for the lysis of antibody coated cells.

Complement as a single immunological entity had a very short life. Already in 1907 Ferrata demonstrated that, as both the euglobulin and the pseudoglobulin fractions of serum were required for the haemolytic activity of complement, complement could not be a single substance. By the time of the second world war four components had been defined and partially characterized (see Pillemer *et al* 1941). These were: the heat-labile euglobulin factor, C'_1; the heat-labile pseudoglobulin factor, C'_2; a heat stable-factor inactivated by yeast cell walls, C'_3 and a heat-stable factor inactivated by hydrazine or ammonia, C'_4. In the

last 10 years further multiplication has taken place and currently (*vide infra*), C'_1 is known to be made up of three sub-components C'_{1q}, C'_{1r} and C'_{1s}; and what was known as C'_3 ('classical C'_3') is known to comprise not less than six separate factors—$C'_{3,\ 5,\ 6,\ 7,\ 8\ \text{and}\ 9}$. Thus, eleven factors in all are, as of now, known to be required for complement activity, at any rate as measured by the haemolysis of sheep erythrocytes sensitized with rabbit antibody.

The study of the nature of complement has passed through a number of phases. The earlier investigations were concerned largely with establishing its existence and by means of partial inactivation and fractionation procedures, dividing it into components. At this stage complement was entirely an activity of various serum fractions although Heidelberger (1941) did demonstrate quantitatively that complement fixation was accompanied by the uptake of protein nitrogen on to the immune complex. During the next phase, complement was still predominantly an activity but an activity whose mechanism was becoming much better understood. This was due in the main to the work of Mayer and his collaborators (see Mayer 1961) who undertook detailed kinetic studies of the major steps of the haemolytic reaction. From these they were able to deduce that the lysis of the red cell did not require cumulative damage at many sites but could result from a 'single hit' of complement activity. Simultaneously the work of Levine (1955), Becker (1956) and Lepow (1959) showed that the activity of at least one complement component—the first—could be correlated with a defined enzymatic activity.

The present phase, which is still very much in progress, is that of converting complement components from the activities of serum fractions to isolated, well-characterized proteins. This phase was started as recently as 1960 by the isolation of human C'_3 (β_{1C}-globulin) by Müller-Eberhard & Nilsson; and Müller-Eberhard and his colleagues have played a leading part in this work. With the availability of pure components it is becoming possible to analyse the reactions of the complement components in biochemical terms. The attempt to understand the manifold functions of complement in terms of the structure of its components is starting and will doubtless keep the study of complement active for some time to come.

DEFINITION OF COMPLEMENT

Hand in hand with the growing appreciation of the complexity of the complement system has come a certain difficulty in giving the system an exact definition. The question was recently discussed (CIBA symposium 1965) and the various points of view are to be found there.

It is possible to define complement entirely in the context of the immune haemolytic reaction. This has the merit of clarity but raises difficulties when complement is regarded biologically. As will be seen in later sections, not only

do many of the biologically significant functions of complement not require all of the components of haemolytic complement but there is now at least one example of a function (conglutination) where a component is required that does not appear to participate in haemolysis. Other divergent branches from the complement sequence in haemolysis may still be found.

The second possibility is to define complement in terms of function where these functions are known to be mediated by at least some of the characterized complement components. This definition again has difficulties of its own, mainly that it cannot yet be stated in precise terms for those functions where the role of complement is not wholly worked out.

IMMUNOCHEMISTRY OF COMPLEMENT AND THE IMMUNE HAEMOLYTIC REACTION

The lysis of sheep erythrocytes (E) sensitized with rabbit antibody (A) by guinea-pig or human complement has served as the model for the great bulk of investigation on complement. The stages involved appear to be essentially the same for both species of complement. The terminology used is that recommended by the Complement Workshop (1966). The haemolytic sequence is shown schematically in Fig. 14.1.

$$EA + C'_{1q,r,s} \xrightarrow{Ca^{++}} EAC'_{1a}$$

The first component of complement (C'_1) occurs in fresh serum (or its euglobulin fraction) as a macromolecular complex with a sedimentation coefficent of around 18S (Naff, Pensky & Lepow 1964). Calcium ions are needed to hold this macromolecular complex together and if these are chelated by EDTA, the complex splits into its three subcomponents, C'_{1q}, C'_{1r} and C'_{1s}. If calcium ions are added again the macromolecular complex reforms. Whereas native C'_1 in serum is enzymatically inactive, partially purified C'_1 spontaneously generates the esterase activity characteristic of activated C'_1 (C'_{1a}) on incubation. This is believed to be associated with purification from the C'_1 inhibitor described below.

C'_{1q} is identical with the 11S component (also called C'_0) described by Müller-Eberhard (1961). This sub-component carries the binding site for the immune complex and the combination of antigen-antibody complex and C'_{1q} will take place in the presence of EDTA. In fact an immune complex binds far more C'_{1q} from serum in the presence of EDTA than it does from normal fresh serum. It seems that the reaction of subsequent complement components in some way inhibits further C'_{1q} binding, and this may be one of quite a number of homeostatic mechanisms controlling complement fixation. Relatively little is

known of C'_{1r}. It is very heat labile, has a sedimentation coefficient of about 7S and is essential for C'_1 activity (Lepow, Naff & Pensky 1965). It has not yet been obtained in pure form.

C'_{1s} with a sedimentation coefficient of 4S is the pro-esterase and after activation can by itself exhibit enzymatic activity. This enzymatic activity is demonstrated by the hydrolysis of esters such as TAME (tosyl arginine methyl ester) or

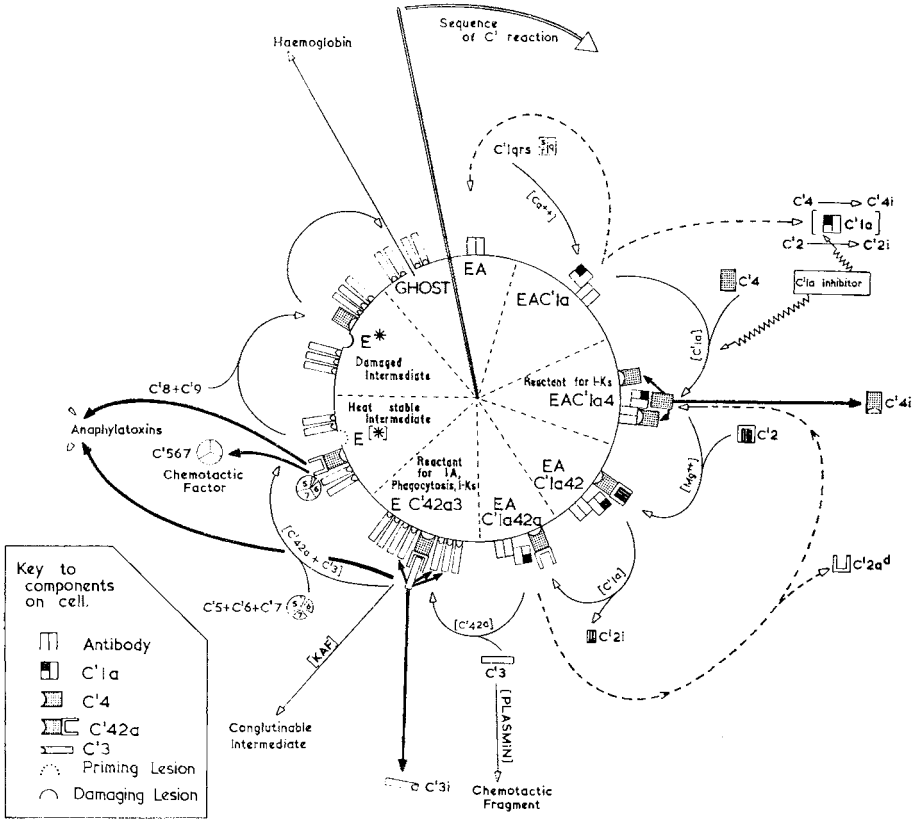


FIG. 14.1. Schematic representation of complement fixation sequence on an antibody-treated erythrocyte.

ATEE (acetyl tyrosine ethyl ester) and is inhibited by such esterase inhibitors as DFP. The enzyme has therefore been called 'C'_1-esterase'. However, it is generally believed that in its activity as a complement component the enzyme is probably acting as a peptidase. Its only known natural substrates are C'_2 and C'_4 (*vide infra*) and it has not been found to have any effect on other complement components or on the cell membrane. C'_1-esterase also increases vascular perme-

ability (Ratnoff & Lepow 1963). This may depend on the production of anaphlatoxin (q.v.) as an eventual result of the formation of a little active C'_{42a} in solution.

The reaction between EA and C'_1 occurs rapidly, the C'_1 apparently binding to the antibody molecule. The bound C'_1 is in its activated form and is now known as C'_{1a}. The complex EAC'_{1a} is quite stable at low ionic strength. In physiological conditions it is rather stable at 37° but dissociates rapidly—still as active enzyme—in the cold (De Looze & Leon 1963). This surprising observation seems at first sight to be incompatible with the second law of thermodynamics, and probably means that either the C'_1 or its receptor site on EA undergoes a temperature-dependent alteration affecting its binding site.

C'_1 inhibitor (see Lepow, Naff & Pensky 1965).
Although not strictly relevant to the haemolytic reaction it is convenient to mention here that an inhibitor of C'_1 esterase normally occurs in serum. This is an α_2 globulin with a sedimentation coefficient of 3S and combines stoichiometrically with the enzyme, apparently at its active site, inhibiting both the enzymatic and haemolytic function. There is no reaction with unactivated C'_1. The inhibitor does not inhibit the haemolytic reaction produced by whole complement because its reaction rate is slower that that of C'_{1a} on C'_4 and C'_2. However, attempts to make EAC'_1 or EAC'_{14} intermediates with reagents containing inhibitor are often unsuccessful. The inhibitor is known to play an important part in inactivating any C'_1 esterase formed in solution where it would destroy C'_4 and C'_2 and give rise to increased capillary permeability. It is interesting that the inhibitor is active against Kallikrein and PF/Dil, two other mediators of increased capillary permeability, as well as against C'_1 esterase (Kagen & Becker 1963).

$$EAC_{1a} \xrightarrow{C'_4 \quad C'_{4i}} EAC'_{1a4}$$

C'_4 (βIE-globulin) has been obtained in a high state of purity from human serum (Müller-Eberhard & Biro 1963). It is a glycoprotein with a sedimentation coefficient of 10S. Its serum concentration is in the region of 50μgm/ml. The characteristic property by which C'_4 was originally recognized is its destruction as a complement component by ammonia or hydrazine (or other primary amines). These agents have no effect on the function of fixed C'_4, so it may be inferred that it is the binding site of C'_4 which is affected.

The reaction of EAC'_{1a} with C'_4 occurs optimally at 37° but some reaction goes on even at 4°.

It is thought (Müller-Eberhard & Lepow 1965) that a result of the action of C'_1-esterase, C'_4 achieves a short lived 'activated' state during which it is cap-

able of combining with receptors on the cell membrane, or with IgG molecules or with other C'_4 molecules. If no reaction has occurred quite quickly it 'decays' into an inactive form. The C'_4 taken up by EAC'_{1a} is less than 10% of that offered (Müller-Eberhard, Dalmasso & Calcott 1966). It is largely bound to the cell membrane but antibody-C'_4 complexes have also been shown to occur and to be active in producing haemolysis (Willoughby & Mayer 1965). The attachment of the C'_4 to the cell membrane is believed to be by hydrophobic bonding (Dalmasso & Müller-Eberhard 1965). This type of bonding would be compatible with the great stability of the EC'_4 bond; and it would also make it unnecessary to postulate specific receptor sites for C'_4 common to the cell membrane, IgG molecules and C'_4 molecules themselves.

The uptake of C'_4 on to EAC'_1 has been reported to increase the sodium flux and to be associated with an increase in the water volume of the cell (Zappacosta, Rossi & Zappacosta 1965).

$$EAC'_{1a4} + C'_2 \xrightarrow{Mg^{++}} EAC'_{1a42} \xrightarrow{C'_{1a}} EAC'_{1a42a} + C'_{2i}$$

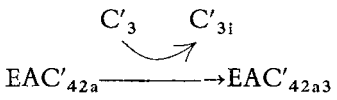
C'_2 is the heat-labile, pseudoglobulin component. Although C'_2 has not yet been well characterized as a protein, a high degree of purification has been achieved for the human component (Polley & Müller-Eberhard 1967) and functional purity for the guinea-pig component. (Borsos, Rapp & Cook 1961). C'_2 is present in very small quantities, less than 10 μgm/ml and its sedimentation coefficient is estimated to be in the region of 6S.

Human C'_2 is inactivated by p-hydroxymercurybenzoate (Leon 1965) suggesting that an -SH group is important for its activity. Oxidation of human C'_2 with low concentrations of iodine (the product is called C'_{oxy-2}) on the other hand markedly potentiates its activity (Polley & Müller-Eberhard 1966). While the biological significance of this observation is obscure, it is a boon for complement research.

The reaction of EAC'_{1a4} with C'_2 requires the activity of C'_1-esterase and the presence of magnesium ions. The reaction is thought to occur in two steps (Mayer 1965). The C'_2 combines with the C'_4 and there acts as substrate for C'_1-esterase which splits it into two parts: C'_{2a} which remains attached to C'_4, and C'_{2i} which goes into the fluid phase.

The complex EAC'_{1a42a} is unstable, the C'_{2a} rapidly eluting off the complex in a haemolytically inactive form C'^{d}_{2a} (Stroud *et al* 1966). The half-life of EAC'_{1a42a} at 37° is approximately 10 min. If human C'_{oxy-2} is used to make EAC'_{1a42a} the half-life is increased about ten-fold (Polley & Müller-Eberhard 1966). The rapid decay of EAC'_{1a42a} may be pictured as another homeostatic mechanism in complement fixation, limiting the C'_3 fixation at any particular complement fixation site (*vide infra*). After the EAC'_{1a42a} stage C'_{1a} serves no

further necessary function and EAC'_{42a} (prepared by eluting C'_{1a} with EDTA) behaves similarly to EAC'_{1a42a}. There is also evidence that fixed C'_1 activity is inhibited during the C'_3 fixation step (Lachmann 1966a)—which may be part of yet another homeostatic mechanism. In any case the symbol C'_{1a} will be deleted from the subsequent complement fixation steps.



C'_3 is by far the most abundant of the complement components, its serum concentration being in the range 500–1000 μgm/ml. It was first identified as β_{1C}-globulin in human serum by Müller-Eberhard & Nilsson 1961, and has more recently been obtained in very pure form by the same workers (Nilsson & Müller-Eberhard 1965). Guinea-Pig C'_3 has also been purified (Nishioka & Linscott 1963; Klein & Wellensiek 1965). It is a glycoprotein with a sedimentation coefficient of 9.5S and is a euglobulin. Like C'_4 it is inactivated, at least to a considerable extent, by ammonia or hydrazine. Although C'_3 shows marked resistance to heating, it undergoes spontaneous decay to an inactive form on storage.

The reaction of C'_3 with EAC'_{42a} requires the activity of the C'_{42a} complex and occurs optimally at 32°. The C'_{42a} complex appears to act as an enzyme—given the name C'_3-convertase by Müller-Eberhard, Polley & Calcott 1966—which has C'_3 as its substrate. The nature of the reaction catalysed is not known but as a result C'_3 is considered to become 'activated'. It may then either become bound on the intermediate complex or if it fails to do this it decays rapidly to an inactive form, C'_{3i} which appears in solution. C'_{3i} can be distinguished from C'_3 on immunoelectrophoresis by its faster mobility. It is however not markedly smaller than C'_3. C'_{3i} in serum, on the other hand, is converted further to β_{1A}-globulin which, with a sedimentation coefficient of 6.8S, is probably about half C'_{3i}, and to a further product, d_{2D} (West *et. al.* 1966)

C'_3 binding, like that of C'_4, is believed to be by hydrophobic bonding to the cell membrane. Perhaps the most significant feature of C'_3 binding is the large amount that is bound. Each C'_{42a} site can lead to the fixation of several hundred molecules of C'_3 although the efficiency of uptake is not greater than 20% (Müller-Eberhard, Dalmasso & Calcott 1966). This loading of the cell with C'_3 is an important aspect of the complement reaction but its main significance seems to be in manifestations of complement activity other than haemolysis.

C'_3 is therefore discussed further in the sections on these other manifestations. It is in fact quite possible that the haemolytic function of C'_3 resides in only that portion of the C'_3 bound in proximity to the C'_{42a} complex (Lachmann & Liske 1966).

The intermediate EAC'_{42a3} shows peptidase activity towards peptides containing aromatic amino acid residues (Cooper & Becker 1966), though it is not yet clear if this activity is involved in subsequent haemolytic steps.

The existence of a natural inhibitor of fixed C'_3 has been reported by Tamura & Jensen (1966). Another factor (the conglutinogen-activating factor) that reacts with fixed C'_3 is discussed further in the section on conglutination.

C'_3 can be inactivated *in vitro* and *in vivo* by a purified factor from cobra venom in conjunction with a specific normal serum β-globulin (Müller-Eberhard *et al* 1966).

$$EAC'_{42a3} \xrightarrow{C'_5 + C'_6 + C'_7 \;\rightarrow\; C'_{567}} \text{Heat Stable Intermediate.}$$

Of the three components reacting at this stage, only C'_5 (β_{1F}-globulin) is so far a well-characterized protein (Nilsson & Müller-Eberhard 1965). C'_6 and C'_7 have been obtained in 'functionally purified' form (Nilsson & Müller-Eberhard 1967). The corresponding guinea-pig components have also been obtained functionally pure (Klein & Wellinsiek 1965; Wellensiek & Klein 1965; Nelson *et al* 1966).

There is evidence that in their reaction with EAC'_{42a3}, C'_5, C'_6 and C'_7 under physiological conditions act as a functional unit (Nilsson & Müller-Eberhard 1967). Most, if not all, the C'_5, C'_6 and C'_7 is not fixed on the alexinated cell but is released as a definite complex into the fluid phase where it acts as a chemotactic factor (see section on Phagocytosis).

The nature of the reaction on the cell is obscure. The reaction requires C'_{42a} complex as well as C'_3 and C'_5, C'_6 and C'_7. The half life of EAC'_{42a3} for haemolytic reactivity is thus as short as that of EAC'_{42a}. The alexinated complex resulting from this interaction is however heat stable and can be lysed by the subsequent complement components even after some hours at 37°. It can be tentatively inferred from experiments involving complement in free solution (q.v.) that the lysis of the heat-stable intermediate does not require the presence of any of the preceding complement components on the cell. If this is indeed so, the formation of the heat-stable complex presumably involves some change at the cell membrane, which 'primes' the membrane for the activity of the subsequent components. However no evidence as to the nature of such a 'priming' lesion is as yet available. It is also difficult to give the heat-stable intermediate a suitable abbreviation. $EAC'_{42a3567}$ seems inappropriate as well as cumbersome; perhaps $E^{(*)}$ (E-semi-star) would be better.

$$\text{Heat-stable intermediate} + C'_8 + C'_9 \longrightarrow E^*$$

Two further components, C'_8 and C'_9 are, as of now, known to be required to complete the haemolytic reaction. They have been obtained in functionally

pure form from guinea-pig serum (Wellensiek & Klein 1965; Nelson *et al* 1966). C'_9 has been highly purified from human serum (Hadding, Müller-Eberhard & Dalmasso 1966).

Little is so far known of the way in which these components act,* but when their reaction is completed the membrane is damaged and the cell proceeds spontaneously to lysis in the absence of any serum factors.

$$E^* \longrightarrow \text{Lysis of Cell.}$$

This final phase of immune lysis has also turned out to be rather less than simple. The spontaneous lysis of E* can be inhibited by cold (Mayer & Levine 1954); by strong (0.09%) EDTA (Frank, Rapp & Borsos 1965) and by 25% albumin (Green, Barrow & Goldberg 1959). It transpires that these inhibitors act on different steps. First a temperature dependent step; then the step that can be inhibited by strong EDTA, finally the step that can be inhibited by 25% albumin.

The nature of the first two steps is unknown, but at the end of the second step, the lytic process is complete as far as the small molecule (e.g. potassium ion) content of the cell is concerned. The final step leading to the leakage of haemoglobin appears to be osmotic in nature; the high colloid osmotic pressure within the cell drawing in water till sufficient stretching has occurred to allow the haemoglobin out (Green, Barrow & Goldberg 1959).

The chemical nature of the actual lytic process remains unclear. It has been suggested (see Fischer 1964) that lysolecithin is produced by the action of a lecithinase, activated in the final stages of the complement reaction, on serum phospholipid. There are a number of difficulties in regarding this as the mechanism of lysis of E*: it does not readily account for the multiple stages in the lysis of E* that occur in the absence of any serum factors; it does not obviously require a 'priming' lesion on the membrane; and it has recently been shown that serum free of all lipid is fully active haemolytically (Dalmasso & Müller-Eberhard 1966). If a phospholipase is the final mediator of complement—and there is as yet no experimental evidence that it is—then it must presumably be one that can act directly on the cell wall phospholipids. On the other hand there is some evidence that lysolecithin is formed during complement activation, and even if it plays no necessary part in the usual model system for immune haemolysis it could nevertheless be important in cell lysis under other conditions or in other manifestations of complement activity.

In contrast to the obscurity that surrounds the chemical nature of the final lytic process, a lot of information on the structural nature of the lesions has been obtained by electron microscopy (Humphrey & Dourmashkin 1965).

*Recent work has shown that the Fe^{++}—chelating substance phenanthroline can abolish the requirement for C'_9 (Hadding and Müller-Eberhard, 1967), suggesting that C'_8 is the important mediator of the last stage of complement lyses, C'_9 perhaps being some form of activator.

These workers examined membranes of red cells lysed by guinea-pig complement by negative staining and found characteristic membrane lesions of mean diameter 88Å, which appeared to be holes surrounded by clear rings.

The number of such lesions per cell was shown to conform with the number of sites of damage predicted by the 'single-hit theory' for particular amounts of complement (Borsos, Dourmashkin & Humphrey 1964). This conformity however depends on using rabbit IgM antibody and guinea-pig complement. If rabbit antibody of the IgG class is used even with guinea-pig complement clusters of lesions are found instead of single ones (Humphrey 1967). The lesions produced in human red cells sensitized with human antibody by human complement are larger (mean diameter 103Å) and occur in very large numbers (Rosse, Dourmashkin & Humphrey 1966). At a complement level giving 12% lysis more than 10,000 lesions were found per cell. At this level of haemolysis the one hit theory predicts on average of about 0.2 hits per cell. The explanation for these disparities is unclear, but they emphasize the dangers of applying conclusions from a particular model system even to systems apparently not markedly different.

The lesions could be produced in formalin fixed membranes and they resisted treatment with trypsin and acid; their appearance was distinct from those produced by saponin, streptolysin O or phospholipase C. Humphrey & Dourmashkin consider that the lesions most probably represent localized changes in the lipid layer of the cell membrane.

The haemolytic reaction mediated by complement is seen to be surprisingly complex even compared with an analogous system such as blood clotting. The cascade phenomenon which is seen in blood clotting—a series of reactions where the product of one reaction serves as a catalyst of the next—is found also in complement. But whereas the cascade of clotting is 'linear'—each product catalysing just the next reaction, the complement sequence is not. Thus C'_{1a} is needed for the fixation of both C'_4 and C'_2; C'_{42a} is needed for both the fixation of C'_3 and the reaction of C'_5, C'_6 and C'_7. The tendency of the reaction to reverse (the change from EAC'_{1a42a} to EAC'_{1a4}) and the possibility that the eventual membrane lesion involves both a priming and a final phase are further complications that blood clotting is spared.

'Free Solution Haemolysis'

The activation of complement leading to immune haemolysis is normally found at specific sites of initiation on the surface of a cell, and the complement action is finely localized to this site. It has, however, recently become possible to mimic complement activation in solution. Thus, C'_1-esterase can be prepared from purified C'_1 or by elution from EAC'_{1a} as already discussed. The interaction of C'_1-esterase, C'_4 and C'_2 to form active C'_{42a} can also take place in solution (Müller-Eberhard *et al* 1966). The C'_{42a} can be detected by the conver-

sion of C'_3 to C'_{3i} in solution and is therefore given the name C'_3-convertase. If C'_3-convertase' C'_3, C'_5, C'_6 and C'_7 are incubated together in the presence of unsensitized erythrocytes, a small proportion of erythrocytes reach the 'heat-stable' intermediate stage in as much as that after washing and incubation for some time at 37°, they can be lysed by C'_8 and C'_9. This reactivity is acquired without any complement components being bound in detectable amounts on the cell membrane (Müller-Eberhard 1967).

THE INITIATION OF COMPLEMENT FIXATION

Complement has as one of its most basic properties the capacity to interact with most, though by no means all, antigen-antibody combinations. The reasons why certain antigen-antibody systems will not fix complement are not wholly understood but a number of factors are known to play a part.

The Immunoglobulin Class of the Antibody

IgM antibodies are, with certain exceptions, very good at initiating complement fixation. IgG antibodies vary in their ability to fix complement. In some species (e.g. guinea-pig, mouse, rabbit) it is possible to separate a distinct complement fixing subfraction of IgG from a non-complement fixing but skin sensitizing subfraction by its slower electrophoretic mobility (see Benacerraf 1964). That these two activities are alternatives and do not coexist on one molecule presumably protects the target cells in skin from complement lysis. IgA antibodies have generally been found not to be complement fixing although Adinolfi *et al* (1966) have reported that IgA antibodies to *E. Coli* mediate 'complement dependent bacteriolysis' (*vide infra*).

There is evidence from the relationship of the amount of antibody on a erythrocyte to the number of electron microscopic lesions produced by complement that a single molecule of rabbit IgM antibody on a sheep cell is sufficient to initiate a site of guinea-pig complement activation, whereas it probably requires two adjacent molecules of IgG (Humphrey & Dourmashkin 1965). Borsos & Rapp (1965) came to a similar conclusion on the basis of calculations on the amount of C'_{1a} bound.

This observation would go far to explain the great superiority (molecule for molecule) of IgM over IgG in initiating complement fixation. It must be appreciated that these relationships are well-established only for rabbit antibody and guinea-pig complement acting on sheep erythrocytes. In other species combinations the situation may not be the same (see next section). At the molecular level the site for complement activation seems to be on the Fc fragment; Fab made with papain being non-complement-fixing and Fab'2 made with pepsin showing much reduced complement binding.

IgG aggregates prepared by heating or by such chemical means as bis-diazobenzidine fix complement as well as antigen-antibody aggregates. It is less likely that this activity results merely from bringing two IgG molecules into close opposition than that there is also a configurational change in the molecules produced both by antigen-antibody interaction and by the other forms of aggregation.

Although a configurational change in IgG seems to be necessary for complement activation, it has been shown (Müller-Eberhard & Calcott 1966) that native IgG and C'_{1q} form a loose interaction that can be detected in the ultracentrifuge. This loose complex can be made up of as many as four IgG molecules per C'_{1q} molecule.

The influence of the immunoglobulin class of antibody in complement reactions is surprisingly not restricted to the initiation of complement fixation. It also affects in an ill-understood fashion subsequent steps of the reaction. Thus Sell & Spooner (1966) have found that while IgG anti-Forssman antibody fixes complement less well than IgM, the fixed complement is highly lytic for both nucleated cells and erythrocytes. On the other hand the complement fixed by IgM antibody, though highly lytic to erythrocytes was relatively ineffective in producing lysis of nucleated cells. Further Humphrey (1967) has shown that whereas sheep red cells sensitized with IgM rabbit antibody and lysed with guinea-pig complement have one lesion (as seen in the electron microscope) per complement activation site, those sensitized with IgG antibody have a cluster of lesions.

The Nature of the Antigen

Immune complexes involving certain antigens fail to fix complement. The Rhesus antigens on red cells are a particularly good example, although one example of a complement-fixing anti-Rh(D) antiserum has been described (Waller & Lawler 1962). In the case of IgG antibodies it is possible to give an explanation in terms of the antigens being too far apart for suitable adjacent pairs to be formed. However, this explanation seems insufficient for the IgM antibodies which also fail to fix complement unless it is postulated that for complement fixation IgM needs to react through two or more combining groups There is no convincing alternative explanation. It is possible to postulate a constraint on the structure of the antibody, i.e. antibodies in order to be capable of reacting with D antigen requiring to be without the complement binding site. However since the antigen combining site is on Fab and the complement activating site on Fc this hypothesis is somewhat unattractive.

Complexes between thyroglobulin and the (IgG) auto-antibodies found in Hashimoto's disease are another example of a generally non-complement fixing system. Here spatial considerations may be responsible; as they may also be for the finding that complement fixation is maximal on antigen-antibody aggregates if these are formed near equivalence (Goldsworthy 1928).

The Relationships between the Species of Origin of the Antibody and that of the Source of Complement

Not all species of complement are fixed equally well by all species of antibody. In fact a considerable degree of selectivity is found to occur, which in the *in vitro*, if not in the *in vivo*, situation is of some importance.

An extreme case is that certain avian antibodies, notably chicken and duck, fail to fix mammalian complement at all. The inter-relationships between various mammalian species have been studied by Coombs and his colleagues (Blomfield, Coombs & Hole 1949; Hoet, Blomfield & Coombs 1954). These workers found that it was not in general true that antibodies of one species fixed their own complement the best. For example human antibody is relatively poor at fixing human complement and pig antibody poor at fixing pig complement. The complements of horse and rabbit were fixed particularly well by all the eleven species of mammalian antibody tested. Antibodies from these same two species were also capable of fixing well all the six species of complement tested. Guinea-pig complement, although it is so commonly used, is rather selective, being particularly badly fixed by cat and pig antibodies and indifferently by human antibodies.

It is possible that some part of the selectivity may reflect differencies in the immunoglobulin composition of the various antisera. Thus the preference of guinea-pig complement for IgM as opposed to IgG antibodies (of the rabbit or mouse) is not shared by rabbit or mouse complement (Winn 1965) which are more readily activated by IgG antibody.

'Non-Specific' Fixation of Complement

When complement fixation is initiated in the absence of antigen-antibody interaction this is often termed 'non-specific' activation. Aggregation of IgG is certainly the principal phenomenon that brings this about. This can be induced at will by heating or by various chemical procedures. This phenomenon is believed to be responsible for the complement fixation which takes place on kaolin or similar particulate matter when put into fresh serum; and for the binding of complement on to red cells put into serum either at low ionic strength (Mollison & Polley 1964) or (after trypsin treatment) into serum in the presence of polyethylene glycol (Dalmasso & Müller-Eberhard 1964). A certain amount of complement fixation occurs on fibrin clotted from fresh plasma and this again is believed to arise in the same way (Pepys, Lachmann & Coombs, 1965).

The extent to which 'non-specific' aggregation of IgG is important *in vivo* is unknown.

The Complement Fixation Test

The detection of antigen-antibody interaction by the fixation of complement goes back to Bordet & Gengou (1901) and remains a most widely used technique. Many techniques for carrying out this test have been devised (see Mayer 1961; Hole & Coombs 1947) relying on the disappearance of the haemolytic or conglutinating activity of complement in the reaction mixture.

Analogous are techniques where the fixation of complement is shown antigenically usually by the presence of C'_3 and C'_4 at the site of the original antigen-antibody reaction. This is the basis of the 'complement-antiglobulin reaction' (Rosenfield, Haber & Gilbert 1960; Pondman *et al* 1960; Harboe *et al* 1963) which is used in the demonstration of low avidity blood group antibodies. This is also the method used for carrying out complement fixation tests on tissue sections (Goldwasser & Shepherd 1958; Burkholder 1961), fluorescein—conjugated antisera to complement (again generally to C'_3 and/or to C'_4) being used to localize the fixed complement. This method can be extended in the study of tissues to localize complement components which have been fixed *in vivo* (Lachmann *et al* 1962). This will be discussed further in a later section.

It may be noticed that C'_4 and C'_3 are the complement components detected in these methods. These are the components known to be directly bound to cell membranes and are those which are found in the 'long-term' complement fixation site. It seems likely that there is a biological significance to this 'long term complement fixation' and it is these two complement components that are likely to be involved.

PHENOMENA ASSOCIATED WITH COMPLEMENT ACTIVITY

Lysis of Antibody-Sensitized Cells

Haemolysis

The process of haemolysis has been dealt with above. Reactions of this kind are involved in certain haemolytic anaemias (e.g. the Donath-Landsteiner type, (Hinz, Picken & Lepow 1961)) particularly where haemolysis occurs predominantly intravascularly—as shown by haemoglobinuria. In haemolytic states mediated by non-complement fixing immune systems (e.g. haemolytic disease sue to Rh incompatibility) abnormal sequestration of cells by the reticulo-endothelial system is the main pathogenic process and intravascular lysis and haemoglobinuria are not prominent. It has been shown (see Mollison 1965) that red blood cells sensitized with small quantities of a non-complement fixing antibody are cleared from the circulation predominantly in the spleen while cells sensitized with small quantities of a complement fixing antibody are sequestrated in the liver.

Cytolysis of Nucleated Cells

Lysis of nucleated cells *in vitro* appears to follow the same complement sequence as haemolysis (Ross & Lepow 1960). There are probably certain differences, as shown for example by the variation found by Sell & Spooner (1966) between the haemolytic and cytolytic activities of guinea-pig complement (in association with IgM antibodies) and by the generally high cytolytic activity of rabbit complement. It may be that owing to the ability of nucleated cells to repair lesions (Chambers & Fell 1931) the lysis of these cells is not always a single hit phenomenon.

The lysis of nucleated cells by antibody and complement *in vivo* can be demonstrated by such procedures as injecting guinea-pigs with anti-Forssman serum.

A reaction of this type may play some part in auto-allergic disease (e.g. Hasimoto's disease) where complement fixing antibodies to cell membrane antigens have been demonstrated (Irvine 1960). The lysis of testicular cells by normal autologous serum (Spooner 1964) is again a similar phenomenon and may become active *in vivo* if the testicular basement membrane is damaged. This could be the case in diseases like the orchitis following mumps.

The lysis of certain bacteria (e.g. *Salmonella typhi*) by antibody and complement is brought about by an analogous process (Muschel & Treffers 1956). A further serum factor, lysozyme, is however, necessary in many cases for bacteriolysis (Inoue *et al* 1959). The lysozyme can act either before or after the complement (Muschel 1965). In the bacteriolysis of *E. coli.*, complement alone will produce some lysis if IgM antibody is used to sensitize the bacteria, whereas if IgA is used the requirement for lysozyme is absolute (Aldinolfi *et al* 1966).

The Treponemal Immobilization Test (Nelson and Mayer, 1949) for syphilis is also a complement-dependent cytotoxic reaction. An *in vivo* example is the lysis of such bacteria as *Vibrio cholerae* in the circulation of an immune animal.

The Effects of Antibody and Complement in Organ Culture

Although isolated tissue culture cells exposed to antibody reacting with cell membrane antigens undergo cytolysis, when such experiments are done in organ culture where the spatial relationships are quite different, other effects have been found (Fell, Coombs & Dingle 1966).

These authors have grown organ cultures of chick bones in the presence of anti-Forssman serum and complement. In this system cytolysis is limited to the outermost cell layer, but there is a degradation and disorganization of matrix structure over the whole bone rudiment. This has been shown to be associated with lysosomal activity (Dingle, Fell & Coombs 1967).

These findings obviously have important implications. Although there is no well-substantiated homologous situation in human disease it has been suggested

(Dingle, Fell & Coombs 1967) that the 'Long Acting Thyroid Stimulator' acts on thyroid in the same way.

Conglutination

The serum of cattle and certain other ruminants but not of other types of mammal contains a substance which will powerfully aggregate immune complexes having complement bound on them. This material was given the name 'conglutinin' by Bourdet & Streng (1909). Conglutinin was at one time regarded as a naturally occurring anticomplement antibody but this is now known not to be the case. Conglutinin has been highly purified and is a specific serum protein, unrelated antigenically to the immunoglobulins. It shows a high degree of molecular assymetry having a sedimentation coefficient of 7.8S, a molecular weight of about 75,000 and a frictional ratio of around 4 (Lachmann & Coombs 1965).

Substances analogous to conglutinin were produced in other species by injection with alexinated material and were for this reason given the name 'immuno-conglutinins' (Streng 1930; Wartiovaara 1932). The phenomenon was studied extensively by Coombs and his colleagues (see Coombs, Coombs & Ingram 1961) who were able to show that immunoconglutinins were formed in response to the antigenic stimulus of an animal's own fixed complement and that therefore these 'auto-stimulated' immunoconglutinins were true auto-antibodies. Physicochemically immunoconglutinins are immunoglobulins predominantly of the IgM class (Lachmann & Coombs 1965; Bienenstock & Bloch 1965). Immunoconglutinins are found in all species that have been tested.

The reactant for conglutinin (the conglutinogen) is found not only in fixed complement but also, remarkably enough, in the cell walls and in the microsomal fraction of yeasts. It is predominantly polysaccharide in composition, mannose being the predominant sugar, and contains some peptide material (Lachmann & Coombs 1965; Leon, Yokohari & Itoh 1966). The reaction of conglutinin with conglutinogen requires calcium ions (Leon 1957) and conglutinin may be purified by absorption on to zymosan (yeast cell walls) and elution with EDTA (Lachmann & Coombs 1965).

The conglutinogen in fixed complement is found in fixed C'_3, but only when the fixed C'_3 has reacted with a further serum factor—to which the name 'conglutinogenactivating-factor' (or KAF) has been given (Lachmann & Müller-Eberhard 1967). KAF appears to play no essential role in immune haemolysis and for this reason it is a matter of opinion whether it should be considered a complement component. It is a β-globulin with a sedimentation coefficient of about 5.5S and a molecular weight in the region of 100,000. It is present in very small amounts and probably acts enzymatically upon bound C'_3. As a result the reactant for conglutinin appears on the alexinated complex and the bound C'_3 also becomes very much more susceptible to proteolytic attack.

As far as is known the conglutinogen in C'_3 from different species is identical.

The reactants for immunoconglutinins are found as far as is known only in *fixed* complement and the reaction of immunoconglutinins with fixed complement does not require calcium ions. The typical immunoconglutinins which produce powerful aggregation are all directed against determinants in fixed C'_3 —the complement components fixed in large amount. There is considerable heterogeneity in these determinants from species to species and probably even within a species (Lachmann & Coombs 1965). In contrast to conglutinin the reaction of immunoconglutinins with fixed C'_3 does not require the prior reaction of fixed C'_3 with KAF. Using more sensitive methods immunoconglutinins reacting with fixed C'_4 have also been demonstrated (Lachmann 1966b). This is the other complement component firmly bound at the complement fixation site.

The Significance of the Conglutination Phenomenon

Conglutinating systems have been used for a long time to detect complement fixing antibodies *in vitro* (see Chapter 1) but information on their *in vivo* role is less extensive.

Nothing is known of the *in vivo* significance of conglutinin itself. Although it is consumed in complement fixing reactions *in vivo* it is not produced in response to such reactions, and cattle are not known to derive any special susceptibilities or immunities from its presence.

Immunoconglutinins, on the other hand, have been shown in mice to exert a protective effect against infection with small numbers of virulent organisms (Ingram 1959) and they have therefore been described as 'physiogenic' autoantibodies (Coombs, Coombs & Ingram 1961).

Immunoconglutins are consumed in complement fixing reactives *in vivo* and their production is stimulated by such reactions. Immunoconglutinin levels can in fact be regarded as an indicator of *in vivo* complement fixation. In this content it may be mentioned that some anti-C'_4 immunoconglutinins have been found in virtually all human sera tested, whereas the presence of anti-C'_3 immunoconglutinin shows possibly more relationship to disease. Elevated titres of anti-C'_3 immunoconglutinins have been reported in acute and (particularly) chronic, bacterial infections and in viral infections (Marks & Coombs 1957). Patients with trypanosomiasis also show particularly high levels. Among non-infective diseases high levels have been reported in such putative auto-allergic conditions as rheumatoid arthritis, ankylosing spondylitis, systemic LE, Hashimoto's disease, Sjögren's syndrome and myasthenia gravis, as well as in gout and silicosis (Marks & Coombs 1957; Bienenstock & Bloch 1967; Pernis, Gambini & Finalli 1959).

Although the high I-K titres in the two last named diseases seem at first sight

surprising, evidence for *in vivo* complement fixation has in fact been presented for both conditions. Barnett, Bienenstock & Bloch (1966) have reported finding C'_3 with IgM and IgM in macrophages from gouty synovid fluid and Pernis has reported fixed C'_3 in silicotic lungs.

Whether the immunoconglutinin found in human auto-allergic disease augments or diminishes the allergic process or neither, is unclear. In certain circumstances the reaction of immunoconglutinin with fixed C'_3 has been shown to fix further complement but this reaction does not go on 'cycling' till all available complement or immunoconglutinin is fixed. The homeostatic mechanisms involved are unknown.

Immune Adherence

This phenomenon (R.A. Nelson 1953) comprises the adherence of antigen-antibody-complement complexes to primate erythrocytes. Such complexes also adhere in an analogous way to non-primate platelets (Nelson & Nelson 1959). This type of reaction was first observed using trypanosomes as antigens (Leupold 1928; Duke & Wallace 1930) but can be shown with a wide variety of complement fixing antigen-antibody systems whether particulate or soluble (R.A. Nelson 1953; Turk 1958).

The immune adherence reactant in fixed complement appears when C'_3 is bound (Nishioka & Linscott 1963) and is not affected by the presence of other complement components The immune adherence receptor on the erythrocyte membrane appears to be mucopeptide in nature and can be split off the erythrocyte with papain (D.S. Nelson 1965). The formation of the immune adherence bond is temperaturede pendent and it can be inhibited by derivatives of aromatic amino acids (Basch 1965) on which basis it has been suggested that the bond is formed as a result of an enzymatic reaction—the fixed C'_3 being the enzyme and the red cell mucopeptide the substrate.

Immune adherence provides an extremely sensitive way of detecting complement fixation. Its *in vivo* significance is believed to lie in the facilitation of phagocytosis that has been shown to occur when, for example, bacteria are bound to red cells by immune adherence (R.A. Nelson 1956). D.S. Nelson (1965) has also adduced evidence that a process operationally analogous to immune adherence will bind antigen-antibody-complement complexes to guinea-pig polymorphs.

Phagocytosis

Dean in 1907 showed that the opsonins of Wright & Douglas (1903) involved both a heat-stable and a heat-labile factor and that phagocytosis, like lysis, was dependent on an antibody-complement system. A formidable quantity of investigation in the subsequent sixty years has fully confirmed that complement does pay a substantial role in facilitating phagocytosis. However phagocytic processes have proved complex and difficult to analyse and a recent review of

the literature (Boyden, North & Faulkner 1965) came to the conclusion that 'dogmatic statements on the part played by complement in this process are completely unjustified at present'. Nevertheless bearing in mind the reservations that results obtained in any one test system may not be applicable to other phagocytic situations, certain conclusions can now be made.

The phagocytic process can be considered in four stages: chemotaxis, adhesion, engulfment and digestion.

Chemotaxis

The direction of movement of polymorphs is influenced by soluble factors along concentration gradients on which the cells migrate. This is the phenomenon of chemotaxis. This has been investigated by observing migration of polymorphs through millipore filters towards the test substance (Boyden 1962). When antigen-antibody-complement systems were investigated in this way it was found that it was the activated C'_{567} complex that was chemotactically active (Ward, Cochrane & Müller-Eberhard 1965, 1966). Since this complex is formed at the surface of the alexinated complex and then escapes back into the fluid phase the necessary concentration gradient will normally exist in the vicinity of the complement fixation site.

Other chemotactic factors unrelated to complement have also been reported —for example peptone (Keller & Sorkin 1965). It is also possible that other, weaker, chemotactic factors related to complement are found since *in vivo* phagocytosis is normal in C'_5 deficient mice and C'_6 deficient rabbits (see below).*

Attachment and Engulfment

Attachment of erythrocytes treated with antibody and complement to polymorphs has been tested in the presence of inhibitors like sodium azide (which abolish engulfment) (D.S. Nelson 1965). As already quoted these experiments lead to the conclusion that attachment was analogous to immune adherence and a function of fixed C'_3.

Experiments where phagocytosis (including both attachment and engulfment) of erythrocytes treated with antibody and complement was studied (R.A. Nelson 1962) showed that the fixation of C'_3 gave rise to phagocytosis. C'_3, being fixed in substantial amounts, is undoubtedly *a priori* the most suitable candidate among complement components to be the opsonin.

However, Mollison (1965) has shown in man that red cells treated with autologous serum at low ionic strength, although they have C'_4 and C'_3 fixed on them as shown antigenically, survived normally *in vivo* (after an initial disappearance from the serum) whereas red cells treated with antibody and complement are cleared very rapidly. There thus seems to be some quantitative

*The formation of a further chemotactic factor by the action of plasmin on C'_3 has recently been described (Ward, 1967)

or qualitative difference between the presence of C'_3 detectable on the cell by an anti-C'_3 serum and active opsonization.

There are other phagocytic systems that appear to be independent of any serum factors and yet others where antibody alone is a sufficient opsonin (see Boyden *et al* 1965). There is also evidence that attachment of particles to macrophages can be achieved by a cytophilic antibody without the intervention of complement (Howard & Benacerraf 1966). Nevertheless in the *in vivo* situation there seems little doubt that complement plays an important role in phagocytic mechanisms, and this may be one of its most significant biological roles.

Little is known of the effect of complement in intracellular digestion. Fischer (quoted by Boyden 1965) has suggested that lysolecithin may be produced intracellularly and act on lysozomal membranes and facilitate the release of lysozomal enzymes into the phagocytic vacuole.

Formation of Anaphylatoxin

There is now compelling evidence that Type I (anaphylactic) reactions are not complement dependent since they are mediated by non-complement-fixing antibodies. (Ovary, Benacerraf & Bloch 1963; Bloch *et al* 1963). However the formation of anaphylatoxin—a histamine releasing substance produced in serum by antigen–antibody complexes and which may produce symptoms very similar to systemic anaphylaxis—is known to involve complement (Osler *et al* 1959).

It has recently been reported that human anaphylatoxin is a small molecule with a molecular weight certainly less than 60,000 which is produced by the interaction of C'_1-esterase, C'_4, C'_2, C'_3 and possibly C'_5 (Dias da Silva & Lepow 1966). There is evidence that guinea-pig anaphylatoxin is a fragment of C'_5 that is produced either in the course of the complement reactions or by proteolysis with trypsin (Jensen 1966).*

Properdin

Properdin was described by Pillemer and his colleagues in 1954 as a newly discovered serum factor that represented an important mediator of 'non-specific immunity'. It was subsequently shown that some at least of the activities of properdin were antibody and complement dependent (see R.A. Nelson 1961) and doubt was thrown both on the significance of properdin and even on its existence.

The purification and characterization of properdin as a protein (Pensky *et al* 1964; Lepow 1965) put an end to the controversy as to its existence. It is a non-immunoglobulin serum β-globulin with a sedimentation constant of 5.2S and a molecular weight of around 230,000.

*It is now recognised that there are two anaphylatoxius, one a fragment of C'_3 and the other a fragment of C'_5 (Lepow, DaSilva and Eisele, 1968).

In conjunction with various naturally occurring antibodies, components of complement and Mg^{++}, it comprises 'the properdin system' which is believed to play a part in the killing of certain bacteria, the neutralization of certain viruses and the haemolysis of erythrocytes of patients with paroxysmal nocturnal haemoglobinuria (Lepow 1961). The mechanisms of action involved and the significance of properdin as factor in immunity remain to be clarified.

Inflammation in Type III Allergic Reactions

The allergic inflammatory reaction produced by antigen-antibody reactions in fluid phase (Type III reactions) are apparently wholly complement dependent (see Cochrane & Ward 1966). The phenomena of anaphylatoxin formation, immune adherence, chemotaxis for polymorphonuclear leucocytes and opsonization are all probably concerned in this inflammation and it is quite possible that others, not yet well characterized, effector mechanisms contribute as well.

Considerable importance has been shown to attach to increase in vascular permeability—as is produced by anaphylatoxin (and by C'_1 esterase)—in the localization of complexes (Cochrane 1963); and to the presence of polymorphs in producing the damage leading to haemorrhage and necrosis in the Arthus reaction and to the basement membrane destruction in the serum sickness type of nephritis (Cochrane & Aikin 1966).

Type n ('Delayed Hypersensitivity') Reactions

The role of complement in these reactions is not established. Neveu & Biozzi (1965) have shown that decomplementation of rats with antigen–antibody complexes reduces delayed hypersensitivity but themselves point out that there are several possible interpretations of their findings.

Homograft Rejection

It is not certain to what extent complement activity affects graft rejection.

Skin grafts are rejected normally by C'_5-deficient mice (Caren & Rosenberg 1965) and results on C'_6-deficient rabbits are conflicting (*vide infra*). There is some indirect evidence that rejection of kidney grafts is in some circumstances associated with complement activity. Thus C'_2 levels are said to fall after kidney transplantation (Austin & Russell 1966) and fixed complement has sometimes been found on rejected renal grafts in the Cambridge series. Complement levels have also been found to be low in runt disease in rats—a graft v. host phenomenon (Fife, Hook & Muschel, 1962).

Arguments supporting the important role of humoral factors in graft rejection have been put by Stetson (1963) and Gewurz *et al.* (1966a) from studies of complement levels, conclude that complement activity can mediate graft rejection but is not essential for this process to occur.

GENETICALLY DETERMINED COMPLEMENT DEFICIENCIES

Guinea-Pigs

The existence of a strain of guinea-pigs lacking haemolytic complement was reported several decades ago (Moore 1919; Hyde 1923). The deficiency was carried as a single Mendelian recessive character and was shown to be due to lack of 'classical C'_3' (i.e. one of the components C'_3–C'_9). The component concerned did not pass the placenta in either direction. The animals survived well under ideal conditions but were more liable to experimental infections. In time the strain died out and no similar guinea-pigs have been reported since.

Within recent years genetically determined complement deficiencies have been reported in other species.

Mice

Strains of mice have been found that lack complement (Rosenberg & Tachibana 1962; Cinader, Dubiski & Wardlaw 1964). These mice also lack an antigenically identifiable serum protein and the variant mice will make antibody to the missing protein on immunization with normal mouse serum. The complement component involved has been shown to be C'_5 (Nilsson & Müller-Eberhard 1965) both antigenically and by haemolytic activity. Genetically the deficiency behaves as a single Mendelian recessive (Tachibana, Ulrich & Rosenberg 1963).

The C'_5-deficient mice have been found to reject skin grafts normally (Caren & Rosenberg 1965) and to show normal *in vivo* phagocytosis of bacteria (Stiffel *et al* 1964) although *in vitro* chemotactic activity was lacking (Ward, Cochrane & Müller-Eberhard 1965). Glynn and Medhurst (1967) have reported that *in vivo* killing of 'complement sensitive' *E. coli* is greatly impaired in the complement deficient mice. These findings are all compatible with what is known of the role of complement components in these various reactions.

Rabbits

Rabbits deficient in complement have been found independently in Germany (Rother & Rother 1961), in Mexico (Biro & Garcia 1965) and more recently in Cambridge (Lachmann, 1967). In all cases the component affected seems to be C'_6 (Ward *et al* 1965). Besides a deficiency in haemolytic activity the rabbit sera lacked the power to generate chemotactic activity *in vitro*.

Biro (1966) was unable to detect any differences between his normal and complement deficient rabbits in homograft rejection and in Type 4 reactions to human gamma-globulin. However he found that the complement deficient rabbits failed to show haemorrhagic necrosis in Arthus reactions. On the other hand the rabbits of Rother and Rother were unable to produce a passive Arthus reaction at all and in some—but not all—animals there was prolonged survival

of skin grafts and absence of delayed hypersensitivity. (Rother, Rother & Schindera 1964; Volk, Mauersberger, Rother & Rother 1964). The reasons for these discrepancies are unclear. It is puzzling why the equally C'_6 deficient rabbits of the latter investigators should vary in their biological activities so much.

Man

Silverstein (1960) reported a case of essential hypocomplementaemia due to a deficiency of C'_2 and Klemperer *et al* (1965) have found further cases and shown the deficiency to be genetically determined autosomal co-dominant trait. The deficiency is incomplete. The C'_2 level in one subject being in the region of 1% of normal, and the subjects appear quite healthy. Gewurz *et al* 1966b have studied one subject in some detail. They found that whereas haemolytic and *in vitro* chemotactic activities were deficient, bactericidal power was significantly impaired only in the presence of added antibody, i.e. the low complement was not the limiting factor in killing bacteria at the antibody levels found in the subject.

A surprising result was that immune adherence was normal, suggesting that the C'_2 available was able to produce sufficient C'_3 fixation to give this activity.

These findings emphasize the limitations of drawing conclusions from genetic deficiencies unless they are absolutely complete. It is not at all certain that for some complement functions 'enough may not be as good as a feast'; and the finding that for some *in vitro* function a deficient serum has to be used undiluted whereas a normal serum gives a titre of many hundred may be immaterial *in vivo* where the serum is in any case undiluted.

INVESTIGATION OF COMPLEMENT IN RELATION TO DISEASE

Complement Levels

Complement activity in serum is generally measured by its total haemolytic activity on EA under standard conditions (Mayer 1961). Estimations of individual components by haemolytic testing has recently also been quite widely used. In general, complement levels whether of components or of total complement have not proved to be a particularly rewarding estimation in the context of human disease, consistent patterns being usually absent. This is perhaps not surprising since the level of any component reflects the balance between synthesis and sequestration, either or both of which processes may be affected by a disease process and neither of which can be readily measured.

The sequestration of complement may in any case result not only from *in vivo* complement fixation. Besides the normal processes of catabolism, and excretion

of components in the urine of patients with proteinuria (Seifter & Ecker 1946) specific inactivation of components by mechanisms unrelated to complement fixation may occur. Thus in hereditary angioneurotic oedema C'_4 and C'_2 are destroyed in solution by C'_1-esterase (Donaldson & Rosen 1964), and Beeson & Rowley (1959) have provided evidence that ammonia production in the kidney is associated with local destruction of C'_4 and a loss of bacterial activity.

It may be that turnover studies of individual components will prove more informative. With the advent of highly purified complement components that can be radioactively labelled such investigations have become feasible and one such study has been published (Alper, Levin & Rosen 1966).

Notwithstanding these limitations, there have been some general findings. Thus, significant falls in complement level have been found consistently in acute systemic LE, glomerulonephritis and serum sickness. In these diseases complement fixation *in vivo* is believed to account for the depressed complement levels. Although serum complement levels in rheumatoid arthritis are variable and often raised, the complement levels in the synovial fluid are generally lower than in synovial fluids from other joint diseases (Pekin & Zvaifler 1964).

Rises in complement titre appear to occur fairly generally in the 'acute phase' following trauma or tissue damage (Boltax & Fischel 1956).

In mice, but not in men, there appears to be a well-marked effect of sex hormones on complement levels (Weintraub *et al* 1966). Males have a substantially higher level of 'classical C'_3' (i.e. activity of $C'_{3,5,6,7,8 \text{ and } 9}$) than females and this difference can be abolished by castration or oestrogen treatment.

Immuno-Conglutinin Levels

The use of I-K. titre as an index of *in vivo* complement fixation has been discussed in an earlier section.

The Appearance of Inactivated Complement Components in the Circulation

Inactive forms of C'_4, C'_2 and C'_3 appear in the fluid phase during complement fixation and the appearance of these factors in fresh plasma could be taken as good evidence of *in vivo* complement fixation. The inactive form of C'_3—whether as C'_{3i} or β_{1A} or α_{2D} which are formed from C'_{3i} in the circulation—is readily detected by immunoelectrophoresis and this is the only inactivated complement component as yet much studied from this point of view.

Inactive C'_3 products in fresh serum have occasionally been found in active systemic lupus erythematosus (Morse, Müller-Eberhard & Kunkel 1962; Lachmann 1963), but even in the active stage this finding is quite unusual. However in active glomerulonephritis their presence in fresh serum is very common (Soothill 1967; West *et al* 1967). It would seem likely that the altered complement components are rapidly cleared from the circulation, and it is possible that to find

these components it is necessary not only to have substantial *in vivo* complement fixation but also interference with the clearing mechanism.

The Detection of *in-vivo* Bound Complement Components in Tissues

Once complement components were sufficiently purified that mono-specific antibodies could be prepared against them it became possible to trace the presence of complement components by the fluorescent antibody technique.

Using antiserum to human C_3', this component has been found in a variety of human lesions, principally the glomerular and vascular lesions of systemic LE, the lesions of glomerulo-nephritis and amyloid deposits (Lachmann *et al* 1962). Amyloid deposits have subsequently been shown also to stain for C'_4 more strongly than for C'_3 (Hatfield & Müller-Eberhard 1965). This is a curious finding since at complement fixation sites in general there is much more fixed C'_3 than C'_4.

Other human lesions shown to stain for complement components include the synovial membranes in rheumatoid arthritis (Rodman *et al* 1966) and the skin lesions of systemic LE where the complement fixation appears to be on complexes formed between nuclear material, which occurs extra-cellularly at the site of the rash, and antinuclear antibodies (Tan & Kunkel 1966).

Model systems have been used to show that the fixation of C'_3 and C'_4 on sections takes place by the usual reactions of complement fixation (Lachmann *et al* 1962; Hatfield and Müller-Eberhard 1965). It is, however, not impossible that there are other mechanisms that are also capable of causing complement binding in tissues. The case of amyloid where the ratio of C'_4 to C'_3 found is anomalous, might suggest such a possibility.

Even where it is accepted that complement is bound in a lesion by the normal processes of complement fixation this does not necessarily imply either that this fixation was initiated by an antigen-antibody reaction rather than a gamma-globulin aggregate or possibly a 'non-specific sensitiser'; nor that complement was a pathogenetic factor for that lesion. On the other hand it does imply that complement dependent phenomena are likely to have been active at that site, and that the wherewithal of Type II or Type III allergic reactions has been present.

More direct evidence for the pathogenetic role of complement has been obtained from studies on experimental allergic disease. There is substantial evidence that complement plays a pathogentic role in the renal lesions of experimental serum sickness (Dixon 1963), nephrotoxic nephritis (Unanue & Dixon 1964) and the vasculitis of the Arthus reaction (Cochrane & Ward 1966) and these lesions show analogous patterns of complement staining to those found in human glomerulo-nephritis and systemic LE. It is, therefore, plausible to consider complement a pathogenetic factor in these analogous human con-

ditions; and in systemic LE and glomerulo-nephritis there is circumstantial evidence to the same end.

These diseases are dealt with elsewhere in this book and will not be considered further here.

Paroxysmal Nocturnal Haemoglobinuria

In this rare disease a proportion of the patient's red cells undergo spontaneous lysis in fresh human serum particularly at slightly acid pH. The abnormality is acquired and lies in the red cells rather than the serum. The haemolytic mechanism concerned has been known for many years to involve complement (Ham & Dingle 1939).

Recently investigations have shown that the lytic efficiency of complement for the PNH cell is unusually high (Rosse & Dacie 1965), rather than that an unusually large amount of complement was fixed on them. It was also found, surprisingly, that these cells if brought to the EAC'_{142a} stage underwent particularly rapid decay to EAC'_{14}. When examined for the electron-microscopically detectable lesions of haemolysis (Rosse *et al* 1966) lysed PNH cells were found to have no more lesions per membrane than did normal human cells in the presence of the same amount of complement although the percentage of PNH cells lysed by a low dose of complement was much higher.

These authors concluded that while complement may be more efficient at producing the first lesion in the membrane of PNH cells, the subsequent proliferation of lesions in both cell types is the same. It has also been suggested (Yachnin & Ruthenberg 1965; Yachnin 1965) that fluid phase complement activation, may be important in PNH cell lysis.

It is not easy to give any explanation in terms of a membrane abnormality to these very diverse findings. It would be interesting to know if the lack of acetyl cholinesterase in PNH cells (Auditore & Hartman 1959) is in any way related.

Hereditary Angioneurotic Oedema

This disease is known to be associated with an absence of the normal serum inhibitor of C'_1-esterase (Donaldson & Evans 1963). During attacks of oedema there is C'_1-esterase free in the serum and low levels of C'_2 and C'_4; but in remission (in spite of the absence of inhibitor) there is little free esterase and normal complement levels (Donaldson & Rosen 1964). Since C'_1-esterase is known to increase vascular permeability the presence of this substance would explain the oedema. However, Kagen & Becker (1963) have shown that C'_1-esterase inhibitor also inhibits the further permeability factors Kallikrein and PF/Dil. Hence it is possible that kinins may also be pathogenic agents in this disease.

CONCLUSIONS

The progress in complement studies in the last few years has shown the system to be even more complex than had been previously suspected. Nevertheless chemical characterization of complement components and of their interactions has been achieved to a significant if as yet limited extent. It has also become possible to ascribe particular biological functions to particular molecules.

Study of the role that complement plays in immunity and in allergic disease has strengthened the earlier evidence that by its functions as opsonin and bactericidal factor complement plays an important role in immunity and that as a reactant in Type II and Type III allergic reactions it may be an important mediator in allergic disease.

The purified cobra venom factor of Müller-Eberhard *et al* (1966) allows, more or less for the first time, a complement component (C'_3) to be inactivated *in vivo* by a mechanism unrelated to complement fixation. With this method—and it is to be hoped equivalent methods for other components—it may become possible to influence complement mediated allergic reactions experimentally and perhaps eventually even therapeutically.

REFERENCES

ADINOLFI M., GLYNN A.A., LINDSAY M. & MILN C.M. (1966) Serological properties of γA antibodies to *E. coli* present in human colostrum. *Immunology* **10,** 517.

ALPER C.A., LEVIN A.S. & ROSEN F.S. (1966) β_{1C}-globulin metabolism in glomerulonephritis. *Science* **153,** 180.

AUDITORE J.V. & HARTMANN R.C. (1959) Paroxysmal nocturnal haemoglobinuria. II. Erythrocyte acetylcholinesterase defect. *Amer. J. Med.* **27,** 401.

AUSTIN K.F. & BLOCH K.J. (1965) In *Complement*, ed. WOLSTENHOLME G.E.W. & KNIGHT J. CIBA Symposium 1964. London: J. & A. Churchill Ltd.

AUSTIN K.F. & RUSSELL P.S. (1966) Detection of renal allograft rejection in man by demonstration of a reduction in the serum concentration of the second component of complement. *Ann. N.Y. Acad. Sci.* **129,** 657.

BARNETT E.V., BIENENSTOCK J. & BLOCH K.J. (1966) Antinuclear factors in synovia. Possible participants in the rheumatoid inclusion body. J.A.M.A. **198,** 143.

BASCH R.S. (1965) Inhibition of the third component of the complement system by deviations of aromatic amino acids. *J. Immunol.* **94,** 629.

BECKER E.L. (1956) Concerning the mechanism of complement action. Inhibition of complement activity by DFP. II. The nature of the first component of guinea pig complement. *J. Immunol.* **77,** 462 and 469.

BEESON P.B. & ROWLEY D. (1959) The anti-complementary effect of kidney tissue. Its association with ammonia production. *J. exp. Med.* **110,** 685.

BENACERRAF B. (1964) In *Molecular and Cellular Basis of Antibody Formation*, p. 223, ed. STETZL J. Publishing House of the Czech Academy of Science, Prague.

BIENENSTOCK J. & BLOCH K.J. (1966) Some characteristics of human immunoconglutinin. *J. Immunol.* **96,** 637.

BIENENSTOCK J. & BLOCH K.J. (1967) Immunoglutinin in various rheumatic diseases and certain diseases suspected of an autoimmune pathogenesis. *Arthritis Rheum.* **10,** 187.

BIRO C.E. (1966) The role of the sixth component of complement in some types of hypersensitivity. *Immunology* **10,** 563.

BIRO C.E. & GARCIA G. (1965) The antigenicity of aggregated and aggregate-free gamma globulin for rabbits. *Immunology* **8,** 411.

BLOCH K.J., KOURILSKY F.M., OVARY Z. & BENACERRAF B. (1963) Properties of guinea pig 7S antibodies. III. Identification of antibodies involved in complement fixation and haemolysis. *J. Exp. Med.* **117,** 965.

BLOMFIELD A.M., COOMBS R.R.A. & HOLE N.H. (1949) The conglutination phenomenon. V. Further experiments on the importance of the choice of complement when examining antisera for the presence of complement fixing and complement absorbing antibodies. *J. Hyg. Camb.* **47,** 132.

BOLTAX A.J. & FISCHEL E.E. (1956) Serological tests for inflammation: serum complement C-reaction protein and erythrocyte sedimentation rate in myocardial infarction *Amer. J. Med.* **20,** 418.

BORDET J. & GENGOU O. (1901) Sut l'existence de substances sensilibisatrices dans la plupart des serumes antimicrobiens. *Ann. Inst. Pasteur* **15,** 289.

BORDET J. & STRENG O. (1909) Les phenomenes d'absorption de la conglutinine du serum de boeuf. *Zbl. Bakt.* (orig.) **49,** 260.

BORSOS T., RAPP H.J. & COOK C.T. (1961) Studies on the second component of complement. III. Separation of the second component from guinea pig serum by chromatography on cellulose derivatives. *J. Immunol.* **87,** 330.

BORSOS T., DOURMASHKIN R.R. & HUMPHREY J.H. (1964) Lesions in erythrocyte membranes caused by immune haemolysis. *Nature (Lond.)* **202,** 251.

BORSOS T. & RAPP H.J. (1965) Complement fixation on cell surfaces by 19S and 7S antibodies. *Science* **150,** 505.

BOYDEN S.V. (1962) The chemototis effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. exp. Med.* **115,** 453.

BOYDEN S.V., NORTH R.J. & FAULKNER S.M. (1965) Complement and the foundation phagocytes. In *Complement;* ed. WOLSTENHOLME G.E.W. & KNIGHT J. CIBA Foundation Symposium. London: J. & A. Churchill Ltd.

BURKHOLDER P.M. (1961) Complement fixation in diseased tissues. I. Fixation of guinea-pig complement in sections of kidney from humans with membranous glomerulonephritis and rats injected with anti-rat kidney serum. *J. exp. Med.* **114,** 605.

CAREN L.D. & ROSENBERG L.T. (1965) Complement in skin grafting in mice. *Immunology* **9,** 359.

CHAMBERS R. & FELL H.B. (1931) Micro-operations on cells in tissue cultures. *Proc. Roy. Soc. B.* **109,** 380.

CIBA Symposium (1965) Definition of complement. Discussion in *Complement,* p. 54, ed. WOLSTENHOLME G.W. & KNIGHT J. London: J. & A. Churchill.

CINADER B., DUBISKI, S. & WARDLAW A.C. (1964) Distribution, inheritance and properties of an antigen, MuBI and its relation to haemolytic complement. *J. Exp. Med.* **120,** 897.

COCHRANE C.G.(1963) Studies on the localization of circulating antigen-antibody complexes and other macromolecules in vessels. II. Pathogenetic and pharmadynamic studies. *J. exp. Med.* **118,** 503.

COCHRANE C.G. & AIKIN B.S. (1966) Polymorphonuclear leucocytes in immunologic reactions. The destruction of vascular basement membrane. *in vivo* and *in vitro. J. exp. Med.* **124,** 733.

COCHRANE C.G. & WARD P.A. (1966) The role of complement in lesions induced by immunologic reactions. In *Fourth International Symposium on Immunopathology,* p. 433, ed. GRABAR P. & MIESCHER P. Basel: Schwabe & Co.

Complement Workshop (1966) Nomenclature of complement. *Immunochemistry* **3,** 495.

COOMBS R.R.A., COOMBS A.M. & INGRAM D.G. (1961) *The Serology of Conglutination and its Relation to Disease.* Oxford: Blackwell Scientific Publications.

COOPER N.R. & BECKER E.L. (1966) Complement association peptidase activity. Complement workshop abstracts. *Immunochemistry* **3,** 496.

DALMASSO A.P. & MÜLLER-EBERHARD H.J. (1964) Interaction of autologous complement with red cells in the absence of antibody. *Proc. Soc. Exp. Biol. & Med.* **117,** 643.

DALMASSO A.P. & MÜLLER-EBERHARD H.J. (1965) Properties of β1C and β1e-globulin dissociated from complement treated erythrocyte membranes. *Fed. Proc.* **24,** 466.

DALMASSO A.P. & MÜLLER-EBERHARD H.J. (1966) Haemolytic activity of lipoprotein-depleted serum and the effect of certain anions on complement. *J. Immunol.* **97,** 680.

DEAN G. (1907) Phagocytosis and bactericidal action. *Brit. med. J.* **ii,** 1409.

DELOOZE L.L. & LEON M.A. (1963) Reversibility of reaction between human C'_1 and sensitized sheep erythrocytes. *Proc. Soc. Exp. Biol. Med.* **112,** 817.

DIAS DA SILVA W. & LEPOW I.H. (1966) Properties of anaphylatoxin prepared from purified components of human complement. Complement Workshop Abstract. *Immunochemistry* **3,** 497.

DINGLE J.T., FELL H.S. & COOMBS R.R.A. (1967) The breakdown of embryonic cartilage and bone cultivated in the presence of complement-sufficient antiserum. II. Biochemical changes and the role of the lysosomal system. *Int. Arch. Allergy.* In press.

DIXON F.J. (Jr.) (1963) The role of antigen-antibody complexes in disease, p. 21. Harvey Lectures, 1963. New York and London: Academic Press.

DONALDSON V.H. & EVANS R.R. (1963) A biochemical abnormality in hereditary angioneurotic oedema. Absence of serum inhibitor of C'_1-esterase. *Amer. J. Med.* **35,** 37.

DONALDSON V.H. & ROSEN F.S. (1964) Action of complement in hereditory angioneurotic oedema. The role of C'_1-esterase. *J. Clin. Invest.* **43,** 2204.

DUKE H.L. & WALLACE J.M. (1930) Red cell adhesion in trypanosomiasis of man and animals. *Parasitology* **22,** 414.

FELL H.B., COOMBS R.R.A. & DINGLE J.T. (1966) The breakdown of embryonic (chick) cartilage and bone cultivated in the presence of complement sufficient antiserum. I. Morphological Changes, their reversibility and inhibition. *Int. Arch. Allergy* **30,** 146.

FIFE E.H., HOOK W.A. & MUSCHEL L.H. (1962) Tissue antibody and complement in runt disease. *Proc. Soc. Exp. Biol. Med.* **110,** 524.

FISCHER H. (1964) Lysolecithin and the action of complement. *Ann. N.Y. Acad. Sci.* **116,** 1063.

FISCHER H. & HAUPT I. (1961) Des cytolysierende prinzip um serum komplement. *Naturfschg.* **116,** 321.

FRANK M.M., RAPP H.J. & BORSOS T. (1965) Studies on the terminal steps of immune haemolysis. II. Resolution of the E* transformation reaction into multiple steps. *J. Immunol.* **94,** 295.

GEWURZ H., SCOTT CLARK D., FINSTAD J., KELLY W.D., VARCO R.L., GOOD R.A. & GABRIELSON A.E. (1966a) Role of the complement system in graft rejections in experimental animals and man. *Ann. N.Y. Acad. Sci.* **129,** 673.

GEWURZ H., PICKERING R.G.J., MUSCHEL L.H., MERGENHAGEN S.E. & GOOD R.A. (1966) Complement dependent biological functions in complement deficiency in man. *Lancet* **ii,** 356.

GLYNN A.A. & MEDHURST F.A. (1967) Possible extracellular and intracellular bactericidal actions of mouse complement. *Nature (Lond.)* In press.

GREEN H., BARROW P. & GOLDBERG B. (1959) Effect of antibody and complement on permeability control in ascites tumour cells and erythrocytes. *J. exp. Med.* **110,** 699.

GOLDSWORTHY N.E. (1928) Experiments upon the relationship of complement fixation to precipitation. *J. Path. Bact.* **31,** 220.

GOLDWASSER R.A. & SHEPARD C.C. (1958) Staining of complement and modification of fluorescent antibody procedure. *J. Immunol.* **80,** 122.

HADDING U. and MÜLLER-EBERHARD H.J. (1967) Complement substitution of the terminal component in immune haemolysis by 1, 10—Phenanthroline. *Science* **157,** 442.

HADDING U., MÜLLER-EBERHARD H.J. & DALMASSO A.P. (1966) Isolation of the terminal component of human complement. *Fed. Proc.* **25,** 485.

HAM T.H. & DINGLE J.H. (1939) Studies on destruction of red blood cells. II. Chronic haemolytic anaemia with paroxysmal nocturnal haemoglobinuria: certain immunological aspects of the haemolytic mechanism with special reference to serum complement. *J. Clin. Invest.* **18,** 657.

HARBOE M., MÜLLER-EBERHARD H.J., FUDENBERG H., POLLEY M.J. & MOLLISON P.L. (1963) Identification of components of complement participating in the antiglobulin reaction. *Immunology* **6,** 412.

HATFIELD P.M. & MÜLLER-EBERHARD H.J. (1965) Quoted by MÜLLER-EBERHARD H.J. & DALMASSO A.P. In *Complement,* p. 313, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill.

HEIDELBERGER M. (1941) Quantitative chemical studies on complement or alexin Part I. *J. exp. Med.* **73,** 681.

HINZ C.F. (Jr.), PICKEN M.E. & LEPOW I.H. (1960) Studies on immune human haemolysis. II. The Donath-Landsteiner reaction as a model system for studying the mechanism of action of complement and the role of C'_1 and C'_1-esterase. *J. exp. Med.* **113,** 193.

HOET J.J., BLOMFIELD A.M. & COOMBS R.R.A. (1954) The selective absorption of haemolytic complements by antibodies from different species of animals. *Brit. J. Exp. Path.* **35,** 32.

HOLE N.H. & COOMBS R.R.A. (1947) The conglutination phenomenon. II. The technique of the conglutinating complement absorption test compared with the haemolytic complement fixation test. *J. Hyg.* **45,** 490.

HOWARD J.G. & BENACERRAF B. (1966) Properties of macrophage receptors for cytophilic antibodies. *Brit. J. exp. Path.* **47,** 193.

HUMPHREY J.H. & DOURMASHKIN R.R. (1965) *Electron Microscope Studies of Immune Cell Lysis.* In *Complement,* CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill Ltd.

HUMPHREY J.H. (1967) Personal communication.

HYDE R. (1923) Complement deficient guinea-pig serum. *J. Immun.* **8,** 267.

INGRAM D.G. (1959) The conglutination phenomenon XIV. The resistance enhancing effect of conglutinin and immuno-conglutinin in experimental bacteriol infection. *Immunology* **2,** 334.

INOUE K., TANIGAWA Y., TAKUBO M., SATANI M. & AMANO T. (1959) Quantitative studies on immune bacteriolysis. II. The role of lysozyme in immune bacteriolysis. *Biken's J.* **2,** 1.

IRVINE W.J. (1960) An investigation of the pathogenesis of Hashimoto's disease by thyroid tissue culture. *J. Endocrin.* **20,** 83.

JENSEN J. (1966) Formation of anaplylatoxin. Complement Workshop Abstract. *Immunochemistry* **3,** 498.

KAGEN L.J. & BECKER E.L. (1963) Inhibition of permeability globulins by C'_1-esterase inhibitor. *Fed. Proc.* **22,** 613.

KELLER H.U. & SORKIN E. (1965) Studies on chemotaxis. II. The significance of normal sera for chemataxis induced by various agents. *Immunology* **9,** 441.

KLEIN P.G. & WELLENSIEK H.J. (1965) Multiple Nature of the third component of guinea-

pig complement. I. Separation and characterization of three factors a, b and c essential for haemolysis. *Immunology* **8**, 590.

KLEMPERER M.R., WOODWORTH A.C., ROSEN F.S. & AUSTEN K.F. (1965) Hereditary deficiency of the second component of human complement—Transmission as autosomal co-dominant trait. *Proc. Central. Soc. Clin. Research* **38**, 43.

LACHMANN P.J. (1963) The formation of β1a globulin *in vivo*. In *Clinical Aspects of Immunology*, p. 260, ed. GELL P.G.H. & COOMBS R.R.A. Oxford: Blackwell Scientific Publications.

LACHMANN P.J. (1966a) A possible homeostatic mechanism in complement fixation: inhibition of activity of fixed C'_1 by the fixation of C'_{3a}. *Nature (Lond.)* **210**, 140.

LACHMANN P.J. (1966b) A sedimentation pattern technique for measuring conglutination. Its application to demonstrating immuno-conglutinins to C'_4. *Immunology* **11**, 263.

LACHMAN P.J. (1967) Unpublished observations.

LACHMANN P.J., MÜLLER-EBERHARD H.J., KUNKEL H.G. & PARONETTO F. (1962) The localization of *in vivo* bound complement in tissue sections. *J. exp. Med.* **115**, 63.

LACHMANN P.J. & COOMBS R.R.A. (1965) *Complement, Conglutinin and Immuno-conglutinins.* CIBA Symposium on Complement, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill Ltd.

LACHMANN P.J. & LISKE R. (1966) The preparation and properties of alexinated intermediates that react with conglutinin. I. Guinea pig complement. *Immunology* **11**, 243.

LACHMANN P.J. & MÜLLER-EBERHARD H.J. (1967) The description in human serum of conglutinogen-activating factor and its effect on the third component of complement. In press.

LEON M.A. (1957) Rolè of cations in conglutination and formation of properdin-zymosan complex from bovine serum. *Proc. Soc. exp. Biol. (N.Y.)* **96**, 202.

LEON M.A. (1965) Complement: inactivation of second component by p-hydroxymercuribenzoate. *Science* **147**, 1034.

LEON M.A., YOKOHARI R. & ITOH C. (1966) Chemical specificity in the conglutinin system and its relation to complement structure. Complement Workshop Abstract. *Immunochemistry* **3**, 499.

LEPOW I.H. (1959) In *Mechanisms of Hypersensitivity*, p. 267, ed. SHAPFER J.H., LOGRIPPO G.A. & CHASE M.W. Boston: Little, Brown & Co.

LEPOW I.H. (1961) The properdin system: A review of current concepts. In *Immunochemical Approaches to Problems in Microbiology*, p. 280, ed. HEIDELBERGER M. & PLESCIA O.J. Rutgers, New Jersey: The University Press.

LEPOW I.H. (1965) In *Complement*, p. 278, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill.

LEPOW I.H., DA SILV AW.D. and EISELE J.W. (1968) Nature and biological properties of human anaphylatoxin IV. International Symposium of Immunopathology, Basle: Schwabe and Co.

LEPOW I.H., NAFF G.B. & PENSKY J. (1965) Mechanisms of activation of C'_1 and inhibition of C'_1-esterase. In *Complement*, p. 74, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill.

LEUPOLD F. (1928) Unter suchungen über Rezidivstamme bei Trypanosomen mit Hilfe des Riechenberg—Phanomens. *Z. Hyg. Infekt—Kr.* **109**, 144.

LEVINE L. (1955) Inhibition of immune haemolysis by di-isopropyl fluorophosphate. *Biochim. Biophys. Acta.* **18**, 283.

MARKS J. & COOMBS R.R.A. (1957) The conglutination phenomenon XI. Immunoconglutinin in human sera. *J. Hyg. Camb.* **51**, 81.

MAYER M.M. (1961) Complement and complement fixation. In *Experimental Immuno-Chemistry*, p. 133, 2nd ed. by KABAT E.A. & MAYER M.M. Springfield, Illinois: Charles C. Thomas.

MAYER M.M. (1965) Mechanism of haemolysis by complement. In *Complement*, p. 4, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. &. A. Churchill.

MAYER M.M. & LEVINE L. (1954) Kinetic studies of immune haemolysis. III. Description of a terminal process which follows the Ca^{++} & Mg^{++} reaction steps in the action of complement on sensitized erythrocytes. *J. Immunol.* **72,** 511.

MOLLISON P.L. (1965) The role of complement in haemolytic processes *in vivo*. In *Complement*, p. 323, CIBA Symposium, London: ed. WOLSTENHOLME G.E.W. & KNIGHT J. J. & A. Churchill.

MOLLISON P.L. & POLLEY M.J. (1964) Uptake of gamma globulin and complement by red cells exposed to serum at low ionic strength. *Nature* **203,** 535.

MOORE H.D. (1919) Complementary and opsonic functions in their relation to immunity. A study of the serum of guinea-pigs naturally deficient in complement. *J. Immunol.* **4,** 425.

MORSE, J.H., MÜLLER-EBERHARD H.J. & KUNKEL H.G. (1962) Antinuclear factors and serum complement in systemic Lupus erythematosus. *Bull. N.Y. Acad. Med.* **38,** 641.

MÜLLER-EBERHARD H.J. (1961) Isolation and description of proteins related to the human complement system. *Acta Soc. Med. upsalien.* **66,** 152.

MÜLLER-EBERHARD H.J. (1967) Personal communication.

MÜLLER-EBERHARD H.J. & NILSSON U. (1960) Relation of a β_1-glycoprotein of human serum to the complement system. *J. Exp. Med.* **111,** 217.

MÜLLER-EBERHARD H.J. & BIRO C.E. (1963) Isolation and description of the fourth component of human complement. *J. Exp. Med.* **118,** 447.

MÜLLER-EBERHARD H.J. & LEPOW I.H. (1965) C'_1-esterase effect of activity and physicochemical properties of the fourth component of complement. *J. exp. Med.* **121,** 819.

MÜLLER-EBERHARD H.J. & CALCOTT M.A. (1966) Interaction between C'_{1q} and γ_G-globulin. Complement Workshop Abstract. *Immunochemistry* **3,** 500.

MÜLLER-EBERHARD H.J., DALMASSO A.P. & CALCOTT M.A. (1966) The reaction mechanism of β_{1c}-globulin (C'_3) in immune haemolysis. *J. exp. Med.* **123,** 33.

MÜLLER-EBERHARD H.J., NILSSON U.R., DALMASSO A.P., POLLEY M.J. & CALCOTT M.A. (1966) A molecular concept of immune cytolysis. *Arch. Path.* **82,** 205.

MÜLLER-EBERHARD H.J., POLLEY M.J. & CALCOTT M.A. (1966) Formation of the $C'_{4,2_a}$ complex in cell free solution and its effect of C'_3. Complement Workshop Abstract. *Immunochemistry* **3,** 500.

MUSCHEL L.H. (1965) Immune bactericidal and bacteriolytic reactions. In *Complement*, p. 155, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill Ltd.

MUSCHEL L.H. & TREFFERS H.P. (1956) Quantitative studies on the bactericidal actions of serum and complement, I, II and III. *J. Immunol.* **76,** 1, 11 and 20.

NAFF G.B., PENSKY J. & LEPOW I.H. (1964) The macromolecular nature of the first component of human complement. *J. exp. Med.* **119,** 593.

NELSON D.S. (1965) Immune adherence. In *Complement*, p. 222, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill Ltd.

NELSON R.A. (Jr) (1953) The immune adherence phenomenon. An immunologically specific reaction between micro-organisms and erythrocytes leading to enhanced phagocytosis. *Science* **118,** 733.

NELSON R.A. (Jr) (1956) The immune adherence phenomenon. A hypothetical role of erythrocytes in defence against bacteria and viruses. *Proc. Roy. Soc. Med.* **49,** 55.

NELSON R.A. (Jr) (1961) The properdin system: methods of measurement and inactivation of human complement components. In *Immunochemical Approaches to Problems in Microbiology*, p. 295, ed. HEIDELBERGER M. & PLESCIA O.J. Rutgers, N.J.: The University Press.

NELSON R.J. (Jr) (1962) Immune-adherence. In *Mechanism of Cell and Tissue Damage produced by Immune Reactions*, ed. GRABAR P. & MIESCHER P. Basel: Schwabe & Co.

NELSON R.A. (Jr) & MAYER M.M. (1949) Immobilization of treponema pallidum *in vitro* by antibody produced in syphilitic injection. *J. exp. Med.* **89,** 369.

NELSON R.A. (Jr) & NELSON D.S. (1959) On the mechanism of immune adherence. II. Analogy to mixed aggregation of sensitized antigens in the presence of complement; immune adherence with animal platelets. *Yale J. Biol.* **31,** 201.

NELSON R.A. (Jr), JENSEN J., GIGLI I. & TAMURA N. (1966) Methods for the separation, purification and measurement of nine components of haemolytic complement in guinea-pig serum. *Immunochemistry* **3,** 111.

NEVEU T. & BIOZZI G. (1965) The effect of decomplementation on delayed type hypersensitivity reactions to a conjugated antigen in rats. *Immunology* **9,** 303.

NILSSON U. & MÜLLER-EBERHARD H.J. (1965a) Immunologic relation between human β_{1F}-globulin and mouse MuB1 (HC). *Fed. Proc.* **24,** 620.

NILSSON U. & MÜLLER-EBERHARD H.J. (1965b) Isolation of β_{1F}-globulin from human serum and its characterization as the fifth component of complement. *J. exp. Med.* **122,** 277.

NILSSON U. & MÜLLER-EBERHARD H.J. (1967) Studies on the mode of action of the fifth, sixth and seventh components of human complement in immune haemolysis. *Immunology*, **13,** 101.

NISHIOKA K. & LINSCOTT W.D. (1963) Components of guinea-pig complement. I. Separation of a serum fraction essential for immune haemolysis and immune adherence. *J. exp. Med.* **118,** 767.

OSLER A.G., RANDALL H.G., HILL B.M. & OVARY Z. (1959) Some relationships between complement, passive cutaneous anaphylaxis and anaphylatoxin. In *Mechanisms of Hypersensitivity*, p. 281, ed. SHAFFER J.H., LOGRIPPO G.A. & CHASE M.W. Boston: Little, Brown & Co.

OVARY Z., BENACERRAF B. & BLOCH K.J. (1963) Properties of guinea pig 7S Antibodies. II. Identification of antibodies involved in passive cutaneous and systemic anaphylaxis. *J. exp. Med.* **117,** 951.

PEKIN T.J. & ZVAIFLER N.J. (1964) Haemolytic complement in synovial fluid. *J. Clin. Invest.* **43,** 1372.

PENSKY J., HINZ C.F.J., TODD E.W., WEDGEWOOD R.J. & LEPOW I.H. (1964) Properties of highly purified human properdin. *Fed. Proc.* **23,** 505.

PEPYS M., LACHMANN P.J. & COOMBS R.R.A. (1965) Unpublished observations.

PERNIS B., GAMBINI G. & FINALLI M. (1959) Immunoconglutinins in the blood of silicotics. *Med. Lavaro* **50,** 250.

PILLEMER L. ECKER E.E., ONCLEY J.L. & COHN E.J. (1941) The preparation and physicochemical characterization of the serum protein components of complement. *J. exp. Med.* **74,** 297.

PILLEMER L., BLUM L., LEPOW I.H., ROSS O.A., TODD E.W. & WARDLAE A.C. (1954) The properdin system and immunity. I. Demonstration and isolation of a new serum protein, Properdin, and its role in immune phenomena. *Science* **120,** 279.

POLLEY M.J.& MÜLLER-EBERHARD H.J. (1966) The effect of iodine on the haemolytic activity

of the second components of human complement. Complement Workshop Abstracts. *Immunochemistry* **3,** 501.

POLLEY M.J. & MÜLLER-EBERHARD H.J. (1967) Isolation of the second component of human complement and enhancement of its activity by oxidation. *J. exp. Med.* In press.

PONDMAN K.W., ROSENFIELD R.E., TALLAL L. & WASSERMAN L.R. (1960) The specificity of the complement antiglobulin test. *Vox Sang.* **5,** 297.

RATNOFF O.D. & LEPOW I.H. (1963) Complement as a mediator in the flammation. Enhancement of vascular permeability by purified C'_1-esterase. *J. exp. Med.* **118,** 681.

RODMAN W.S. *et al* (1966) Quoted by Müller-Eberhard *et al.* (1966).

ROSENBERG L.T. & TACHIBANA D.F. (1962) Activity of mouse complement. *J. Immunol.* **89,** 861.

ROSENFIELD R.E., HABER G.V. & GILBERT H.S. (1960) Complement and the antiglobulin test. *Vox Sang.* **5,** 182.

ROSS A. & LEPOW I.H. (1960) Studies on immune cellular injury. I. Cytotoxic effects of antibody and complement. *J. exp. Med.* **112,** 1085.

ROSSE W.F. & DACIE J.V. (1965) Complement and the P.N.H. red cell. In *Complement,* CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill Ltd.

ROSSE W.F., DOURMASHKIN R.R. & HUMPHREY J.H. (1966) Immune lysis of normal human and paroxysmal nocturnal haemoglobinuria (PNH) red blood cells. III. The membrane defects caused by complement lysis. *J. exp. Med.* **123,** 969.

ROTHER V. & ROTHER K. (1961) Über eine angeboren Komplementdefekt bei Kaninchen. *J. Immun. Forch.* **121,** 224.

ROTHER K., ROTHER V. & SCHINDERA F. (1964) Passive Arthus-Reaktion bei Komplementdefekten Kaninchen. *Z. Immun. Allergieforsch.* **126,** 473.

SEIFTER S. & ECKER E.E. (1946) Complement and isohaemagglutinins in urinary proteins. *J. clin. Invest.* **25,** 809.

SELL K.W. & SPOONER R.L. (1966) Differential effectiveness of complement fixed by IgG and IgM antibodies in the cytolysis of dog kidney tissue culture cells. *Immunology* **11.** 533.

SILVERSTEIN A.M. (1960) Essential hypocomplementemia: report of a case. *Blood* **16,** 1338.

SOOTHILL J.F. (1967) Altered complement component C'_3 (β_{1C}-β_{1A}) in patients with glomerulonephritis. *Clin. & Exp. Immunol.* **2,** 83.

SPOONER R.L. (1964) Cytolytic activity of the serum of normal male guinea-pigs against their own testicular cells. *Nature (Lond.)* **202,** 915.

STETSON C.A. (1963) The role of humoral antibody in the homograft reaction. *Adv. Immunology* **3,** 92.

STIFFEL C., BIOZZI G., MOUTON D., BOUTHILLI E.R. & DECREUSEFOND C. (1964) Studies on phagocytosis of bacteria by the reticulo-endothelial system in a strain of mice lacking hemolytic complement. *J. Immunol.* **93,** 246.

STRENG (1930) ImmunoKonglutinin-antiKomplement. *Acta Path. Microbiol. Scand. Supp. III.* **20,** 411.

STROUD R.M., MAYER M.M., MILLER J.A. & MCKENZIE A.T. (1966) C'_{2ad}, an inactive derivative of C'_2 released during decay of EAC'_{42a}. *Immunochemistry* **3,** 163.

TACHIBANA D.K., ULRICH M. & ROSENBERG L.T. (1963) The inheritance of haemolytic complement activity in CF_1 mice. *J. Immunol.* **91,** 230.

TAMURA N. & JENSEN J. (1966) Purified components of guinea-pig complement; reaction of erythrocytes with antibody and the first four components. Complement Workshop Abstracts. *Immunochemistry* **3,** 504.

TAN E.M. & KUNKEL H.G. (1965) An immunofluorescent study of the skin lesions in systemic lupus erythromatosus. *Arthr. & Rheum.* **9,** 37.

TURK J.L. (1958) Immune adherence with soluble antigens. *Immunology* **1**, 305.

UNANUE E. & DIXON F.J. (1964) Experimental glomerulonephritis. VI. Participation of complement in nephrotoxic nephritis. *J. exp. Med.* **119**, 965.

VOLK H., ROTHER K., MAUERSBERGER D. & ROTHER V. (1964) Prolonged survival of skin homografts in rabbits defective in the third component of complement. *Am. N.Y. Acad. Sci.* **120**, 26.

WALLER M. & LAWLER S.D. (1962) A study of the properties of the rhesus antibody (Ri) diagnostic for rheumatoid factor and its application of GM grouping. *Vox Sang.* **7**, 591.

WARTIOWAARA T.W. (1932) Über die Entwicklung der Konglutinierenden eigenschaft bei der immunisierung. *Acta. Soc. Med.* '*Duodecim*'. Ser A, **14**, No. 15. 1.

WARD, P.A. (1967) 'A plasmin split fragment of C'_3 as a new chemotactic factor'. *J. exp. Med.* **126**, 189.

WARD P.A., COCHRANE C.G. & MÜLLER-EBERHARD H.J. (1965) The role of serum complement in chemotaxis of leucocytes *in vitro*. *J. exp. Med.* **122**, 327.

WARD P.A., COCHRANE C.G. & MÜLLER-EBERHARD J.H. (1966) Further studies on the chemofactic factor of complement and its formation *in vivo*. *Immunology* **11**, 141.

WEINTRAUB R.M., CHURCHILL W.H. (Jr.), CRISLER C., RAPP H.J. & BORSOS T. (1966) Mouse complement: influence of sex hormones on its activity. *Science* **152**, 782.

WELLENSIEK H.J. & KLEIN P.G. (1965) Multiple nature of the third component of guinea-pig complement. II. Separation and description of two additional factors and d; preparation and characterization of four intermediate products. *Immunology* **8**, 604.

WEST, C.D., DAVIS, N.C., FORRISTAL, J., HERBST, J. and SPITZER, R. (1966) 'Antigentic determinants of human βIC and βIG globulins'. *J. Immunol.*, **96**, 650.

WEST, C.D., WINTER, S., FORRISTAL, J., MCCONVILLE, J.M. and DAVIS, N.C. (1967) 'Evidence for *in vivo* breakdown of βIC globulin in hypercomplementerric glomerulo-nephritis. *J. clin. Invest.*, **46**, 539.

WILLOUGHBY W.F. & MAYER M.M. (1965) Antibody-complement complexes. *Science* **150**, 907.

WINN H.J. (1965) Effects of complement on sensitized nucleated cells. In *Complement*, p. 133, CIBA Symposium, ed. WOLSTENHOLME G.E.W. & KNIGHT J. London: J. & A. Churchill.

WRIGHT A.E. & DOUGLAS S.R. (1903) An experimental investigation of the role of the blood fluids in connection with phagocytosis. *Proc. Roy. Soc. B.* **72**, 357.

YACHNIN S. (1965) The haemolysis of red cells from patients with P.N.H. by partially purified subcomponents of the third complement component. *J. Clin. Invest.* **44**.

YACHNIN S. & RUTTENBERG J.M. (1965) The initiation and enhancement of human red cell lysis by activators of first component of complement and by first component esterase; studies using normal red cells and red cells from patients with P.N.H. *J. Clin. Invest.* **44**, 518.

ZAPPACOSTA S., ROSSI G. & ZAPPACOSTA C. (1965) Effet de la fixation de C'_1 et C'_4 sur les mouvements du sodium dans les erythrocytes de mouton sensibilises. *G. Microbiol.* **13**, 1.

SECTION III

THE ALLERGIC STATE AND IMMUNITY

CHAPTER 15

THE ALLERGIC RESPONSE AND IMMUNITY

R.R.A. COOMBS & H. SMITH

INTRODUCTION

Immunology deals with many phenomena quite unrelated to immunity, but one should remember that the science derived its origin from the study of immunity to infective diseases and it is still today vitally concerned with immunity, not only in the bacterial and viral diseases, but also in the protozoal and helminthic diseases.

It is perhaps no exaggeration to say that immunoprophylaxis is one of the most important branches of medicine responsible for saving countless lives.

Furthermore, with the better understanding of the allergic mechanisms underlying immunity and with the availability of human immunoglobulins (World Health Organization Report, 1966), it is possible that immunotherapy will yet fulfill the early hopes it engendered at the beginning of this century.

In the early forties, the great impact of chemotherapy and antibiotic treatment diverted attention from the necessity of studying further the mechanisms of immunity in the infective diseases amenable to such treatment. However, it was soon realized that in many infections there was an interdependence between the action of the drug and the clearance of bacteria by the allergic reactions. In other diseases—for instance, typhoid, scrub typhus and streptococcal infections—it is now recognized that too rapid administration of antibiotics results in a lack of immunization against a subsequent attack. Moreover, the possibility of a world-wide spread of Resistance Transfer Factor, which renders bacteria resistant to a wide spectrum of antibiotics (Anderson 1967) warns us of the folly of neglecting modern studies aimed at harnessing the immunity mechanisms for therapeutic use.

In this book, immunity (and the epithet immune) is considered as defining a clinical state (see also Chapter 20 which deals with the clinical state of hypersensitivity). At the cellular and molecular level, iminunity is established by a combination of mechanisms 'non-specified' in the immunological sense on the one hand and other mechanisms 'specific' or allergic on the other hand. Both types of mechanisms are briefly discussed in this chapter. The state of enhanced protection or 'acquired immunity' is mostly dependent on allergic mechanisms.

When pathogenic micro-organisms invade a host the clinical outcome depends on an interplay of the reactions of the parasites to the conditions *in vivo* and the reaction of the host to foreign intrusion. The great variety of reactions of different parasites and their hosts can lead to almost any clinical outcome, from complete subjugation of the host by the parasite to complete destruction of the parasite by the host or indeed a position of near stalemate as occurs in chronic infections. It must be remembered however, that, although the reactions of both parasite and host are many and variable, the behaviour of the parasite is to some extent pre-determined by the conditions *in vivo* and that the repertoire of allergic reactions of the host is also limited. These reactions are not so adaptable as implied in the often used notion of parasitic attack on host defence with its corollary that both processes are under commanders-in-chief capable of changing the deployment of their forces according to how the battle proceeds.

Survival during the first few hours within the host's tissues is crucial for invading micro-organisms (Miles, Miles & Burke 1957). The clinical outcome of infection is largely decided by the degree of microbial survival during this primary lodgement stage, when the protective reactions of the host are weighted against the relatively few invading micro-organisms. At this stage, host resis-

tance depends mainly on certain non-specific mechanisms, notably inflammation and its sequelae, which operate immediately micro-organisms enter and irritate the tissues (Paz & Spector 1962; Spector & Willoughby 1963). A possible specific (allergic) mechanism, operative at this stage, is mentioned later in the chapter. If some microbes survive during the primary lodgement and begin to grow, the spread of infection from the original site is opposed by the action of the fixed phagocytes of the reticulo-endothelial system.

These protective mechanisms operate in the non-allergized as well as in the allergized host. However, in the latter the mechanisms are usually of increased efficiency. This strengthening and elaboration of the normal mechanisms of host resistance against a particular pathogenic micro-organism coupled with the production of specifically allergized cells and of serum antibodies capable of direct neutralization of noxious microbial products, forms the basis of the clinical state of acquired immunity.

In some infections the allergic responses, when well developed, may damage host tissues and even contribute to the spread of infection, e.g. in tuberculosis (Middlebrook 1965). However, the more usual result of allergic responses to vaccination, to latent infection and to invading organisms a few days after initial infection is an increased resistance of the host.

In this chapter we briefly discuss some of the microbial factors responsible for virulence and diseases, the 'non-specific', i.e. non-allergic processes of host resistance and the allergic mechanisms which may result in a specific increase in resistance. Main illustrations are taken from infections caused by pathogenic bacteria but special circumstances relevant to infections caused by viruses, protozoa and helminths are also mentioned.

MICROBIAL FACTORS RESPONSIBLE FOR DISEASES

This is a brief summary of the mechanisms whereby micro-organisms counteract the resistance of the host and produce disease. Bacteria are the main examples taken and the near synonymous terms 'pathogenicity' and 'virulence' are used as suggested by Miles (1955), i.e. the former in respect to species (some strains of which may be avirulent) and the latter in respect to strains within the species.

Virulent micro-organisms have various attributes which allow them to establish themselves in a host and produce disease. First, they must be able to penetrate the anatomical barriers to infection except when these barriers are traumatized. How virulent organisms survive on the skin and mucous surfaces in competition with the multitude of commensals, and eventually penetrate into the tissue is largely unknown. Together with other factors this process is involved in the communicability of pathogenic micro-organisms about

which so little is known, e.g. we have no idea why measles and brucellosis are more contagious than mumps and typhoid fever.

Once within the tissues, virulent micro-organisms must have the inherent ability to grow and multiply in the nutritional conditions which exist there. The avirulence of some strains of a pathogenic species may well arise from the simple inability of the organisms to grow and divide in this environment (Burrows 1955). Differences in nutritional conditions in different conditions in different hosts and in different tissues within the same host may be responsible (together with differential distribution of inhibitory factors—see later) for host and tissue specificities of pathogenic micro-organisms. Recently, the predilection of *Brucella spp.* for certain foetal tissues of susceptible species (ox, sheep, goat and pig) was correlated with the presence only in these tissues of a growth stimulant for *Brucella spp*—erythritol (Keppie 1964). Such nutritional considerations may be responsible for virulent organisms localizing in areas where they may be relatively inaccessible to resistance mechanisms of the host either specific or non-specific, e.g. in foetal tissues of certain species.

In addition to an ability to grow in the host tissue, virulent organisms must also produce factors which act in a positive manner against the host. The factors may be grouped in either or both of two classes. First, there are the so-called *aggressins* or *auxiliary pathogenic factors* (Miles 1955) which although not necessarily toxic, inhibit the resistance mechanisms of the host and allow the organisms to grow freely in the host tissues. Second, there are the compounds which cause the disease symptoms or death of the host; in most cases the compounds (*toxins*) directly harm the host. In yet other instances (e.g. tuberculosis) the disease is the result not so much of a toxin but of an allergic reaction of hypersensitivity to the organism and its products (see introductory chapter) together with an inability of the host to destroy the organism effectively.

The role of the *auxiliary pathogenic factors* in allowing microbial growth is more obvious in those diseases, such as anthrax, where the harmful effect attributable to each organism is low, since the host tolerates so many organisms before finally succumbing to the disease. Organisms producing diseases of this type, rather than those characterized by a single powerful exotoxin (e.g. *Clostridium tetani*) present the major problems in the study of pathogenicity. Here virulence is due to the possession of an armoury of products all of which have to be formed *in vivo* for maximum virulence and some of which may be difficult to recognize because they are absent when the organism is grown *in vitro* (Smith 1958). The activity of these virulence factors may be largely interdependent so that the absence of one results in a striking loss of virulence, e.g. capsular polyglutamic acid and the toxin of *Bacillus anthracis* (Keppie, Harris-Smith & Smith 1963). On the other hand, the activity of the virulence factors of some organisms, e.g. *Staphylococcus aureus* may be more additive and the absence of one component may result in relatively small reductions in viru-

lence. Examples of *auxiliary pathogenic factors* are summarized below with a brief reference to various *toxins*.

Auxiliary Pathogenic Factors

These factors inhibit the resistance mechanisms of the host which primarily react with the surface of the invading organism. It is not surprising therefore that many of these factors are surface products which may or may not be present as recognizable capsules. However, the fact that a micro-organism is capsulated does not automatically mean that the organism is virulent. There are numerous capsulated strains of pathogenic species (*B. anthracis*, *Str. pneumoniae*, *P. pestis*) which are avirulent as well as many capsulated strains of non-pathogenic species. It is the nature of the surface material which is important, not the capsule *per se*.

(i) *Inhibitors of serum and tissue (non-antibody) bactericidins*
Capsular polyglutamic acid and the anthrax toxin acting together inhibit the action of 'anthracidal substance' in horse serum and white blood cell extracts (Keppie *et al* 1963). A cell wall product from *Brucella abortus* which is also liberated into the surrounding medium inhibits the bactericidal action of bovine serum on *B. abortus* (Smith, Keppie, Pearce & Witt, 1962).

(ii) *Surface and capsular products that inhibit ingestion by phagocytes*
A number of such products are known, for example: capsular polysaccharides of pneumococci: capsular polyglutamic acid of *B. anthracis*; capsular polysaccharide of *Haemophilus influenzae*; M. protein and capsular hyaluronic acid of *Streptococcus pyogenes*; the envelope substance of *P. pestis*; and the Vi antigen of *Salmonella typhi*. However, their mode of action is often not clear. Interferences with phagocytosis may be purely mechanical but more specific mechanisms may be involved, e.g. interference with adsorption of opsonin (Keppie *et al* 1963).

(iii) *Extracellular products that inhibit phagocytic function*
Example of these products are the anthrax toxin (Keppie *et al* 1963) and the various leucocidins of the staphylococci (Morse 1965); they seem to have a direct toxic action on the phagocytes.

(iv) *Inhibitors of the intracellular bactericidal action of phagocytes*
The majority of pathogenic organisms are handled effectively by the phagocytes once they are ingested. However, there are a number of parasites, e.g. the tubercle bacillus, *Brucella spp.* and *Listeria monocytogenes* which survive phagocytosis and grow intracellularly. Phagocytosis tests on these organisms *in vitro* reflect their behaviour *in vivo*. Virulent strains of these pathogenic species survive and grow intracellularly; avirulent strains either grow more slowly or

are gradually destroyed (Mackaness 1964a; Smith & FitzGeorge 1964a). At present, however, the chemical basis for the survival and growth of intracellular micro-organisms is unknown. Recent attempts to investigate the problem using *B. abortus* and bovine buffy coat cells have indicated that the difference in intracellular behaviour between virulent organisms and avirulent ones is not due to an enhanced capacity of the former to use the nutritional conditions within the phagocyte (Burrin, Keppie & Smith 1966), nor is it due to the greater catalase content of the virulent bacteria which would inactivate hydrogen peroxide, a possible destructive agent in phagocytes (FitzGeorge, Keppie & Smith 1965). It may however be connected with the production of a protective cell wall material by the virulent organisms under the conditions of growth *in vivo* (Smith & FitzGeorge 1964a; FitzGeorge & Smith 1966).

Toxins

Pathogenic species can be divided roughly into three categories as regards the production of toxins.

1. Organisms which produce *in vitro* and *in vivo* well characterized exotoxins responsible for practically the whole disease syndrome, e.g. *Cl. tetani* and *Corynebacterium diphtheriae.*

2. Organisms which produce a number of toxins *in vitro*; often it is difficult to determine which of these toxins are produced *in vivo* and are of prime importance in the disease, e.g. *Str. pyogenes* and *Staphylococcus aureus.*

3. Organisms which produce a fatal or serious disease syndrome yet have not been shown to produce a significant toxin *in vitro*, e.g. *Str. pneumoniae.*

NON-SPECIFIC MECHANISMS OF HOST RESISTANCE

In the non-allergized host, it is sometimes difficult to distinguish between truly non-specific phenomena involved in resistance to infection and those phenomena mediated by naturally occurring antibody. Nevertheless, non-specific antimicrobial mechanisms do operate. Space will not permit a full description of all these mechanisms. Attention will be concentrated on the phagocytic activity of the reticulo-endothelial system for two reasons. First, together with the important, but little understood protection afforded by the mucous membranes and skin (which excludes a multitude of potentially pathogenic commensals), phagocytosis forms the main protective mechanism of the body. Second, the efficiency of the phagocytic system can be much increased by the allergic responses of the host, which are the main subject of this chapter. However, a few *naturally occurring antimicrobial substances* should be mentioned briefly, since they contribute to host resistance and their presence or absence may to some extent explain the different susceptibilities of different host-species to the same in-

fection; for example, the resistance of some animal-species to anthrax has been correlated with the presence of 'anthracidal' substances in their sera and tissues (Bloom & Prigmore 1952). In our preoccupation with mechanisms of acquired immunity, we may forget that differences in susceptibility to infection between host-species often exceed those between allergized and non-allergized hosts of the same species.

Non-Specific Antimicrobial Factors

β lysins

These heat-stable substances are bactericidal for Gram-positive bacteria; they are found in sera of most animals and increase in infection (Petterson 1926; Naff *et al* 1958; Myrvik & Leake 1960).

Lysozyme

This enzyme is found in many body fluids; it lyses Gram-positive organisms and enhances the bactericidal action of antibody and complement for Gram-negative organisms (Muschel 1963).

Antibacterial substances in tissues

These bactericidal substances are found in many tissues; most of them are basic polypeptides (Bloom & Prigmore 1952; Skarnes & Watson 1957).

'*Interferon*'

A virus inhibitor indistinguishable from virus-induced interferon appears quickly in the circulation of mice and rabbits soon after injecting bacterial endotoxin (Hallum, Youngner & Stinebring 1965). Substances of this type and others in serum and tissues (Ginsberg 1960) may contribute to resistance to virus infections.

Complement

Micro-organisms might be sensitized to the action of complement by substances other than antibody (Hirsch 1965; see also Müller-Eberhard 1965). The very significant role complement plays in association with the allergic reactions is discussed in the next section.

Phagocytosis by Cells of the Reticulo-Endothelial System

Later in this chapter, the influence of the allergic reactions on phagocytic mechanisms will be described. However, phagocytosis is not entirely dependent on specific factors; it occurs in the absence of serum (Hirsch & Strauss 1964; Shayegani & Mudd 1966; Brogan 1966) and in the presence of serum factors which are not antibodies (Hirsch & Strauss 1964; Brogan 1966).

Apart from their power of phagocytosis the most important fact about phagocytes is the variety of types which differ in origin, morphology, constituents and possibly in bactericidal function. A full description of the different varieties of phagocytes cannot be undertaken here. Briefly, there are two main types—the polymorphonuclear neutrophils (and some would include the eosinophils) and the blood monocytes and tissue macrophages. The polymorphonuclear phagocytes are end-cells with a short life; they are derived from different stem cells from those of the mononuclear phagocytes which are capable of prolonged life *in vivo* and *in vitro*. All four varieties of phagocytes are found in the blood and in inflammatory responses to infection; in most responses, polymorphonuclear cells predominate at first but then die, leaving the mononuclear phagocytes ascendent. Macrophages form almost the whole of the fixed phagocytic system in the lymph nodes, spleen, liver, etc.

There are three stages in the phagocytosis of micro-organisms, contact, ingestion and intracellular killing and digestion.

Contact with micro-organisms is effected by random hits, by chemotaxis due to non-specific (Florey 1962) and specific mechanisms (see later) and by trapping on uneven surfaces in confined tissue spaces (Wood 1960). The chance of contact with the fixed phagocyte is increased by filtration systems in the lymph nodes, spleen and liver which bring the micro-organisms near to the phagocytes.

Ingestion of micro-organisms appears to involve a preliminary stage of adhesion to the surface of the phagocyte (Lockwood & Allison 1966). After the preliminary stage, the surface of the phagocyte invaginates and the micro-organism is engulfed within a phagocytic vacuole—the phagosome, the membrane of which is derived from the inverted cytoplasmic membrane of the phagocyte. Ingestion, although occurring without serum factors, is increased as we shall see in the next section by antibody and even more so by antibody and complement. However, it is also increased by the so-called non-specific 'heat-labile opsonins' (Hirsch & Strauss 1964) which may be important at a stage before antibody develops. The fixed phagocytes of the reticulo-endothelial system behave in these respects like the wandering phagocytes (Cohn & Hirsch 1965). Once inside phagocytes, susceptible micro-organisms such as pneumococci, streptococci, coliforms and anthrax bacilli are *killed and digested* quickly. Non-susceptible organisms, e.g. *Brucella spp.* or tubercle bacilli survive and grow at rates related to their virulence.

Knowledge of the intracellular bactericidal mechanisms is fragmentary. Neutrophils contain lysozyme and phagocytin (a basic protein bactericidal for a wide range of Gram-positive and Gram-negative organisms) associated with granules which discharge into the phagocytic vacuoles containing the micro-organisms. On the other hand, peritoneal macrophages and blood eosinophils contain no phagocytin or lysozyme and alveolar macrophages contain lyso-

zyme but no phagocytin (Cohn & Hirsch 1965). Practically nothing is known of the bactericidal mechanisms of these phagocytes and similarly of the fixed macrophages of the reticulo-endothelial system. Lactic acid (Dubos 1954) and hydrogen peroxide (Iyer, Islam & Quastel 1961) have been suggested as possible intracellular bactericidal agents but have not been proven so. Despite the paucity of knowledge, there is sufficient to suggest that the bactericidal capacities of different types of phagocytes may differ and even vary with the species of micro-organisms being ingested; for example one type might be more efficient for removing Gram-negative organisms and another for Gram-positive species.

There are reports that the bactericidal mechanisms of mononuclear phagocytes are less effective than those of polymorphonuclear phagocytes. *Pasteurella pestis* survives and grows within mouse and guinea-pig monocytes yet is killed when ingested by polymorphonuclear cells (Cavanaugh & Randall 1959). Ingested staphylococci are killed more effectively by rabbit polymorphonuclear cells than by macrophages (Mackaness 1960). Avirulent strains of *Brucella abortus* are killed and the growth of virulent strains inhibited more by polymorphonuclear phagocytes than by monocytes of bovine blood (Smith, Harris-Smith & FitzGeorge 1962). A period of survival within monocytes might be an advantage to an invading micro-organism since it would be protected from the bactericidal action of serum factors (e.g. *B. abortus* in bovine blood, Smith & FitzGeorge 1964b) or from the action of the more destructive polymorphonuclear phagocytes. Indeed, after a period within monocytes, *P. pestis* becomes resistant to subsequent ingestion by polymorphonuclear cells (Cavanaugh & Randall 1959) and *B. abortus* becomes more resistant to the bactericidal action of serum (Stinebring 1962). In some granulomatous diseases polymorphonuclear cells have been found to lose their bactericidal but not their ingestive function thus providing protection for the ingested organisms against serum factors and antibiotics (Holmes *et al* 1966). In addition to differences between mononuclear and polymorphonuclear cells macrophage populations may show a heterogeneity with respect to bactericidal capacity; members of populations of rabbit and mouse macrophages differed in their capacities to kill staphylococci and *Salmonella typhimurium* respectively (Mackaness 1960; and Rowley 1966). Furthermore, despite the species differences it is interesting that mouse peritoneal macrophages were more effective for killing *Salm. typhimurium* and staphylococci than were guinea-pig lung macrophages (Pavillard & Rowley 1962).

It is apparent from the discussion above that in carrier states and in chronic infections, micro-organisms may persist intracellularly in the less bactericidal phagocytes where they are protected from other more destructive mechanisms both specific (allergic) and non-specific.

The possible effect of opsonins* on subsequent intracellular killing is a matter

* An opsonin is any factor promoting phagocytosis.

of controversy. Most studies have involved specific opsonins and are discussed in the next section.

The reticulo-endothelial system can be stimulated or depressed by non-specific stimuli leading to increased or decreased resistance to infection. Injection of bacterial endotoxin is a common method of stimulating the system; it induces metaplasia providing more phagocytes and also appears to increase their phagocytic and bactericidal capacities (Cohn & Morse 1960; Rowley 1962, 1966). The action of endotoxin is discussed later. Cortisone depresses the activity of the reticulo-endothelial system mainly by decreasing the inflammatory response; whether or not the activities of phagocytes are affected is a matter of debate (Germuth 1956; Hirsch & Church 1961).

SPECIFIC (ALLERGIC) MECHANISMS OF HOST RESISTANCE

The specific mechanisms or processes which are initiated following the introduction of foreign antigenic substances constitute the allergic response, and much of the biology of this response has been outlined in Chapter 11. Besides the serum antibodies produced by plasma cells, other cells or possibly earlier forms of the same cell line are developed with inbuilt specific mechanisms, about which we are still largely ignorant. These cells, as well as the serum antibodies, probably also enter into reactions with the inciting antigens in the tissues —whether these antigens be in a free molecular state, bound to tissue cells or still forming an integral part of the invading parasite.

Most of the macromolecular substances of micro-organisms and their soluble-products act as antigens after the organisms gain access into the body and each antigen produces its own specific allergic response. Disregarding for the moment the many attributes of the cellular responses, the nature of the serum antibody to each antigen is far from simple, as was once expressed in the Unitarian hypothesis (Zinsser 1921). Directed against any one antigen the stimulated antibodies may be found in any one or in all of the different classes (i.e. IgG, IgA, IgM, IgD or IgE) or sub-classes within these classes of immunoglobulins. The significance of this is that the biophysical properties differentiating these classes of antibody may determine differences in biological behaviour of the antibodies with a consequent increased complexity in the pattern of reaction.

Instead of attempting a comprehensive account or analysis of the form of immunity in different infective diseases, which would be outside our capabilities, we intend simply to put forward a way of grouping the various reactions arising out of the allergic responses, which could alone, or in concert, bring about the acquisition of immunity. Having these modes of reaction and their limitations before us and knowing that this is the extent of our present knowledge, should give us a greater boldness in analysing the immune mechanisms

in any particular infective disease. The point is that the allergic processes themselves are intricate but certainly fairly limited. The immense variety of pattern of interaction, the understanding of which requires a detailed study of each disease, is more a reflection of the natural history of the micro-organisms in the body and the consequences resulting from the locus of infection.

The following scheme (see Fig. 15.1) gives a grouping of allergic reactions into ways in which they are able to assist in establishing immunity.

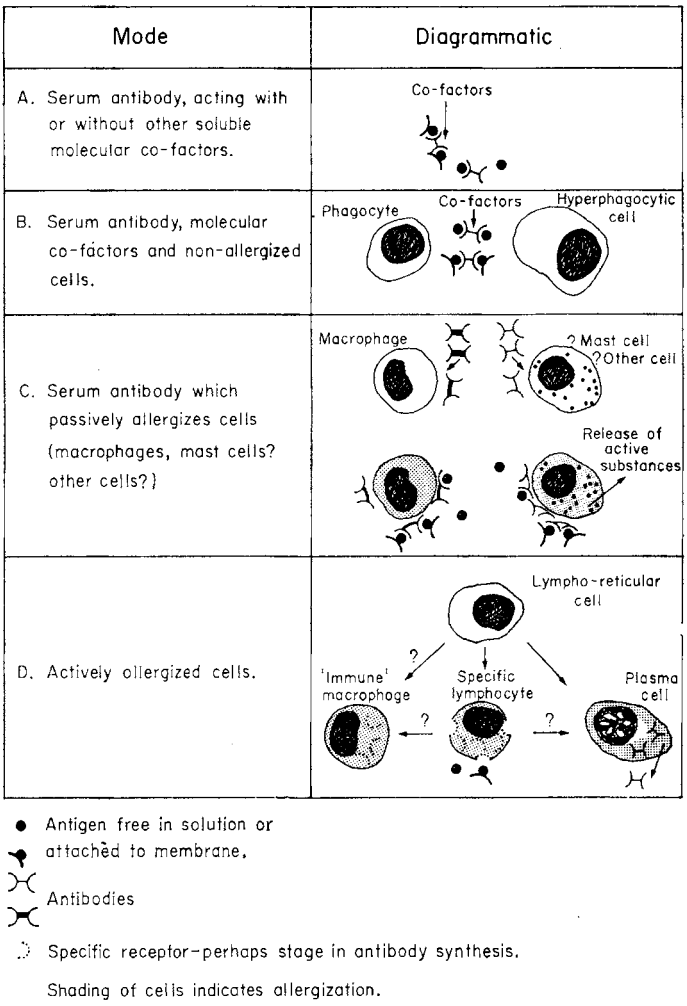


FIG. 15.1. Grouping of allergic reactions into ways or modes in which they are able to assist in establishing immunity.

Mode A: Serum antibody acting with or without other soluble molecular co-factors.

Mode B: Serum antibody acting with or without other soluble molecular co-factors, together with 'non-allergized' cells. It follows that pretreatment of micro-organisms with antibody in the presence or absence of co-factors will render the organisms more prone to phagocytosis and destruction by non-allergized phagocytes in a medium lacking antibody.

Mode C: Serum antibody 'passively allergizing' cells. Thus in a medium lacking antibody micro-organisms may be expected to be destroyed more efficiently by the cells (macrophages, mast cells or other cells) after the latter have been pre-treated with antibody.

Mode D: Reaction of 'actively allergized cells'. Here no humoral antibody, as we know it, is involved. The cells themselves must display as a result of allergization an enhanced capacity to destroy micro-organisms, neutralize their noxious products or initiate an inflammatory response unconducive to the survival of the organisms. The main difficulty in demonstrating unequivocally the presence of 'actively allergized cells' is the lack of certainty that all influence of humoral antibody has been excluded.

The above scheme is thought to embrace all the components of allergic reactivity constituting the immune response.

Mode A—Serum Antibody Acting with or Without other Soluble Molecular Co-Factors

Antibody Acting as Antitoxin

This is a straight-forward reaction — combination of antibody either with the active site on the toxic molecule or in such a way that it neutralizes its toxic action, e.g. by steric hindrance or allosterism. This is the all important mechanism of immunity in toxin-dependent diseases such as diphtheria, tetanus and other clostridial infections. If the toxin (or toxins) can be neutralized, the micro-organisms can be dealt with without much difficulty. Immunity in these diseases is directly related to antitoxin production. In streptococcal and staphylococcal infections antibodies play a role in neutralizing toxins, but the latter may not be the major factors determining the disease. Antitoxic immunity is discussed at greater length in Section V of the book (see also Wilson & Miles 1964).

Antibody neutralization of toxic substances can operate during the early stages of infection when the toxic substances are acting as aggressins in inactivating phagocytes, e.g. the anthrax toxic complex or the staphylococcal leucocidins. Immunity to anthrax is largely based on the neutralization of the toxin by antibody when the toxin is acting as aggressin in the Decisive Period of Miles (see later).

Antibody Neutralizing Viruses

This again is an uncomplicated reaction — combination of antibody with the protein or lipid coat of the virus, which does not 'kill' the virus, but prevents either its adsorption on to or penetration into cells. As free antibody does not gain entrance to cells (except possibly in certain pinocytotic vacuole) such antibodies can only serve a useful role where complete virus, after intracellular multiplication, is released into the tissue fluids before infecting other cells (e.g. in influenza and poliomyelitis). Such a mechanism of immunity may be useless in infections such as herpes simplex where virus is able to pass from cell to cell without emerging into the tissue fluids (Black & Melnick 1955). It has also been suggested that virus antibody found in mucus of the mucosa and in intestinal contents (called copro-antibody) may be a factor reducing contagion in poliomyelitis (Lipton & Steigman 1963).

Neutralizing antibody is probably more important in preventing a second attack of a viral disease or attack after vaccination than in overcoming a primary infection where cells are likely to be parasitized before an allergic response is mounted.

Antibody Inhibiting Important Enzyme Systems of the Parasite

The main role of antibody against bacterial surface antigens is in initiating complement activity and promoting phagocytosis. However, it is possible that certain bacteria may have a vital enzyme system as their Achilles' heel, which could be blocked by antibody combination. The possibility of such an enzyme acting as antigen may be one of the circumstances underlying the better immunizing capacity of live as opposed to dead vaccines.

Also to be mentioned in this context is the evidence that antibodies to the excretory and soluble (ES) antigens of larval helminths migrating through tissues lead to a stunting of growth and interference in migration. It is conjectured that antibody acting alone may inhibit enzymes of the oral secretions and in precipitating with other secretory and excretory products may inhibit and block physiological activities. Together these may result in stunted growth (Taliaferro & Sarles 1939).

The antibody preventing multiplication of *Trypanosoma lewisi* in rats described by Taliaferro (1929) may also exemplify this role of antibody.

Reaction of Antibody with Soluble but Possibly Non-Toxic Microbial Products

Such reactions probably accompany most infections as the organisms must elaborate many antigenic macromolecules which are soluble and which may not be toxic. Antibody reacting with these antigenic molecules may trigger off further reactions which promote local inflammation and bring polymorpho-

nuclear leucocytes to the site. These leucocytes may accumulate as part of a general inflammatory response or as a result of a more specific chemotactic differential set up by antigen-antibody complexes and complement involvement (see later).

With mounting antigen and antibody concentration more definite Arthus-type lesions may develop. These will certainly affect, either retarding or aiding, the spread of infection. On the other hand, they may create a tissue environment unfavourable to sustained growth of the infecting organism. Dubos (1954) has suggested such a circumstance as playing a role in immunity to tuberculosis.

In another circumstance, potentially opsonizing antibody might be neutralized by the release of soluble antigenic aggressins of the bacterium—an example of this would be the capsular material of the pneumococcus.

Antibody Initiating the Killing and Lysing of Organisms by Complement

Just as complement haemolyses red cells sensitized with antibody, so some (Gram-negative), but by no means all, bacteria may be killed and even lysed by a combination of complement and antibody. Lysozyme may also be an essential reactant in this process acting on a mucopeptide substrate possibly exposed by the action of complement on the cell wall (Wardlaw 1962). Surprisingly perhaps, this process is not considered to be an important reaction of immunity against bacteria, but with commonly occurring levels of 'natural' antibody, it may play a role against Gram-negative organisms in the very early stages of infection or lodgement—the 'Decisive Period' of Miles (Miles, Miles & Burke 1957). *Vibrio cholerae* is very susceptible to the destructive action of antibody and complement, and everyone is familiar with the Pfeiffer phenomenon in the peritoneal cavity of the guinea-pig. However, in cholera most of the organisms remain in the gut where there is not likely to be much active complement.

Many protozoa such as trypanosomes are very susceptible to lysis by antibody and complement. This process would be a most effective immune mechanism in trypanosomiasis were it not for the fact that antigenically distinct variants constantly arise bringing about a new parasitaemia until they, in their turn, are lysed by developing antibody.

Mode B—Serum Antibody, Acting with or without other Soluble Molecular Co-Factors, Together with Non-Allergized Cells

The essential component characteristic of this mode of reaction is the phagocytic cell. Phagocytosis is undoubtedly the most important immunity mechanism of the body and it has already been discussed at some length (see earlier in this

chapter and Chapters 12 and 14). In the present section we are mainly concerned with the role of antibody and of antibody and complement in concert acting as opsonins in promoting phagocytosis. Although phagocytosis may proceed in the absence of antibody and other co-factors, each stage of the process may be powerfully influenced by the molecular interactions of the allergic response. The effectiveness of phagocytosis of organisms opsonized by antibody and complement *in vivo* is evident from studies of Biozzi *et al* (1961) who found phagocytic clearance of organisms from the blood stream to be an extremely sensitive gauge for antibody. In this connection, it must be remembered that antibodies of the different immunoglobulin classes may differ widely in their ability to act as opsonins.

Before phagocytosis can take place, phagocytic cells must be brought to the area. Over and above any influx due to general inflammatory chemotactic forces a *chemotactic effect* on polymorphonuclear leucocytes can be initiated by antigen-antibody complexes in the presence of unheated serum (Boyden 1962). Ward, Cochrane & Müller-Eberhard (1966) showed that following adsorption of β1C during complement fixation C'5, 6 and 7 interacted and were given off as a trimolecular complex into the medium. The concentration gradient of this complex gives a directional attractive force to polymorphonuclear leucocytes. This gradient is specific for polymorphonuclear cells and has no attraction for macrophages (Boyden, personal communication).

The next stage in phagocytosis is *contact and ingestion* of the organism. Opsonic substances may react with selective receptors on the cell membrane of the phagocytes. In the case of antibody, certain classes of immunoglobulins or subfractions of a class (e.g. γ_2 in the guinea-pig) are known to have a very high and selective affinity from the macrophage membrane, but not for that of the polymorphonuclear leucocytes. Other opsonins may show other preferences; the determinant on fixed complement with special affinity for the phagocyte membrane has been shown to reside on the β1C molecule of fixed C'3 (Nelson 1962).

The *intracellular events* following ingestion of bacteria have already been briefly discussed. Whether combined antibody with or without other co-factors can influence the enzymic and other degradation processes inside the phagosomes is not really known. Jenkin & Rowley (1963) consider that opsonin ingested with the organism aids in this killing, but other investigators do not agree with this (Mackaness 1960; Thorpe & Marcus 1964; Smith & FitzGeorge 1964b; Blanden, Mackaness & Collins 1966). Not all organisms are killed inside the phagocytes and this is especially so with regard to facultative intracellular parasites (e.g. brucella) in macrophages. Here, far from being killed, the organisms may actually gain sanctuary from the extracellular destructive humoral factors. This brings us up against one of the major problems of immunity, namely, what further mechanisms, allergic or otherwise, can be envisaged,

which may assist in the destruction of facultative intracellular pathogens surviving inside macrophages.

At this point it is necessary to remind the reader that in this section we are discussing phagocytes as 'non-allergized' cells. In considering further non-allergic mechanisms for combating facultative intracellular parasites, it should be recalled that macrophages the body over are not of uniform type and indeed may possess quite different biological mechanisms for microbial destruction.

It is also known that the phagocytic cells of the reticulo-endothelial system can be made more efficient and brought up, as it were, to 'Olympic standards'. Administration of endotoxin apparently increases (by whatever means it acts) the number, size and activity of reticulo-endothelial cells. The enhanced activity may be shown by a better clearance of organisms from the blood stream. Here we would seem to have an example of 'non-allergized' phagocytes *primed non-specifically* (if action of endotoxin is non-specific in the immunological sense)* for a *non-specific role*. Such cells have been called 'hyperphagocytic cells'. Metaplasia may even be involved in the action of endotoxin as seen in activation of non-phagocytic endothelial cells of the liver (Howard 1959).

In studies on acquired cellular resistance Mackaness (1964b) provided evidence that macrophages can be primed for a greater and more efficient *non-specific effect* by a *specific induction*, i.e. two properly spaced doses of the same antigen. It will be extremely important to analyse the mechanism by which these macrophages have attained their greater efficiency in killing virulent organisms; but in that the effect is not specific as between the priming stimulus and the organism against which the increased resistance is effective, it would seem better to regard the type of cell in question as a 'hyperphagocytic cell' rather than as an 'immune phagocyte'.

The need for this slight pedantry is that an 'immune phagocyte' or what we would call an 'actively allergized macrophage or phagocyte' may exist, i.e. a phagocyte with a built-in specific mechanism (see later and Fig. 15.1). Such a cell could well have a more efficient yet specific mechanism for killing organisms intracellularly within its phagosomes. Already, as discussed in the following subsection, we have certain knowledge of what we call a 'passively allergized macrophage'.

Mode C—Serum Antibody 'Passively Allergizing' Cells

If a cell is able to absorb an antibody passively on to its membrane (see Fig. 15.1) it may be said to be 'passively allergized' and two examples will be mentioned.

* It is still held by some that the endotoxin reaction is specific in the immunological sense and is in fact an allergic reaction (Stetson 1964).

The significant point is whether these cells are more effective in killing micro-organisms or counteracting their effect.

Guinea-Pig Macrophage-Cytophilic Antibody

From original studies on the rabbit Boyden & Sorkin (1960, 1961) drew attention to a type of antibody with the property of cytophilia, i.e. it was capable of passively sensitizing the cells. In the guinea-pig there is a corresponding type of antibody which passively sensitizes macrophages (Boyden 1964) and it has been shown (Jonas *et al* 1965) that this passive adsorption is selective for the macrophage in the guinea-pig; it is not even adsorbed on to the membrane of the polymorphonuclear leucocyte.

As yet, insufficient is known about these special antibodies to indicate their biological role. It has been suggested that they may be involved in delayed-type hypersensitivity, but this seems unlikely. Nor is this particular type of antibody, which is a γ_2 immunoglobin in the guinea-pig, concerned in anaphylaxis. One may conjecture that it would enhance specifically the contact- and engulfment-phase of phagocytosis by macrophages, in situations where free serum opsonins are not readily available, i.e. in tissue spaces and on serous surfaces and mucous membranes. The selective adsorption of this particular immunoglobulin to the macrophage membrane might convey to the cell very powerful and specific opsonizing powers (Berken & Benacerraf 1966). Such antibody attached passively to the macrophage membrane would be taken into the cell as the membrane invaginates to form the phagosome. These special antibodies are being studied in other species also. According to Rowley, Turner & Jenkin (1964) the 'cell-mediated immunity' to *Salm. typhimurium* in the mouse can be explained on the basis of a 19S cytophilic antibody passively allergizing cells.

One can safely prophesy that the next few years will see many publications on the activity and function of these antibodies in the reactions of immunity.

The cellular affinities of cytophilic antibodies have not been extensively worked out as yet, and, although in the guinea-pig the so-called macrophage cytophilic antibody is selective for the macrophage, it is possible that a cytophilic antibody selective for the polymorphonuclear leucocyte may be found. Indeed Fitzpatrick *et al* (1967) consider that the reaginic antibody in man is cytophilic for these cells and not simply for the blood basophils. There would be great interest in the acquired properties of passively allergized polymorphonuclear cells, since even when unallergized they are the most effective destroyers of microbes during the 'Decisive Period' of Miles.

'Anaphylactic', Reagin and Reagin-Like Antibodies

This type of antibody also passively allergizes cells although we are still not

absolutely certain which cells are involved. Much evidence points to mast cells and basophils, but other cells also may be implicated. When such passively allergized cells or tissues come into contact with antigen, a sequence of events leads to liberation of pharmacological mediators from the cellular lysosomes. Histamine is the main pharmacological mediator, but there are many others and their effects vary in the different species. There may be relaxation or constriction of smooth muscle and increased vascular permeability, as is well exemplified in various clinical states of hypersensitivity. However, these self-same reactions under certain conditions, may also play a role in immunity.

Localized oedema following increased vascular permeability may allow complement molecules and the larger antibody molecules to gain access to the tissue spaces and their bactericidal action there may play a vital role during the early 'Decisive Period' of an infection (Miles *et al* 1957). Also, it is conceivable as postulated by Jancso (1947) that histamine liberated by such an allergic reaction promotes latent phagocytic powers of endothelial cells and possibly of fibroblasts also. Intracellular antibacterial substances such as basic polypeptides, lysozyme and even interferon-like substances might also be liberated by such a mechanism.

Perhaps a more spectacular illustration is the 'self-cure' mechanism by which sheep rid themselves of heavy infestations of the stomach worm *Haemonchus contortus* (Soulsby & Stewart 1960). There is evidence to suggest that antigenic material in the anti-coagulatory oral secretions or other excretions of the nematode reacts with cells in the gastric wall which are passively allergized with specifically stimulated reagin-like antibody; this results in local liberation of histamine. Either because of, or following this Type I allergic reaction (see Chapter 20) the worms are expelled from the gut. There is also evidence that reagin-like antibodies with their characteristic property of passively sensitizing cells have an important role to play in immunity to *Nippostrongylus braziliensis* in the rat (see later).

Also meriting consideration are colostral antibodies, that are passed to the newborn calf (Pierce & Feinstein 1965; Feinstein & Pierce 1967) and whose biological role is assumed to be that of conveying passive *immunity* over the neonatal period. Amongst these γ_1 globulins are to be found the antibodies capable of passively allergizing cells in the skin for histamine release by antigen. Thus, there may be some association between antibodies which passively allergize cells and immunity.

The passive uptake of antibody may be tied up with the mechanisms of transport of γ-globulin molecules over the epithelial surface of the gut as has been discussed by Brambell (1966). The point we wish to make is that the type of allergic reaction which we see clinically in hypersensitivity as anaphylaxis may, under other conditions, contribute to the build up of immunity.

Mode D—Actively Allergized Cells

During allergization (immunization) cellular events are initiated involving reticulum cells, small lymphocytes and their differentiation into plasma cells (see Chapters 11 and 12). Once committed by antigenic stimulus to some specific synthetic activity the cells may be referred to as being 'actively allergized'. If such actively allergized cells (as either terminal effector cells or cells at some stage in their differentiation) encounter antigen again, as may well happen if antigen is still present in the body or is reintroduced into the body, some cellular reaction can be expected. It must be confessed however that, as yet, we have very little precise knowledge about such reactions at either the molecular or cellular level.

'Cellular immunity' is a very vague term and may well involve reactions of Modes B, C or D (see Fig. 15.1). There is of course also a cellular reaction consequent on the antigen-antibody interactions outlined in Mode A, so that no absolutely clear-cut differentiation is possible between cellular and humoral immunity as such. In Mode B the phagocytic cells involved are non-allergized. In Mode C the cells are passively allergized by humoral antibody, while in Mode D it is believed that there is no humoral antibody involved and that the primarily reacting cells are the actual actively allergized cells themselves. In this latter case, however, until the interactions of actively allergized cells are better characterized and understood it may be difficult to exclude the possibility that the supposed actively allergized cells are not really passively allergized as in Mode C.

Evidence for the direct participation of *actively allergized small lymphocytes* in reactions with 'target antigens' comes from studies such as those of Rosenau & Moon (1961, 1962) and Koprowski & Fernandes (1962) in which lymphocytes from actively sensitized animals are added to target cells growing as monolayers in culture. Again, many of the spleen or lymph node cells from mice injected with sheep red cells which can form rosettes *in vitro* with added sheep red cells (Nota *et al* 1964; Zaalberg, van der Meul & van Twisk 1966) are small lymphocytes as shown by fixing the red cell rosettes and staining the reacting tissue cell inside (McConnell & Coombs 1968). This may be considered to be a reaction *in vitro* of actively allergized small lymphocytes.

From experiments involving rejection of homografts of ascites tumour cells, Granger & Weiser (1964) plead the case for the truly 'immune macrophage'—equivalent to what we would call the *actively allergized macrophage*. However, no specificity of reaction has, as yet, been demonstrated for the 'immune phagocyte' discussed by Suter & Ramseier (1964) nor, as already mentioned for the specifically stimulated 'immune macrophage' described by Mackaness (1964b). But if the findings of Coulson, Gurner & Coombs (1967) that some lymphocytes undergoing transformation in mixed cell cultures *in vitro* (see Chapter 1)

take on macrophage-like properties, are confirmed in other systems and that this also occurs consequent on antigen-induced transformation, then this would be fairly direct evidence for the existence of actively allergized macrophages (see Fig. 15.1). The question of whether or not such macrophages have an increased capability compared with normal macrophages to remove microbes or neutralize their products could then be investigated.

As antigen-antibody interactions are characterized in serology, so the need is now to characterize the interactions of antigen and supposedly actively allergized cells *in vitro* as a prelude to understanding such reactions in the body, especially, in our present context, as regards reactions which may play a role in immunity. One needs evidence of specific receptors in or on such cells and a full understanding of the consequences of reaction with antigen. Relevant phenomena here are:

(i) Transformation of allergized small lymphocytes into blast forms and mitotic stimulation by antigen. This could result in antibody being formed locally.

(ii) Evidence from the experiments of David and his colleagues (David *et al* 1964; David 1966) that antigen acting on allergized lymphoid cells causes the release of a factor, which in the particular experimental system studied reduces the migrating activity of macrophages.

(iii) The indication that some small lymphocytes undergoing blast transformation may acquire macrophage-like properties. As regards immunity, such a cell, endowed with a built-in specific mechanism could have a powerful as yet unknown anti-bacterial mechanism for killing ingested organisms.

(iv) The liberation of a Lymph Node Permeability Factor (LNPF), which follows the action of antigen on supposedly actively allergized mononuclear cells (Schild & Willoughby 1967). Besides increasing vascular permeability, this promotes diapedesis of leucocytes and the deposition of fibrinoid material around vessel walls.

Until these events are fully investigated it will not be possible to unravel the various mechanisms by which these particular allergic reactions may act in establishing immunity in various diseases, especially those caused by the facultative intracellular parasites such as the *salmonellae*, *brucellae* and tubercle bacilli.

What we have attempted to do in this section is to display the repertoire of the allergic reactions of the host and to illustrate the different pathways by which these reactions could possibly contribute to immunity or protection of the host against infective organisms. As with reactions underlying clinical states of hypersensitivity (see Chapter 20) it is helpful for our understanding to be able to do this. However, it must be remembered that in most infections, all of these modes of interaction may be simultaneously engaged although to a different degree and effect.

SPECIAL CONSIDERATIONS INFLUENCING THE EFFECTIVENESS OR OTHERWISE OF THE ALLERGIC REACTIONS IN ACHIEVING THE IMMUNE STATE

Virulence Factors and 'Protective Antigens'

Many products of virulent organisms are antigens and often they form the basis of diagnostic tests. However, only a few of these antigens are protective or immunogenic, i.e. actively immunize against the disease. These immunogenic antigens are important for vaccination and some may be produced only by live vaccines *in vivo* (see later). Immunogenic antigens are necessarily factors involved in virulence, either toxins, e.g. the toxins of *C. diphtheriae* or auxiliary pathogenic factors, e.g. the polysaccharide of *Str. pneumoniae* or the envelope substance of *P. pestis* (Macleod & Bernheimer 1965). However, all antigenic virulence factors need not be immunogenic, e.g. the murine toxin of *P. pestis* (Pollitzer 1954). Finally some virulence factors are not even antigenic, e.g. the capsular hyaluronic acid of *Str. pyogenes* (Macleod & Bernheimer 1965). The latter situation might account for the lack of immunity that occurs in some cases even after overt disease (e.g. staphylococcal infections).

The above considerations also apply to viruses. Many antigens are produced during the course of infection, but with only certain of these is the corresponding antibody protective. Neutralization can be achieved only by antibodies to the surface coat. Other antibodies such as the antihaemagglutinin of vaccinia or the antibody to the ribonucleo-protein of the influenza virus are mere accompaniments of infection.

Even with the so-called protective antigens, the effectiveness of the allergic response will be influenced by the class of immunoglobulins stimulated, the biological properties of each class being so different.

Changes of Microbial Behaviour *in vivo* Which Might Render Ineffectual the Allergic Response of the Host

The nutritional conditions under which virulent micro-organisms survive and grow *in vivo* are different from those in artificial culture and possible sources of natural infection such as water, sewage and soil. If infection results from transmission from another host (of the same or different species), the environment for the micro-organism in the new host may be different from that of the old host; and almost certainly will be different from the conditions existing during transmission, e.g. the organism may have been enclosed some time in dried sputum. Even inside the host nutritional conditions will continually change as infection proceeds (Smith 1958, 1960). At first, in inflamed tissues conditions of

low oxygen tension and low pH will exist (Dubos 1954), whereas later in the blood stream the oxygen tension will be high and the pH neutral. Eventually the infection may localize and the micro-organisms will be affected by the particular nutritional conditions in the localized site.

The changes in nutritional conditions outlined above will affect microbial metabolism and hence the production of virulence factors. Lacey (1961) has shown how the nature of the surface antigens and the virulence of pathogenic organisms can be influenced by changes in environment. Also, Meynell (1961) has demonstrated the profound effect environment can have on the production of toxins and auxiliary pathogenic factors by taking examples from studies of *Bordetella pertussis*, the enterobacteriaceae, *P. pestis*, *B. anthracis*, *Str. Pyogenes*, *Str. pneumoniae*, *C. diphtheriae*, *Cl. tetani* and *Cl. welchii*. Hence, the virulence factors produced by an invading organism may change as infection proceeds and probably the greatest change will occur during the first crucial hours of infection when the organism is adapting to the conditions within a new host (Burrows 1955; Meynell 1961).

Phenotypic variation is not the only mechanism which can produce types of micro-organisms different from those originally entering the host. Selection of more virulent genotypes can also occur *in vivo* and together with phenotypic variation accounts for the classical increase of virulence on animal passage. The possibility of genetic transfer must also be borne in mind; this could occur not only between types of the same species as in the classical experiments of Griffiths but between bacteria of different species in view of the recent work on transference of Resistance Factors (Anderson 1965, 1967).

If, at a particular stage of infection, the allergic responses of the host have been largely provoked by micro-organisms grown in a different environment (either *in vitro* or *in vivo* at a different stage during infection), a loophole in the acquired resistance of the host can exist. The most obvious example is infection of a host after vaccination with a dead preparation produced *in vitro* which does not contain all the antigenic virulence factors that are formed *in vivo*. *The superiority of some live vaccines*, e.g. BCG or *B. abortus* S19, over corresponding killed preparations may be due to the production by the former *in vivo* of protective antigens not formed to any significant extent *in vitro*; and also to the activation of processes discussed in a previous section by the live organisms multiplying *in vivo*.

Returning to the question of variation *in vivo*, this is the way trypanosomes and probably also malarial parasites (see later and Chapter 46) are able to survive in the blood stream in the face of the mounting allergic response of the host. The trypanosome is easily lysed and killed by antibody and complement, but as antibody mounts a new variant with a quite distinct membrance antigen is formed and accounts for the next parasitaemia. In bacterial infections also, antigenic variation *in vivo* can contribute to the persistence of the infection;

Lacey (1961) has discussed this question in relation to *Bordetella* spp. It may well occur also in *Salmonella* infections.

Antigenic sub-types, exemplifying variation at some time, but not necessarily during an infection, are of course well recognized and often account for the apparent ineffectiveness of certain vaccines.

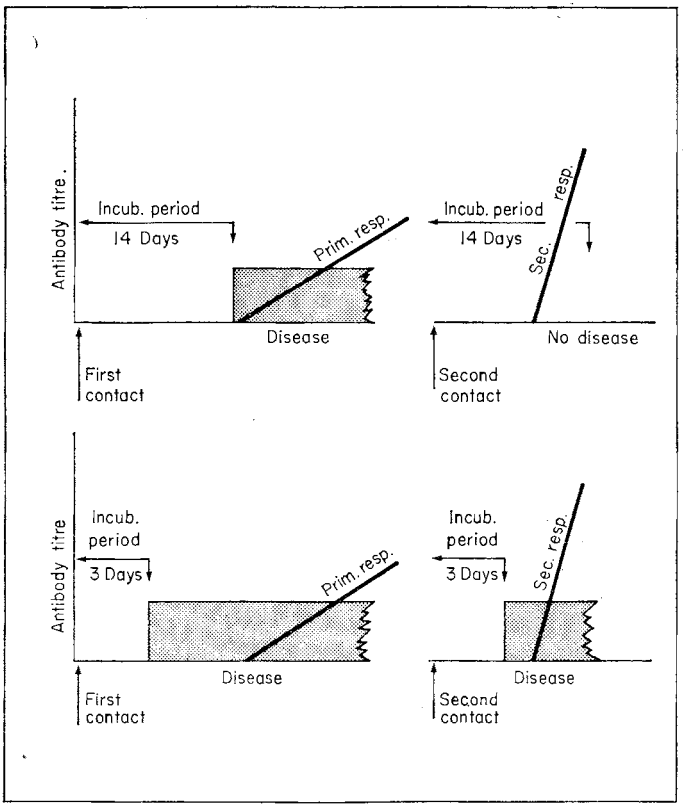


FIG. 15.2. Graphic representation of the relationship of the secondary antibody response and incubation period to permanent immunity. Redrawn from Macleod. (1953).

THE LENGTH OF THE INCUBATION PERIOD OF THE DISEASE

There are many factors both of the microbe and of the host which determine the length of the incubation period in different diseases. In virus diseases long ubation periods occur in those diseases where haematogenous spread is required to set up the characteristic disease picture away from the site of primary

infection. Macleod (1953) has emphasized the importance of the length of this period in determining whether a good lasting immunity can be established against the disease. As may be seen in Fig. 15.2, a normal secondary allergic response has the best opportunity of providing an effective immunity to a second attack of the same disease if the incubation period of the disease is sufficiently long. In diseases with a short incubation period, the secondary allergic response may mount too late to prevent the disease.

Neonatal Period

For the first month or two after birth in both man and domestic animals the allergic reactions of resistance to infective diseases are effected mainly by passive transfer of humoral antibodies (see Chapters 12, 13 and 44) from the mother; no allergized cells are transferred. The newborn must therefore rely for its immunity on the allergic reactions mentioned under Modes, A, B, and C, and on the immune status of the mother. While at most times showing adequate resistance, the young are known to be very susceptible to enteric infections with *E. coli*, *Salmonella spp.* and *Shigella spp.* It is possible that adequate quantities of effective antibody do not have access to intestinal and other mucous surfaces and IgA, which may be more effective than IgG in this situation, is not transmitted from the mother; nor is IgM, which may be especially effective for bacteriolysis (see Chapters 13 and 19).

In hypogammaglobulinaemia on the other hand the allergic reactions under Modes A, B and C must be greatly enfeebled, leaving the reactions of actively allergized cells to fulfil what role they can. It is interesting that immunity to tuberculosis and to many virus diseases is not imperilled in these cases, while resistance to other infections is diminished (see Chapter 19).

EXAMPLES OF INFECTIONS AND THE MECHANISMS OF IMMUNITY TO THEM

Bacterial Diseases

Although each disease and the host's allergic reactions to it is a study in itself, there are similarities between different infections which allow the rough classification given below. However some diseases straddle across the classes, e.g. infections with the gas gangrene organisms *Cl. welchii*, *Cl. oedematiens*, and *Cl. chauvei* are primarily toxaemias but tissue invasion is also important.

Acute Toxaemias: Immunity Based on Antitoxin Production

In infections caused by *C. diphtheriae* and *Cl. tetani*, the causative organisms remain localized and do not multiply. Tissue invasion is minimal. The disease

symptoms and death of the host are caused by a single, powerful toxin which diffuses from the infection area. Immunity depends on the production of specific antitoxin by the host, either naturally as the result of an infection, or artificially as the result of immunization with the appropriate toxoid.

Acute Invasive Diseases: Immunity Based on Allergic Reactions Against Auxiliary Pathogenic Factors

In diseases such as pneumonia, plague and anthrax the causative organisms must grow to large numbers in the host to produce their damaging effects. Prevention of microbial growth is the prime mechanism of immunity; this depends on the production of antibodies to the auxiliary pathogenic factors especially those which prevent phagocytosis. Antibodies to capsular polysaccharides are the basis of acquired immunity to pneumonia (Macleod & Bernheimer 1965). Immunity to plague does not depend on antibodies to the murine toxin of *P. pestis* but on antibodies neutralizing anti-phagocytic products notably the envelope substance and the V and W antigens (Pollitzer 1954; Burrows 1963). In anthrax, immunity depends on the production of antibodies to the toxic complex. This complex, which together with capsular polyglutamic acid prevents the destruction of *B. anthracis* by phagocytosis and by the 'anthracidal substance' (see above) is made up of three components only one of which is adequately protective when injected alone.

Chronic Intracellular Infections: Immunity Depends on Antibody and as yet Ill-Defined Cellular Mechanisms

These diseases are typified by brucellosis and tuberculosis which have been used as examples in the sections above. The microbial factors responsible for the intracellular survival and growth of the causative organisms are unknown. Similarly, the cellular mechanism of acquired immunity and their interplay with humoral factors are not clear; nor is the connection between acquired immunity and hypersensitivity which contributes to host tissue damage and, possibly, to spread of infection. The lack of knowledge is emphasized by the fact that live vaccines (BCG and S19) must still be used in the field. Although some dead preparations have immunizing activity, they are not so effective as the live vaccines (Middlebrook 1965; Roux 1962). However, if the microbial products responsible for intracellular survival could be recognized, they might prove to be potent protective antigens by influencing the mechanisms of cellular resistance in a manner comparable to that achieved with live vaccine (Smith & FitzGeorge 1964a; FitzGeorge & Smith 1966).

Virus Diseases

The allergic reactions are of great importance in preventing a second attack of a virus disease or in preventing an attack following immunoprophylaxis which may be in the form of a vaccine or immune γ-globulin. The significance of the allergic responses in the recovery from a truly primary infection is less certain, and other non-allergic factors may be of more importance (Baron 1963).

Mainly because of the intracellular habitat of the virus pathogen, infections present problems for the fruitful participation of the allergic reactions in establishing immunity. None of the allergic reactions is able, as far as is known, to proceed against truly intracellular parasites if the cell membrane is not altered antigenically in some way as a result of the parasitization. Exceptions to this, perhaps, are possibly unselective uptake by pinocytosis and intake of cytophilic antibody attached to the membrane of macrophages during the formation of a phagosome. Emperipolesis (see Roitt, Jones & Doniach 1962) by an actively allergized lymphocyte is likely to demand primary combination of specific receptors on the lymphocyte with a corresponding determinant on the virus-parasitized cells.

The virus is of course susceptible to allergic reactions when extracellular, e.g. when it first enters the body, when passing from cell to cell or during haematogenous spread. It is thus not surprising that neutralizing humoral antibody has been found to be very important in the development of immunity to virus diseases. Evidence for this is seen in the protective value of immune γ-globulin for contacts in measles and of maternal antibody against herpes simplex in human infants (Anderson & Hamilton 1949) and against ectromelia in newborn mice (Fenner 1948). To abort influenzal infections a level of humoral antibody is needed in the secretions of the mucous membrane of the lower respiratory tract and, as has already been mentioned, Lipton & Steigman (1963) consider that contagion in poliomyelitis can be reduced by antibody in the mucous secretions of the alimentary canal.

That actively allergized cells also make an important contribution to immunity in virus diseases is suggested by the fact that persons with hypogammaglobulinaemia (see Chapter 19) but with normal delayed-type hypersensitivity reactions are capable of developing adequate immunity to many virus diseases, e.g. vaccinia, mumps, measles, chicken pox and poliomyelitis. It must be remembered however that such persons are not completely devoid of γ-globulin and small amounts of neutralizing antibody may be present (Baron *et al* 1962).

Emphasizing the significance of actively allergized cells is the commonly adopted practice of gauging the resistance of a person to smallpox by the local skin response to the vaccinia virus. The formation of a non-vesiculating papule on the second day and which probably represents a delayed-type hypersensitivity response (Type IV reaction, see Chapter 20) is accepted as the 'reaction of im-

munity'. Again delayed-type hypersensitivity skin reactions (reactions of actively allergized cells) have been suggested as a measure of immunity in both influenza (Beveridge 1952) and mumps (Angle 1961).

For a fuller discussion of mechanisms of immunity in virus diseases the reader is referred to Beveridge (1963) and Fenner (1965).

Protozoal Diseases

Under natural conditions there is ample evidence for the existence of a degree of immunity in protozoal diseases although in most cases this seems to be a '*non-sterilizing immunity*', i.e. a clinical state of immunity maintained by a continuing low grade infection. Sergent described this state or concept as one of *premunition* (see Sergent 1963).

In many protozoal infections there is a very powerful allergic response. In malignant tertian malaria, for instance, the γ-globulin level and turnover is many times the normal (Cohen & McGregor 1963) and the 'malarial antibodies', it seems, when passively transferred across the placenta from mother to the newborn are able to confer to infants of the endemic area a passing protection against a parasitaemia. Parasites in the exoerythrocytic tissue stage in the liver are thought to be unaffected and for this and other reasons (see below) relapses occur.

There are presumably two main circumstances which make the allergic reactions ineffective in achieving a solid immunity in most protozoal infections. These are, firstly, the sanctuary achieved by the parasites during their intracellular or tissue stages and secondly, the ability of many of the parasites to change completely the antigenic structure of their delicate plasma membrane. Evidence for this variability in antigenic structure during the course of an infection is seen perhaps most clearly and has been studied to the greatest extent in trypanosome infection. These delicate organisms living in the blood plasma are easily lysed and killed by antibody and complement as may be seen by suitable tests *in vitro* (Lourie & O'Connor 1937; see also Le Page 1968). A parasitaemic infection would be rapidly eliminated were it not that before complete lysis of the whole trypanosome population (A) antigenic distinct variants (B) form and multiply to establish the next parasitaemia before sufficient specific antibody (anti-B) is produced. This latter antibody then eliminates population B, but not before a subsequent variant C arises and so on. In African trypanosomiasis, although the parasites gain access to the tissue spaces there is not thought to be an intracellular form in the mammalian host.

There is now evidence for such sequential antigenic variation in malarial parasites (Brown & Brown 1965) but here the difficulties of achieving effective immunity are further increased by the intracellular habitat of the parasites and again by the existence of the various forms in the complicated life cycle. Even discounting formation of variants, sporozoites, merozoites, trophozoites

and gametocytes probably have different antigenic structure from each other and there may also be insufficient antigenic material of the one vital stage to stimulate sufficient antibody or other form of specific allergic response.

Evidence for an effective participation of allergized cells (type unspecified) where, experimentally, serum antibodies were ineffective is presented by Pierce & Long (1965) in a study of acquired immunity to *Eimeria tenella* in fowls. Birds in which the development of the bursa of Fabricius was suppressed by hormonal treatment and which failed to develop serum antibodies were nevertheless successfully immunized by sporulated oocysts *per os*.

All these complicating factors and the fact that, as yet, it is still impossible to cultivate for any period *in vitro* the infective forms of most of the parasitic protozoa perhaps explain why little advance has, as yet, been made in immunoprophylaxis (see Chapter 46).

However the realization that many of the remaining scourges of the world today are protozoal diseases, and the increasing evidence of the failure of chemotherapy due to drug resistance and again the scientific challenge itself makes the study of immunity to protozoal infections a very important and attractive field of scientific research. Such research requires the fullest knowledge, skills and imagination of the protozoologist, biochemist and immunologist.

As background literature, the reader is referred to the classic book of Taliaferro (1929), the symposium (Garnham, Pierce & Roitt 1963) organized by the British Society for Immunology, a recent World Health Organization Report (1965) on 'Immunology and parasitic diseases' and Chapter 46 of this book.

Helminth Diseases

Although many helminths are to be found in the alimentary canal where they are relatively free from allergic influences, most parasites have a migratory phase travelling through the tissues of the host. The special circumstances to be taken into consideration in helminth infections is not an intracellular abode but rather the structure and size of the migrating form, often armoured like a tank or armadillo, with a thick cuticle against which antibody and complement have insignificant effect. However, even tanks have their vulnerable points and nematode larvae, as already mentioned, have orifices where secretory and excretory products get precipitated by antibody with consequent stunting of growth and curtailment of the life cycle.

A most important immunological finding associated with helminth infection in animals is the regular stimulation of a type of antibody comparable in many ways with the reagin of man. In man, this type of antibody is recognized by its heat-lability and ability to give the weal and flare reaction in primate skin; and in animals by this same lability and ability to give the skin reaction of passive cutaneous anaphylaxis in the homologous species. This antibody has been reported in the rat (Ogilvie 1964; Ogilvie 1967) and in the rabbit (Hogarth-

Scott 1967)—animals which don't easily produce 'skin-sensitizing antibody'. Such antibodies are not easily produced by injecting an extract of the worms, but are produced by infection. Of interest is whether this reagin-like antibody has any special role to play in acquired immunity to helminth infection. As mentioned in an earlier section the disengagement of *Haemonchus contortus* from the stomach wall and expulsion of the worms in the so-called 'self cure' mechanism of sheep (Soulsby & Stewart 1960) may reflect just such a role. Jones & Ogilvie (1967) have investigated the role of such antibodies in immunity in the rat to *Nippostrongylus braziliensis* infection.

The importance of cellular factors in immunity to metazoan parasites has also long been stressed. The significant role of allergized 'lymphoid' cells in infection of guinea-pigs with *Trichostrongylus colubriformis* has recently been shown in passive cellular transfer experiments by Dineen & Wagland (1966).

The important advance achieved by immunizing animals with X-irradiated larvae, which are unable to complete their life cycle in the definitive host, yet which are capable of stimulating an effective immunity is described in Chapter 47.

With helminth infections as with protozoal diseases, major advances in our knowledge of immunity mechanisms and their exploitation can be confidently anticipated in the near future (see World Health Organization Report, 1965).

REFERENCES

ANDERSON E.S. (1965) A rapid screening test for transfer factors in drug sensitive Enterobactériaceae. *Nature (Lond.)* **208,** 1016.

ANDERSON E.S. (1967) Facteurs de Transfert et Resistance aux antibiotiques chez les Entérobactéries. *Ann. Inst. Past.* **112,** 547.

ANDERSON S.G. & HAMILTON J. (1949) The epidemiology of primary herpes simplex infection. *Med. J. Aust.* **1,** 308.

ANGLE (1961) The use of mumps skin test in adults. *J. Amer. med. Ass.* **177,** 650.

BARON S., NASOU J.P., FRIEDMAN R.M., OWEN G.M., LEVY H.B. & BARNETT E.V. (1962) Antibody production by hypogammoglobulinaemic patients. *J. Immunol.* **88,** 443.

BARON S. (1963) Mechanism of recovery from viral infection. *Adv. Virus Res.* **10,** 39.

BERKEN A. & BENACERRAF B. (1966) Properties of antibodies cytophilic for macrophages. *J. exp. Med.* **123,** 119.

BEVERIDGE W.I.B. (1952) Immunity to viruses. A general discussion with special reference to the role of allergy. *Lancet* **ii,** 299.

BEVERIDGE W.I.B. (1963) Acquired immunity: viral infections. In *Modern Trends in Immunology*, p. 130, vol. 1, ed. CRUIKSHANK R. London: Butterworth.

BIOZZI G., STIFFEL C., HALPERN B.N., LE MINOR L. & MORTON D. (1961) Measurement of the opsonic effect of normal and immune sera on the phagocytosis of *Salmonella typhi* by the reticulo-endothelial system. *J. Immunol.* **87,** 296.

BLACK E.L. & MELNICK J.L. (1955) Micro-epidemiology of poliomyelitis and herpes B infection. *J. Immunol.* **74,** 236.

BLANDEN R.V., MACKANESS M.B. & COLLINS F.M. (1966) The mechanism of acquired resistance in mouse typhoid. *J. Exp. Med.* **124,** 585.

BLOOM W.L. & PRIGMORE J.L. (1952) A method for preparation of antibacterial basic proteins of normal tissues. *J. Bact.* **64,** 855–8.

BOYDEN S.V. & SORKIN E. (1960) The adsorption of antigen by spleen cells previously treated with antiserum *in vitro*. *Immunology* **3,** 272.

BOYDEN S.V. & SORKIN E. (1961) The adsorption of antibody and antigen by spleen cells *in vitro*. Some further experiments. *Immunology* **4,** 244.

BOYDEN S.V. (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. exp. Med.* **115,** 453.

BOYDEN S.V. (1964) Cytophilic antibody in guinea-pigs with delayed-type hypersensitivity. *Immunology* **7,** 474.

BRAMBELL F.W. ROGERS (1966) The transmission of immunity from mother to young and the catabolism of immunoglobulins. *Lancet* **ii,** 1087.

BROGAN T.D. (1966) Mechanisms of phagocytosis in human polymorphonuclear leucocytes. *Immunology* **10,** 137.

BROWN K.N. & BROWN I.N. (1965) Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature (Lond.)* **208,** 1286.

BURRIN D.H., KEPPIE J. & SMITH H. (1966) The isolation of phagocytes and lymphocytes from bovine blood and the effect of their extracts on the growth of *Brucella abortus*. *Brit. J. exp. Path.* **47,** 70.

BURROWS T.W. (1955) The basis of virulence for mice of *Pasteurella pestis*. In *Mechanisms of Microbial Pathogenicity*, p. 152. Cambridge University Press.

BURROWS T.W. (1963) The virulence of *Pasteurella pestis* and immunity to plague. *Ergab. Mikrob. Immun. exp. Therap.* **37,** 59.

CAVANAUGH D.C. & RANDALL R. (1959) The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-borne plague. *J. Immunol.* **83,** 348.

COHEN S. & MCGREGOR I.A. (1963) Gammaglobulin and acquired immunity to malaria. In *Immunity to Protozoa*, p. 123, ed. GARNHAM P.C.C., PIERCE A.E. & ROITT I. Oxford: Blackwell Scientific Publications.

COHN Z.A. & HIRSCH J.G. (1965) Phagocytic cells. In *Bacterial and Mycotic Infections of Man*, p. 215. London: Pitman Medical Publishing Co. Ltd.

COHN Z.A. & MORSE S.I. (1960) Functional and metabolic properties of polymorphonuclear leucocytes. II. The influence of lipopolysaccharide endotoxin. *J. exp. Med.* **111,** 689.

COULSON A.S., GURNER B.W. & COOMBS R.R.A. (1967) Macrophage-like properties of some guinea-pig transferred cells. *Int. Arch. Allergy.* **32,** 264.

DAVID J.R. (1966) Delayed hypersensitivity *in vitro*: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc. nat. Acad. Sci., Wash.* **56,** 72.

DAVID J.R., AL-ASKARI S., LAWRENCE H.S. & THOMAS L. (1964) Delayed hypersensitivity *in vitro*. *J. Immunol.* **93,** 264.

DINEEN J.K. & WAGLAND B.M. (1966) The cellular transfer of immunity to *Trichostrongylus colubriformis* in an isogenic strain of guinea-pig. *Immunology*, **11,** 47.

DUBOS R.J. (1954) *Biochemical Determinants of Microbial Diseases*, p. 37. Cambridge: Harvard Univ. Press.

FEINSTEIN A. & PIERCE A.E. (1967) Structure and activity of bovine immune globulins. *Immunology*. In press.

FENNER F. (1948) The epizootic behaviour of mouse-pox (infectious ectromelia). *Brit. J. exp. Path.* **29,** 69.

FENNER F. (1965) Immune mechanisms in viral infections. In *Viral and Rickettsial Infections of Man*, p. 356, ed. HORSFALL F.L. & TAMM I. 4th edition. Philadelphia: J. B. Lippincott.

FITZGEORGE R.B., KEPPIE J. & SMITH H. (1965) The relation between resistance to hydrogen peroxide and virulence in the brucellae. *J. Path. Bact.* **89,** 745.

FITZGEORGE R.B. & SMITH H. (1966) The chemical basis of the virulence of *Brucella abortus* VII. The production *in vitro* of organisms with an enhanced capacity to survive intracellularly in bovine phagocytes. *Brit. J. exp. Path.* **47,** 558.

FITZPATRICK M.E., CONNOLLY R.C., LEA D.J., O'SULLIVAN S.A., AUGUSTIN R. & MACAULEY M.B. (1967) *In vitro* detection of human reagins by double layer leucocyte agglutination: method and controlled blind study. *Immunology* **12,** 1.

FLOREY H.W. (1962) Inflammation. In *General Pathology*, p. 40. Philadelphia: Saunders.

GARNHAM P.C.C., PIERCE A.E. & ROITT I. (1963) *Immunity to Protozoa*, ed. Oxford: Blackwell Scientific Publications.

GERMUTH F.G. (Jr.) (1956) The role of adrenocortical steroids in infection, immunity and hypersensitivity. *Pharmacol. Rev.* **8,** 1.

GINSBERG H.S. (1960) Serum and tissue inhibitors of virus. *Bact. Rev.* **24,** 141.

GRANGER G.A. & WEISER R.S. (1964) Homograft target cells: specific destruction *in vitro* by contact interaction with immune macrophages. *Science* **145,** 1427.

HALLUM J.V., YOUNGNER J.S. & STINEBRING W.R. (1965) Interferon activity associated with high molecular weight proteins in the circulation of mice injected with endotoxin or bacteria. *Virology* **27,** 429.

HIRSCH J.G. (1965) Host resistance to infectious disease. In *Bacterial and Mycotic Infections o Man*, p. 170, ed. DUBOS R.J. & HIRSCH J.G. London: Pitman Medical Publishing Co.

HIRSCH J.G. & CHURCH A.B. (1961) Adrenal steroids and infection: the effect of cortisone administration on polymorphonuclear leukocytic functions and on serum opsonins and bactericidins. *J. clin. Invest.* **40,** 794.

HIRSCH J.G. & STRAUSS B. (1964) Studies on heat-labile opsonin in rabbit serum. *J. Immunol.* **92,** 145.

HOGARTH-SCOTT R.S. (1967) Rabbit reagin-like antibodies. *Int. Arch. Allergy.* **32,** 201.

HOLMES B., QUIE P.G., WINDHORST D.B., POLLARA B. & GOOD R.A. (1966) Protection o phagocytized bacteria from the killing action of antibiotics. *Nature (Lond.)* **210,** 1131

HOWARD J.G. (1959) Activation of the reticulo-endothelial cells of the mouse liver by bacterial lipopolysaccharide. *J. Path. Bact.* **78,** 465.

IYER G.Y.N., ISLAM D.M.F. & QUASTEL J.H. (1961) Biochemical aspects of phagocytosis. *Nature (Lond.)* **192,** 535.

JANCSO M. (1947) Histamine as a physiological activator of the reticulo-endothelial system. *Nature (Lond.)* **160,** 227.

JENKIN C.R. & ROWLEY D. (1963) Basis for immunity to typhoid in mice and the question of 'Cellular Immunity'. *Bact. Rev.* **27,** 391.

JONAS W.E., GURNER B.W., NELSON D.S. & COOMBS R.R.A. (1965) Passive sensitization of cells. Passive sensitization of macrophages by guinea-pig cytophilic antibody. *Int. Arch. Allergy* **28,** 86.

JONES V.E. & OGILVIE B.M. (1967) Reaginic antibodies and immunity to *Nippostrongylus braziliensis* in the rat. Some properties of the antibodies and antigens. *Immunology.* In press.

KEPPIE J. (1964) Host and tissue specificity. In *Microbial Behaviour in vivo and in vitro*, p. 44. Cambridge University Press.

KEPPIE J., HARRIS-SMITH P.W. & SMITH H. (1963) The chemical basis of the virulence of *Bacillus anthracis*. IX. Its aggressins and their mode of action. *Brit. J. exp. Path.* **44,** 446.

KOPROWSKI H. & FERNANDES M.V. (1962) Auto-sensitization reaction *in vitro*. *J. exp. Med.* **116,** 467.

LACEY B.W. (1961) Non-genetic variation of surface antigens in Bordetella and other micro-organisms. In *Microbial Reaction to Environment*, p. 343. Cambridge University Press.

LE PAGE R.W.F. (1968) Immune lysis of trypanosomes: description of the method and its use for the detection of antigenic variation in *T. congolense*. *Ann. trop. Med. Parasit.* In press.

LIPTON M.M. & STEIGMAN A.J. (1963) Human coproantibody against polioviruses. *J. infect. Dis.* **112**, 57.

LOCKWOOD W.R. & ALLISON F. (1966) Electron microscopy of phagocytic cells. III. Morphological findings related to adhesive properties of human and rabbit granulocytes. *Brit. J. exp. Path.* **47**, 158.

LOURIE E.M. & O'CONNOR R.S. (1937) Trypanolysis *in vitro* by mouse immune serum. *Ann. Trop. Med. Parasit.* **30**, 365.

MCCONNEL I. & COOMBS R.R.A. (1968) To be published.

MACKANESS G.B. (1960) The phagocytosis and inactivation of staphylococci by macrophages of normal rabbits. *J. exp. Med.* **112**, 35.

MACKANESS G.B. (1964a) The behaviour of microbial parasites in relation to phagocytic cells *in vitro* and *in vivo*. In *Microbial Behaviour in vivo and in vitro*, p. 213. Cambridge University Press.

MACKANESS G.B. (1964b) Thei mmunological basis of acquired cellular resistance. *J. exp. Med.* **120**, 105.

MACLEOD C.M. (1953) Relation of the incubation period and the secondary immune response to lasting immunity to infectious diseases. *J. Immunol.* **70**, 421.

MACLEOD C.M. & BERNHEIMER A.W. (1965) Pathogenic properties of bacteria. In *Bacterial and Mycotic Infections of Man*, p. 146. London: Pitman Medical Publishing Co.

MEYNELL G.G. (1961) Phenotypic variation and bacterial infection. In *Microbial Reaction to Environment*, p. 174. Cambridge University Press.

MIDDLEBROOK G. (1965) The Mycobacteria. In *Bacterial and Mycotic Infections of Man*, p. 490. London: Pitman Medical Publishing Co.

MILES A.A. (1955) The meaning of pathogenicity. In *Mechanisms of Microbial Pathogenicity*, p. 1. Cambridge University Press.

MILES A.A., MILES E.M. & BURKE J. (1957) The value and duration of defence reactions of the skin to the primary lodgement of bacteria. *Brit. J. exp. Path.* **38**, 79.

MORSE S.I. (1965) Staphylococci and other micrococci. In *Bacterial and Mycotic Infections of Man*, pp. 412–39.

MÜLLER-EBERHARD H.J. (1965) The role of antibody complement and other humoral factors in host resistance in infection. In *Bacterial and Mycotic Infections of Man*, pp. 181, 200, ed. DUBOS R.J. & HIRSCH J.G. London: Pitman Medical Publishing Co.

MUSCHEL L.H. (1963) Activity of the antibody-complement system and lysozyme against gram-negative organisms. *Proc. Fedn. Amer. Socs. exp. Biol.* **22**, 673.

MYRVIK Q.N. & LEAKE E.S. (1960) Studies on antibacterial factors in mammalian tissues and fluids. IV. Demonstration of two non-dialyzable components in the serum bactericidin system for *Bacillus subtilis*. *J. Immunol.* **84**, 247.

NAFF, G., MYRVIK Q.N., HENELT E. & SOTO-FIGUEROA E. (1958) Studies on antibacterial factors in mammalian tissue and fluids. III. Serum bactericidin levels for *Bacillus subtilis* in amyocardial infarction, pneumonia, and other diseases of man. *J. Immunol.* **81**, 123–5.

NELSON R.A. (1962) Mechanisms of cell and tissue damage produced by immune reactions. *2nd International Symposium of Immune Pathology*, p. 245, ed. GRABAR P. & MIESCHER P. Basel: Benno Schwabe & Co.

NOTA N.A., LIACOPOULOS-BRIOT M., STIFFEL C. & BIOZZI G. (1964) Immuno-cyto-adhérence: une methode simple et quantitative pour l'étude *in vitro* des cellules productrices d'anticorps. *C. R. Acad. Sci. Paris* **259,** 1277.

OGILVIE B.M. (1964) Reagin-like antibodies in animals immune to helminth parasites. *Nature (Lond.)* **204,** 91.

OGILVIE B.M. (1967) Reagin-like antibodies in rats infected with the nematode parasite *Nippostrongylus braziliensis*. *Immunology*. In press.

PAVILLARD E.R.J. & ROWLEY D. (1962) A comparison of the phagocytic and bactericidal ability of guinea-pig alveolar and mouse peritoneal macrophages. *Aust. J. exp. Biol. med. Sci.* **40,** 207.

PAZ R.A. & SPECTOR W.G. (1962) The mononuclear-cell response to injury. *J. Path. Bact.* **84,** 85.

PETTERSON A. (1926) Über die warmebestandigen keimtotenden Substanzen, die beta-Lysine der Tiersera und die von diesen beeinflussten Batktesien. *Z. Immun. Forsch. exp. Ther.* **48,** 233.

PIERCE A.E. & FEINSTEIN A. (1965) Biophysical and immunological studies on bovine immune globulins. *Immunology* **8,** 106.

PIERCE A.E. & LONG P.L. (1965) Studies on acquired immunity to coccidiosis bursaless and thymectomized fowls. *Immunology* **9,** 427.

POLLITZER R. (1954) *Plague, Chronicle World Health Organization, Monograph No.* 22, p. 137. Palais des Nations, Geneva.

ROITT I.M., JONES H.E.H. & DONIACH D. (1962) Mechanisms of cell and tissue damage produced by immune reactions. In *2nd International Symposium of Immunopathology*, p. 174. Eds. GRABAR P. & MIESCHER, P. Basle: Benno Schwabe & Co.

ROSENAU W. & MOON H.D. (1961) Lysis of homologous cells by sensitized lymphocytes in tissue culture. *J. Nat. Cancer Inst.* **27,** 471.

ROSENAU W. & MOON H.D. (1962) The inhibitory effect of hydrocortisone in lysis of homologous cells by lymphocytes *in vitro*. *J. Immunol.* **89,** 422.

ROUX J. (1962) Les vaccinations contre les brucelloses: immunisation au moyen de fractions antigéniques. *Ann. Inst. Pasteur* **102,** 801.

ROWLEY D. (1962) 'Phagocytosis' *Adv. Immunol.* **2,** 241.

ROWLEY D., TURNER K.J. & JENKIN C.R. (1964) The basis of immunity to mouse typhoid. 3. Cell bound antibody. *Aust. J. exp. Biol. Med. Sci.* **42,** 237.

ROWLEY D. (1966) Phagocytosis and immunity. *Experientia.* **22,** 1.

SCHILD H.O. & WILLOUGHBY D.A. (1967) Possible pharmacological mediators of delayed hypersensitivity. *Brit. Med. Bull.* **23,** 46.

SERGENT E. (1963) Latent infection and premunition. Some definitions of microbiology and immunology. In *Immunity to Protozoa*, p. 39, ed. GARNHAM P.C.C., PIERCE A.E. & ROITT I. Oxford: Blackwell Scientific Publications.

SHAYEGANI M.G. & MUDD S. (1966) Role of serum in the intra-cellular killing of staphylococci in rabbit monocytes. *J. Bact.* **91,** 1393.

SKARNES R.C. & WATSON D.W. (1957) Antimicrobial factors of normal tissues and fluids. *Bact. Rev.* **21,** 273.

SMITH H. (1958) The use of bacteria grown *in vivo* for studies on the basis of their pathogenicity. *Ann. Rev. Microbiol.* **12,** 77.

SMITH H. (1960) Studies on organisms grown *in vivo* to reveal the bases of microbial pathogenicity. *Ann. N.Y. Acad. Sci.* **88,** 1213.

SMITH H. & FITZGEORGE R.B. (1964a) The chemical basis of the virulence of *Brucella abortus*. V. The bases of intracellular growth in bovine phagocytes. *Brit. J. exp. Path.* **45,** 174.

SMITH H. & FITZGEORGE R.B. (1964b) The effect of opsonization with antiserum on the survival of *Brucella abortus* within bovine phagocytes. *Brit. J. exp. Path.* **45,** 672.

SMITH H., HARRIS-SMITH P.W. & FITZGEORGE R. (1962) Unpublished observations.

SMITH H., KEPPIE J., PEARCE J.H. & WITT K. (1962) The chemical basis of the virulence of *Brucella abortus*. IV. Immunogenic products from *Brucella abortus* grown *in vivo* and *in vitro*. *Brit. J. exp. Path.* **43,** 538.

SOULSBY E.J.L. & STEWART D.F. (1960) Serological studies of the self cure reaction in sheep infected with *Haemonchus contortus*. *Aust. J. Agric. Res.* **11,** 595.

SPECTOR W.G. & WILLOUGHBY D.A. (1963) The inflammatory response. *Bact. Rev.* **27,** 117–54.

STETSON C.A. (1964) Role of hypersensitivity in reactions to endotoxin. In *Bacterial Endotoxins*, p. 658, ed. LANDY M. & BRAUN W. Inst. Microbial. Rutgers State Univ.

STINEBRING W.R. (1962) Characteristics of intracellularly grown *Brucella abortus*. *J. infect. Dis.* **111,** 17.

SUTER E. & RAMSEIER H. (1964) Cellular reactions in infection. *Adv. Immunol.* **4,** 117.

TALIAFERRO W.H. (1929) *The Immunology of Parasitic Infections*. New York, The Century Co.

TALIAFERRO W.H. & SARLES M.P. (1939) The cellular reactions in the skin, lung and intestine of normal and immune rats after infection with *Nippostrongylus muris*. *J. infect. Dis.* **64,** 157.

THORPE B.D. & MARCUS S. (1964) Phagocytosis and intracellular fate of *Pasteurella tularensis*. *J. Immunol.* **92,** 657.

WARD P.A., COCHRANE C.G. & MÜLLER-EBERHARD H.J. (1966) Further studies on the chemotactic factor of complement and its formation *in vivo*. *Immunology* **11,** 141.

WARDLAW A.C. (1962) The complement-dependent bacteriolytic activity of normal human serum. *J. exp. Med.* **115,** 1231.

WILSON G.S. & MILES A.A. (1964) *Principles of Bacteriology and Immunity*, p. 1261, ed. TOPLEY & WILSON, 5th edition. London: Edward Arnold & Co.

WOOD W.B. (1960) Phagocytosis, with particular reference to encapsulated bacteria. *Bact. Rev.* **24,** 41.

World Health Organization (1965) Immunology and parasitic diseases. *Wld. Hlth. Org. Techn. Rep. Ser. No.* 315.

World Health Organization (1966) The use of human immunoglobulin. *Wld. Hlth. Org. Techn. Rep. Ser. No.* 327.

ZAALBERG O.B., VAN DER MEUL V.A. & VAN TWISK J.M. (1966) Antibody production by single spleen cells: a comparative study of the cluster and agar-plaque formation. *Nature (Lond.)* **210,** 544.

ZINSSER H. (1921) On the essential identity of the antibodies. *J. Immunol.* **6,** 289.

CHAPTER 16

PROTECTIVE IMMUNITY

G. PAYLING WRIGHT*

INTRODUCTION

Etymologically, the word 'immune' is derived from the Latin legal term '*immunis*', meaning 'not liable for duty', and applied in later Roman times to a group of privileged young men protected by their high social class from the obligation of undertaking the ordinary military service of the state. To this extent, therefore, the expression 'protective immunity' would savour of tautology, and in modern usage, the term immune still carries the connotation of protection, as in the many prophylactic immunization measures involving the use of antigenic material that are now widely employed in the control of certain epidemic diseases. Analysis of the immune condition, however, has made it apparent that a state of protection may not be the only result of exposing the tissues to an antigen, and that 'immunization' must be regarded from a broader point of view: the process involves, as von Pirquet maintained fifty years ago,

* Reprinted by kind permission of his wife, Dr Helen Payling Wright.

the creation of a highly specific altered reactivity, or 'allergy' of the tissues of the host to the antigen concerned (see Appendix).

In the practical study of the possible bearing of allergy upon protection, a distinction must be drawn between the simpler reactions of the exposed tissues to a single antigenic substance, and those of more complex kind that follow an infection with a living organism that possesses an elaborate antigenic structure and is, moreover, capable under certain circumstances of invading and multiplying in the tissues of the host.

Much of our knowledge of allergic reactions has come from the study of the results of the injection of specific, and often highly purified, antigenic proteins and even under these more narrowly defined conditions, two very different types of allergic reaction may develop. In the first, well exemplified by the inoculation of the toxoids of diphtheria and tetanus, specifically modified globulins—antitoxins—are formed in the tissues which when liberated into the circulation are capable of combining promptly with, and neutralizing the injurious properties of, the respective toxins. In such instances, the creation of a state of allergy results in protection. But under other circumstances, the formation of an antibody to an equally well-defined protein antigen may be a source of grave danger, and the state of allergy be precisely the reverse of protective. In these instances, the antigenic stimulus has led to a state of potentially anaphylactic hypersensitivity (Type I reaction) to the particular protein used. In man, an example of this condition is met with occasionally in persons who have been temporarily protected passively against diphtheria or tetanus by the injection of a specific antitoxic horse serum, and who later fall victim to anaphylactic shock when they are injected for a second time, months, or even years, afterwards.

In infections, the relationship of allergic reactions to protective immunity is rendered much more difficult of analysis by reason of the multiplicity and varying toxicity of the antigenic substances that are present in living micro-organisms. This complexity can be illustrated by reference to the *Salmonella* group of bacteria. Some of the antigens in these organisms, the Boivin-type endotoxins, are intrinsically highly toxic, and are probably important in the pathogenesis of the lesions in enteric fever; others, notably those present in the bacterial flagella, although themselves devoid of any toxic properties, are equally antigenic and stimulate the formation of their own distinctive antibodies. It is possible, for instance, to promote the production to a high titre of the antibodies to the flagella of these bacteria by the inoculation of a rough strain of one of these micro-organisms without conferring any appreciable protection on the 'immunized' host should he subsequently become infected with a pathogenic strain of the bacterium. For with the *Salmonellae*, the endotoxic component of the bacillus, against whose injurious action on the host's cells protection is sought, is somatic and not flagellar in situation. Indeed, a purified

preparation of the somatic, Boivin-type, antigen injected into an animal in sublethal doses can confer a high degree of protective immunity to a subsequent infection with a pathogenic strain of the bacterium (Morgan 1945).

Although with the *Salmonellae* it seems likely that some degree of protective immunity will follow closely upon allergy to the somatic antigenic endotoxin, in other kinds of infection it is possible that the state of allergy conferred against the non-toxic surface antigens of the micro-organism may in itself prove beneficial. In such instances, the protection results from the promotion by specific opsonins of prompt phagocytosis of the bacteria and the consequent handicap placed upon their multiplication in the tissues. In pertussis, for instance, it seems likely that in active prophylaxis with vaccines, protection results chiefly from the allergy produced through the formation of agglutinins against the surface antigens of the bacterium, and that the readiness with which these antigens are lost on artificial cultivation has been mainly responsible for the difficulties encountered in developing a satisfactory prophylactic vaccine in this disease (Evans & Perkins 1953).

THE MECHANISMS OF PROTECTIVE IMMUNITY IN ALLERGIC REACTIONS

In the foregoing remarks, several examples have been given of the protective immunity that can be conferred by the presence of circulating antibodies at an appropriately high titre. It is the production of these specific γ-globulins that underlies immunity in a large proportion of human and animal infections. This is well illustrated by the production of virus-neutralizing antibodies after an infection with the viruses of vaccinia (McCarthy, Downie & Bradley 1958) and poliomyelitis (Medical Research Council Report 1961). But although the formation of specific humoral antibodies as an allergic response to some antigens is the most important element in protective immunity against most pathogenic organisms, it is not the only specific mechanism of defence by which the body can protect itself. With some infections, the allergic response seems to act protectively for the host less through the formation of specific circulating humoral antibodies, than through some specific modification in the reactivity of certain of its mesenchymal cells. This form of allergy has long been regarded by many pathologists as the principal defensive mechanism in re-infection in tuberculosis.

PROTECTIVE IMMUNITY AND SPECIFIC CIRCULATING HUMORAL ANTIBODIES

No two diseases of man and animals illustrate better the protective value of circulating humoral antibodies than diphtheria and tetanus—both diseases in

which a highly toxic protein metabolite of the organism appears to be the sole pathogenetic agent. Irrespective of whether the antitoxin is produced actively by the host, in response to the toxin or its toxoid or is introduced passively by an injection of the appropriate hyperimmune animal serum, protection is conferred against the disease. For diphtheria, the early experiments of von Behring and his colleagues on animals soon received notable support from the clinical study on passive antitoxin treatment of children that was carried out by Fibiger in Copenhagen and by Roux and his associates in Paris. The value of passive prophylactic protection against tetanus was first convincingly shown by Nocard (1897) when he reduced the former high mortality of farm animals after castration from this disease by a prior injection of tetanus antitoxin. Unfortunately, the hope that established tetanus might be treated successfully by antitoxin, though having statistical support (Laurence & Webster 1963). has not been wholly fulfilled—a difference between this disease and diphtheria that is probably attributable to the fact that the virtual impermeability of the normal blood-brain barrier to proteins prevents the access of the antitoxin to the site of action of the toxin in the cerebrospinal axis.

In recent years, the belief in the protective value of circulating humoral antibodies as protection against the products of certain pathogenic micro-organisms has been greatly strengthened by observations made on persons suffering from some congenital or acquired inability to synthesize plasma globulins. Most of these studies have been made on infants suffering from hypogammaglobulinaemia, though further supporting knowledge has been gained from adult patients with neoplasms, notably multiple myeloma and chronic lymphatic leukaemia, in whom globulin synthesis is disturbed.

With multiple myeloma, the plasma globulins as a whole are often greatly raised, sometimes to four or five times the normal, but the normal γ component becomes increasingly deficient as the disease progresses, and such patients become unusually susceptible to pyogenic infections (Porges 1956). In chronic lymphatic leukaemia also, the concentration of γ-globulin in the plasma is materially lowered, and the ability of such patients to respond to immunization with various antigens, among them tetanus toxoid, is greatly reduced (Shaw *et al* 1960; Barr & Hamilton-Fairley 1961).

Most of our knowledge of the importance of γ-globulins against infections has come from the study of young children suffering from hypo- or agamma-globulinaemia, a genetically conditioned abnormality of protein synthesis that occurs predominantly in male infants (Squire 1960; see Chapter 20). Immediately after birth, the plasma γ-globulin concentration of the infant is almost the same as that of its mother, but during the ensuing few months it declines more or less logarithmically, and in parallel with the bacteriostatic power of the blood against various *Salmonellae* (Dancis & Kunz 1954), until, in a normal infant, it rises again through its own active synthesis of these pro-

teins. In the congenitally affected infants, no comparable rise takes place, so that during the greater part of childhood the concentration of the plasma γ-globulin is very low—perhaps one-fiftieth of that of a normal child of the same age (Zak & Good 1959).

Children suffering from hypogammaglobulinaemia are known to be very susceptible to certain bacterial infections, notably those caused by pyogenic bacteria. In consequence, they suffer seriously and repeatedly from infectious diseases, particularly from pneumonia, otitis media, sinusitis, pyoderma, meningitis and cystitis, and the childhood mortality of these patients is correspondingly raised as a result (Good, Bridges & Condie 1960). The immunological deficiency of these children is further shown by the fact that inoculation with toxoids and vaccines are of little avail in provoking the formation of specific antibodies, and the general unresponsiveness of the antibody-forming apparatus is further emphasized by the virtual absence of iso-haemagglutinins in the blood of many of these children.

Passive Maternal Immunity: Its Acquisition and Duration

In all mammals studied, the newborn offspring acquires antibodies from its mother, but the routes by which these antibodies are mainly transferred differ notably in different species (Brambell 1958). In man and in certain rodents, their passage takes place wholly before birth. In ruminants, the horse and the pig, the offspring are born without any antibodies in their blood and hardly any γ-globulin in their plasma; both are acquired rapidly after birth by the absorption through the intestinal mucosa of γ-globulins that are present in the colostrum which is ingested during the first few days of extra-uterine life. In other species again, maternal antibodies pass to the young both before and after birth.

In the Rhesus monkey, and hence almost certainly in man whose placental structure is similar, the antibodies are transmitted solely through the placenta, which is of the relatively simple haemochorial type. Formerly, the possibility that some of the maternal antibodies might reach the primate foetus by way of the amniotic fluid had never been excluded, but any such supposition seems no longer likely from experiments on Rhesus monkeys in which isotopically labelled homologous serum proteins were injected into the maternal circulation shortly before parturition (Bangham, Hobbs & Terry 1958). Of all the proteins introduced, only the γ-globulins crossed the placenta and entered the foetal circulation. Moreover, the only protein to be recovered from the amniotic fluid was the labelled albumin. The inference that the placenta and not the amniotic sac provided the route for the passage of antibodies was further strengthened by finding that labelled γ-globulins injected directly into the amniotic fluid failed to reach the blood of the foetus.

Our knowledge of the persistence in the infant of maternally acquired γ-globulins comes mainly from two sources. First, the progressive fall in the concentration of γ-globulins in the blood of hypogammaglobulinaemic infants. In such infants, the half-life of these proteins is about 50 to 60 days—a period that agrees well with that found after an intravenous injection of identifiable homologous γ-globulin either into these or into normal children (Gitlin, Janeway, Apt & Craig 1959). With this rate of turnover, little of the maternal antibody would be left in the circulation of infants aged 6 months or more. The second source of information on the persistence of maternal γ-globulins is their rate of disappearance in an infant whose own γ-globulins belong to a genetically different group from those of the mother (Linnet-Jepsen, Galatius-Jensen & Hauge 1958; Grubb 1959). Among Danish people, over half have γ-globulins of the type Gm(a+), and the rest of the type Gm(a−). At birth, the infant's γ-globulins, being wholly derived from the mother, are of the same type as her own, but if the genotype of the mother and infant differ, the genotypical γ-globulin that is distinctive for the infant begins to appear in its blood in the course of a few months and gradually replaces that of the different type that was acquired during intra-uterine life.

These estimates of the duration of persistence of maternally acquired γ-globulins in the infant agree reasonably with observations made many years ago on the progressive disappearance of diphtheria antitoxin that takes place during the first year of life. In a large study undertaken in Vienna, this antitoxin could be identified in the serum of the mother and of the infant at birth, but in the absence of any overt specific stimulus, it was no longer recognizable in the great majority of the latter 12 months later (Von Gröer & Kassowitz 1919).

The Acquisition of Active Immunity in Childhood

While the decline in the concentration of γ-globulin in the blood of a hypogammaglobulinaemic infant born to a normal mother provides an indication of the rate of loss of the transplacentally acquired antibodies, so the rise in the concentration of this type of protein in the normal offspring of a hypogammaglobulinaemic mother gives some idea of the age at which an infant becomes capable of synthesizing its own γ-globulins. In two infants, born to such an affected mother, that were studied by Zak & Good (1959), γ-globulins began to appear in the child's blood at about the end of the 2nd month, and by 9 months, the concentration of these proteins in their plasma was about the same as that of normal infants born to normal mothers.

How far this early post-natal synthesis of γ-globulins is a normal process of biochemical development and how far it depends upon the impact of the wide

variety of antigens to which the infant becomes exposed soon after birth can be explored by the study of the plasma proteins and antibodies of animals maintained for months in germ-free environments.

When newborn rats are maintained in a germ-free colony and weaned at about 3 weeks, their serum proteins develop normally except for their β- and γ-globulins, the concentrations of both of which remain very much below those found for these proteins in the serum of normal rats of their age kept under ordinary conditions (Gustafsson & Laurell 1959). This state of hypogammaglobulinaemia persists, but if after they have reached maturity, these rats are removed from their germ-free environment and exposed to the microbiological flora usual for this species, γ-globulins begin to appear in their blood after 3 or 4 weeks, at first slowly but then more rapidly until eventually they attain the same concentration as those of the control animals (Gustafsson & Laurell 1959; Wostmann & Gordon 1960). Furthermore, there is evidence that this rise in the concentration of the γ-globulins in the previously germ-free animals parallels the appearance of circulating antibodies to some of the mixed microbial flora to which they have become exposed (Wagner 1959). In view of the supposed formation of γ-globulins by cells of the lymphoreticular system (see Chapter 12), it is of interest that as long as the animals are germ-free, this system remains relatively underdeveloped, but when the animals are exposed to normal colony conditions, the lymphoreticular system undergoes hyperplasia (Gordon & Wostmann 1960).

The results of these experiments on rats, which are supported by others on other species of small laboratory animals, show that the synthesis of plasma γ-globulins takes place as a result of the stimulation of the antibody-forming apparatus of the newborn animal by specific antigens that are present in the wide diversity of micro-organisms with which it ordinarily comes quickly into contact. Even when maintained under sterile conditions, some stimulation of the lymphoreticular system may take place from antigenic material present in the food and absorbed unchanged through the intestinal mucosa, and this may be responsible for the synthesis of the small quantity of γ-globulin found in the serum of the germ-free animals.

Attention was drawn over 40 years ago by Nicolle & Lebailly (1919), during their study of rickettsial infections in North Africa, to a condition to which they gave the name '*infections inapparantes*'. Since then, the combined studies of epidemiologists and immunologists on human communities have made it abundantly clear that in several important infectious diseases (Reimann 1960), subclinical attacks of '*infections inapparantes*' may much outnumber in their frequency those attacks that are diagnosable clinically. Today, as the study of the concentration of immuno-conglutinin in the sera of healthy persons has shown (Marks & Coombs 1957), there seems no doubt that in urban populations particularly much herd immunity is acquired unknowingly through these

unrecognized infections, particularly when social conditions are poor and hygiene is correspondingly defective.

Two diseases, diphtheria and poliomyelitis, illustrate well the epidemiological importance of the subclinical attack as an immunizing experience. In the inter-war period, before the introduction of a national scheme for the active prophylactic immunization of infants, an average of nearly ten thousand cases of clinical diphtheria were notified annually among children under 15 years in the London Metropolitan Boroughs. About the same period, bacteriological studies showed that between 1 and 2 per cent of this child population were carriers of virulent diphtheria bacilli; from what is known of the duration of the carrier state in this disease, it can confidently be assumed that over a hundred thousand of these children would become infected with diphtheria bacilli in the course of a year. This estimate agrees well with one made from the known Schick reactions of the million London children who were then at risk: about 13% of these children became Schick-negative annually. From these statistics, the inference is warranted that well over ten children become infected with virulent diphtheria bacilli—and consequently become Schick-negative—for every child that develops a clinical attack of the disease (Payling Wright & Payling Wright 1942). This provides a measure of the '*infections inapparantes*' for diphtheria that prevailed in this large urban community 30 years ago.

The introduction of tissue culture methods for the isolation of poliomyelitis virus, together with the opportunity that this technique affords for the detection and titration of specific virus-neutralizing antibodies in the serum of exposed persons, has revolutionized our ideas on the epidemiology of this disease. Instead of the infection being apparently sporadic in a community, as was formerly inferred from the occurrence of the occasional paralytic case, it is now known that poliomyelitis viruses spread in a population with great rapidity during epidemics, probably as quickly as that of measles, to judge from the outbreaks of the two diseases in Tahiti in 1951 (Rosen & Thooris 1953), and that the clinical manifestation of poliomyelitis attracts relatively little attention in the majority of persons who become infected. Even with the attenuated variants that are included in Sabin's oral vaccine, the virus strains employed can frequently be recovered from the stools of many of the contacts of the persons to whom the vaccine was originally given (Lennartz & Valenciano 1961; Medical Research Council Report 1961).

The evidence on which the belief in the great frequency of inapparent infections in poliomyelitis is based has come from two sources. First, the virus of the prevailing epidemic type can often be recovered from the stools of persons who never develop the disease in a clinically recognizable form although in close contact with an overt case. In the United Kingdom recently in a collected series of seventy-eight cases of paralytic poliomyelitis, due to virus

strains of all three types, virus was recovered from the stools of about a quarter of the 241 home contacts of all ages, and over a half of those aged 0–4 years (Public Health Laboratory Service, 1958). Comparable estimates for inapparent infections in this disease were obtained in a similar study in Ohio (Horstmann, McCollum & Mascola 1955).

Second, much information has been obtained from the distribution of poliomyelitis virus-neutralizing antibodies in specimens of serum taken at random from the general population of the locality under investigation. Estimates of the frequency and distribution of inapparent infections made from such serological studies are much more instructive than those derived from the recovery of the virus from the stools, for the presence of the virus in the intestines is very transitory, while the antibodies excited by the infection persist at a readily recognizable level for years. In South Africa, in the Bantu people, among whom paralytic poliomyelitis is uncommon, about three-quarters of the infants were found to possess antibodies to the Lansing strain of the virus at birth. This proportion fell to a minimum of about one-third by the age of 2 years, and rose steadily afterwards until specific antibodies were present in the serum of nearly all children between 8 and 10 years (Gear 1955). Very similar figures for the changes in antibody with age for this strain of virus were recorded for a group of mainly Negro children in Baltimore (Turner, Hollander, Buckley, Kokko & Winsor 1950). The epidemiology of poliomyelitis thus bears a similarity to that of diphtheria: it is essentially that of a widespread disease in which relatively few persons who are infected display conspicuous clinical manifestations, and the great bulk of the community acquire their protective immunity through inapparent infections.

That insusceptibility towards the so-called childhood infectious diseases is not dependent upon some change in bodily metabolism that is the result of ageing, but upon a highly specific process of immunization is shown by the epidemics described in certain island populations when, after an interval of many years, the inhibitants become again exposed to infection. Among the numerous records of epidemics that spread in uncontrollable fashion in virgin communities, that described by Panum (1847) for measles in the Faroe Islands in the middle of the last century is among the most notable. When measles was again introduced into the Islands from Copenhagen in 1846 about three-quarters of the population contracted the disease, and few escaped who had not suffered from measles, at the previous outbreak in 1781, 65 years before. As Panum pointed out, old persons 70 or 80 years of age, who had not had measles in the former epidemic, acquired the disease as readily as a child of 7 or 8. Infants, too, were inordinately susceptible, since they had acquired no passive protection from their mothers during intra-uterine life. A comparable epidemic of measles, again introduced from Copenhagen, took place in South Greenland in 1951, in which in 3 months over four thousand persons of all ages—

practically the entire population that had become exposed—contracted the disease (Christensen, Schmidt, Jensen, Bang, Andersen & Jordal 1953).

Loss of Actively Acquired Immunological Protection

Once the antigenic stimulus has ceased to act upon the antibody-forming tissues, the amount of the specific antibody in the blood gradually declines. Some permanent change, however, has taken place in the cells concerned in antibody production, for they remain thereafter highly sensitive to the particular antigen, and respond with promptitude and vigour should they again become exposed to it: this is the well-known 'secondary response', on the basis of which much of our active immunological prophylaxis depends. It is well illustrated in diphtheria, which has been much studied from this standpoint. In this disease, a type of individual has been recognized to whom the name 'Schick-positive-immune' has been given. In these people, the level of the circulating antitoxin has fallen to too low a level to effect the neutralization of the small amount of the toxin that is injected in the Schick test—hence they are Schick-positive. Yet since their antibody-forming tissues have been sensitized previously, they respond so quickly by the formation of antitoxin that should the person become infected with a toxigenic strain of the organism, the intoxication is so rapidly and effectively countered that no clinical manifestations appear.

A remarkable aspect of the epidemiology of measles in London in recent times has been the regular biennial nature of the outbreaks. The factors that may be responsible for this distinctive feature of the disease have been widely discussed, and Stocks's (1930) theory of 'latent epidemization' has been much supported. From his analysis of the differing liabilities to measles of those apparently unaffected children in families in which a case of the disease had, or had not, occurred, Stocks concluded that some degree of immunity had been acquired by the exposure. This immunity was only temporary, lasting for little over a year, so that every second year a large fraction of the child population would again become susceptible and an epidemic develop in consequence.

Apart entirely from the natural decay in specific immunity with time, such as is known to occur in many infectious diseases, protection of a form that was originally immunological may deteriorate as a result of adverse social conditions, and notably of malnutrition. Many of our ideas of the interrelation of nutrition and resistance to infection have come from the common association of epidemics and famines. It is too often overlooked that in times of famine, the social upheaval can affect many factors that can contribute to the spread of an infectious disease, for example, the dispersal of people in search of food in tropical countries, or the concentration of persons seeking warmth in overcrowded houses in less temperate climates. The former can be instanced by cholera in

India and the latter by the rise in respiratory tuberculosis in European countries in the recent wars.

Very few studies have been made on the immunizability of a seriously undernourished population. In 1946, in the Wuppertal district of the Ruhr, Gell (1951) made some observations on the immunity responses of some sixty gravely undernourished persons to three mild antigens with any of which it was unlikely that they had had previous contact. With two of these antigens, tobacco mosaic virus and fowl's red cells, he was able to obtain responses that, though smaller, were not much smaller than those he found in his well-nourished control subjects. It seems from these observations, that in human beings who may literally be famished, the antibody-forming system is maintained with little deterioration in its efficiency. Under conditions of widespread famine, it seems likely that epidemics of highly infectious diseases are more likely to be promoted by the dispersal of the causative micro-organism through the movements of infected persons in search of food, than by any marked loss of herd immunity in the stricken population. Famines provoke social disturbances of great complexity, of which undernutrition is only one component.

PROTECTIVE IMMUNITY AND ALLERGIC TISSUE REACTIONS

In no major disease in occidental countries has the relationship between immunity, expressed as an allergy, and specific protection proved more controversial than in tuberculosis. That a re-infected individual responds differently, and more energetically, to the tubercle bacillus and to some of its products, has been known ever since Koch described his classical 'phenomenon' in 1891. Whether such an accelerated, and at first sight a more severe, tissue reaction proves in any way beneficial to the reinfected individual is crucial to the point at issue. Whether the acute destructive inflammatory reaction that appears at the site of re-inoculation is to be regarded as harmful or not depends upon whether the criterion accepted by the observer is to be the gravity of the local tissue damage or the likelihood of the survival of the invidual.

Evidence for Specific Immunity of a Protective Nature in Tuberculosis

Ever since the pioneer studies of Naegeli in the General Hospital at Zurich about the end of the last century, it has been apparent that in western Europe tuberculosis is an even more widespread disease than was formerly supposed, and that the number of persons, even in ordinarily prosperous communities, who contract tuberculosis greatly exceeds the number whose cause of death can be specifically attributed to it. It was these and other similar observations in necropsy rooms elsewhere in Europe and America that led to the expression

'Jedermann hat ein Bisschen Tuberkulose am Ende'. From such findings as emerged from many post morten rooms of general hospitals, the inference seemed clear that once a person had recovered from a minor, and often subclinical, attack of tuberculosis, he had acquired a degree of immunity which would later give him some protection in a world in which he would often be thrown into contact with tuberculous persons. The subsequent introduction of the tuberculin test as a means of epidemiological analysis soon confirmed the conclusion drawn from morbid anatomical findings.

The first convincing study, the forerunner of many similar ones since, that demonstrated the protective value of some prior tuberculous infection was that carried out over many years by Heimbeck (1936) in Oslo. He kept records of the after-histories of the probationer nurses at the Ulleval Hospital with special reference to the comparative likelihoods of the development of tuberculosis in those who on admission were tuberculin positive or negative respectively. His findings are summarized in Table 16.1. The widely different morbidities for respiratory tuberculosis—6.4 and 46.4 per thousand—in Heimbeck's two groups attracted wide attention, and led shortly to many comparable studies elsewhere. Of these, the largest was the Prophit Survey (1948) carried out before the last world war on nurses and medical students in London. The findings in this survey substantially supported Heimbeck's conclusion that when young persons are placed in circumstances, such as hospital wards and outpatients, in which they are likely to be exposed to open cases of respiratory tuberculosis, a previous infection that has been successfully resisted is succeeded by a decreased susceptibility to subsequent re-infection.

TABLE 16.1

Morbidities from Tuberculosis

Tuberculin test on admission	Number of nurses	Tuberculous morbidity	
		All forms	Respiratory
Positive	625	27	4
Negative	280	96	13

Morbidities from tuberculosis of nurses in the Ulleval Hospital, Oslo, between 1924 and 1935, in relation to their reaction to tuberculin on entry (Heimbeck 1936).

More convincing evidence that some measure of specific protection in tuberculosis is associated with the development of allergy comes from the findings of two small, intensive studies on population groups which had been actively

immunized with attenuated tubercle bacilli (BCG). The first was made by Aronson (1958) upon eight North American tribes in Alaska. Two large groups, each mainly composed of adolescents, were inoculated, one with BCG and the other with saline. The medical histories of the two groups, followed with great care

TABLE 16.2

Mortalities from Tuberculosis and Other Diseases

Group	Number followed up	Died during subsequent twenty years	
		All causes except tuberculosis	Tuberculosis
BCG	1547	91	13
Saline	1448	82	68

Mortalities from tuberculosis and other diseases in a BCG-inoculated and a control group of Alaskan Indians between 1935 and 1955 (Aronson, Aronson & Taylor 1958).

at intervals over the subsequent 20 years, are set out in Table 16.2. The mortalities from all causes apart from tuberculosis were the same in both the BCG and the control groups (fifty-nine and fifty-seven per thousand respectively), but that from tuberculosis was nearly six times greater in the unvaccinated persons.

TABLE 16.3

Morbidities from Pulmonary Tuberculosis

Original test	Received BCG	Total number	Number exposed to infected teacher	Developed pulmonary tuberculosis
Positive	no	130	105	4
Negative	yes	133	106	2
Negative	no	105	94	41

Morbidities from pulmonary tuberculosis in three groups of Danish schoolgirls: (i) originally tuberculin positive; (ii) originally tuberculin-negative but rendered positive with BCG and (iii) still tuberculin-negative at the time of the outbreak (Hyge 1947).

The second piece of evidence comes from the records of an epidemic of tuberculosis in a state school for adolescent girls in Denmark in 1943 (Hyge 1947). The girls in this school could be divided into three roughly equal groups: those that had previously become tuberculin positive as a result of chance

infection; those that had been rendered tuberculin positive, *though formerly negative*, by the inoculation of BCG some months before, and those who were tuberculin negative at the time of the epidemic. Owing to the presence of an open case of tuberculosis among the mistresses in the school, a sharp outbreak of tuberculosis developed in the classes that she taught. The results of the epidemic are given in Table 16.3. In this outbreak, the previous administration of attenuated tubercle bacilli to about half the tuberculin-negative girls exposed greatly reduced the proportion who subsequently contracted the disease—tuberculin allergy and protection were thus correlated.

TABLE 16.4

Dissemination of Tubercle Bacilli

Structures examined	Intervals between inoculation and excision							
	1 hr	3 hr	6 hr	10 hr	1 day	4 days	7 days	10 days
	Control guinea-pigs							
Viscera	+	−	+	+	+	+	+	+
Lymph nodes	+	−	+	+	+	+	+	+
	Re-infected guinea-pigs							
Viscera	−	−	−	−	−	−	+	−
Lymph nodes	−	−	−	−	−	−	+	−

Effect of time of extirpation of the tissues at the site of inoculation upon the dissemination of tubercle bacilli in control and re-infected guinea-pigs (Willis 1925).

THE MECHANISM UNDERLYING PROTECTION IN TISSUE ALLERGY

The accelerated inflammatory reaction that follows a local re-infection with tubercle bacilli has been regarded by many pathologists as a possible protective mechanism ever since Koch described his 'phenomenon'. The possibility was put to the test by Willis (1925) nearly 40 years ago, and his results still seem to have undiminished validity. He compared the relative rapidity of dispersal of tubercle bacilli from an inoculation site in the flank of normal guinea-pigs and of guinea-pigs that had been infected previously with a relatively avirulent strain of the tubercle bacillus and had acquired allergy in consequence. The test for the rapidity of dispersal was undertaken by excising the infected tissues round the site of inoculation at various intervals after inoculation and examining the viscera and regional lymph nodes at necropsy many weeks later to detect the presence of absence of metastatic tuberculosis foci. The results of Willis's experiments, which have been confirmed in experiments carried out by other investigators subsequently, are shown in Table 16.4. Willis's observations showed

that in re-infected guinea-pigs, the dispersal of tubercle bacilli from a site of inoculation is much delayed. Although this delay was less evident when similar experiments were undertaken on rabbits, there is general agreement that in re-infected allergic animals the bacilli become fixed near the site of inoculation (Freund & Angevine 1938). The explanation of this fixation is obscure, and may depend upon vascular and other changes in the local tissues that take place much more rapidly than has formerly been supposed. In guinea-pigs it is known that a fibrin barrier soon forms round the acutely inflamed area (Krause 1926) but while this may be a contributory element in the protective mechanism in this species, the concept cannot be generalized since the same barrier does not develop in rabbits under comparable conditions (Lurie 1950).

Specific Antibodies in Tuberculosis

Numerous studies have convinced many disappointed investigators that specific antibody formation is not a conspicuous feature of tuberculosis. This comparative anergy may be partly attributable to the relatively slight antigenic stimulus provided by the bacilli or to the long clinical course of the infection when compared with the metabolic turnover rates of any specific antibody globulins. But although serum from animals that are either tuberculous, or have been inoculated with dead whole bacilli, has no bactericidal action for tubercle bacilli, it does contain, although only at a low titre (Raffel 1955) specific antibodies that can modify the properties of their surface. The union of such antibodes to the tubercle bacilli has been shown by the altered behaviour of the organisms in an electrophoretic cell and by their enhanced ingestion when they are exposed to phagocytic cells (McCutcheon *et al* 1929).

In an interacting system as complex as that which develops in tuberculous infections it is manifestly unlikely that protection will be correlated with any single variable among the multiplicity of host responses. None the less although neither bactericidal, nor even growth-inhibitory, themselves, it seems likely that the antibodies formed in response to an infection can lead to some degree of protection against bacilli that are free in the circulating fluids, possibly by opsonizing their surface and thus promoting their ingestion by phagocytes.

Mesenchymal Cellular Reactions in Tuberculosis

There is little doubt that protection in tuberculosis depends mainly upon the ability of mesenchymal cells to destroy the bacilli, and that this capacity is materially raised should the animal be re-infected. Lurie (1933) demonstrated this modification in the reaction of the host when he injected living tubercle bacilli intravenously into control and re-infected rabbits. By sampling tissue aliquots at intervals afterwards, he was able to determine the numbers of bacilli that had survived in the substance of the liver, spleen, kidneys and lungs of these animals. Almost invariably, he was able to recover a much smaller number of

bacilli from the organs of the re-infected animals than from those of the controls. Indeed, when the primary infection was still active, though restricted to the site of initial inoculation, the destruction of the bacilli in the four organs he sampled was complete. This accelerated ingestion and intracellular destruction of the circulating tubercle bacilli might well depend upon the presence of opsonizing antibodies in the serum, even though their titre might be low by ordinary serological standards.

Histological examination of the organs of the control and re-infected rabbits showed that the destruction of the circulating bacilli was mainly accomplished by macrophages, which in the animals of the latter group were mobilized more quickly round the sites of lodgement of the bacilli in the tissues. Since tubercle bacilli may sometimes survive ingestion by phagocytic cells, the possibility has been raised on many occasions that in addition to inducing the formation of humoral antibodies, the development of tuberculous allergy may lead to some intracellular change in the macrophages that renders their cytoplasm inhibitory to the growth of, or even bactericidal for, any ingested tubercle bacilli. At present this problem is unsolved, and the relevant literature is controversial (Suter 1954; Mackaness 1954). It is possible, however, as Raffel (1955) has suggested, that any such change in a particular macrophage may occur only after it has itself ingested one or more tubercle bacilli, thus becoming exposed to tuberculoproteins, and may not be a general indiscriminate transformation of all the macrophages throughout the body under the influence of some circulating humoral factor.

GENERAL OBSERVATIONS ON PROTECTIVE IMMUNITY

The majority of infections that are incurred under natural conditions are of the nature of first infections, and for protection the animal must initially rely wholly on defences of an unspecific kind. Success or failure in the struggle between host and parasite depends very largely on the relative speeds with which the two contestants can bring their offensive or defensive armaments to bear in the mutual struggle. Indeed, as Miles (1955) has emphasized, it is during this early phase that the outcome of the infection is often decided. Any lag phase in the multiplication of the organisms allows a longer period for the mobilization of the host's defences. Among these, the series of local tissue changes that comprise the inflammatory reaction are, for many types of infection, the most effective in combating the organism. If the natural development of this mainly vascular reaction is hindered, as it can be experimentally through inducing prolonged vasoconstriction by a local injection of adrenaline, the usual protective mechanisms of the host may be placed at a disadvantage for a sufficiently long period for the organisms to establish themselves and to overrun any defences that may

subsequently be raised against them. Similarly, should the rate of multiplication of the organisms be retarded, as is often possible through the use of bacteriostatic drugs, the balance may often be swung permanently to the advantage of the host.

Each instance of infection has its own unique character—the distinctive features imposed on its course by a combination of the peculiarities of both host and parasite. Any attempt to generalize from one form of infection to another, therefore, is necessarily hazardous, but if any generalization is justified it would seem to be that the value of the form of specific immunity that is regarded as protective is to be attributed to the acceleration of a wide range of allergic reactions in the mesenchymal tissues of the host, most, if not all, of which have some defensive value. Humoral antibodies are formed more promptly and in greater amount by the previously sensitized tissue, while the cellular reaction evoked, with its usual culmination in phagocytosis, is more vigorous and effective, especially when it is fortified by the action of humoral agents. It is the greater promptitude with which it can react to attack by the parasite that places the allergic animal in a position of advantage and endows its specific immunity with protective value.

REFERENCES

ARONSON J.D., ARONSON C.E. & TAYLOR H.C. (1958) A twenty-year appraisal of BCG vaccination in the control of tuberculosis. *Arch. intern Med.* **101,** 881–93.

BANGHAM D.R., HOBBS K.R. & TERRY R.J. (1958) Selective placental transfer of serum-proteins in the rhesus. *Lancet* **ii,** 351–4.

BARR M. & HAMILTON-FAIRLEY G. (1961) Circulating antibodies in reticuloses. *Lancet* **i,** 1305–10.

BRAMBELL F.W.R. (1958) The passive immunity of the young mammal. *Biol. Rev.* **33,** 488–531.

CHRISTENSEN P.E., SCHMIDT H., JENSEN O., BANG H.O., ANDERSEN V. & JORDAL B. (1953) An epidemic of measles in Southern Greenland, 1951. II. Measles in virgil soil. *Acta med. scand.* **144,** 430.

DANCIS J. & KUNZ H.W. (1954) Studies on the immunology of the newborn infant. VI. Bacteriostatic and complement activity of the serum. *Pediatrics* **13,** 339–45.

EVANS D.G. & PERKINS F.T. (1953) An agglutinin-production test in the study of pertussis vaccines. *J. Path. Bact.* **66,** 479–88.

FREUND J. & ANGEVINE D.M. (1938) The spread of tubercle bacilli in the bodies of sensitized and immunized animals. *J. Immunol.* **35,** 271–88.

GEAR J.H.S. (1955) Poliomyelitis in the underdeveloped areas of the world. In *Poliomyelitis. Wld. Hlth. Org. Monogr. Ser. No.* 26, pp. 31–58.

GELL P.G.H. (1951). In *Studies of Undernutrition, Wuppertal, 1946–9. Spec. Rep. Ser. med. Res. Coun. Lond.*, No. 275, pp. 193–203.

GITLIN D., JANEWAY C.A., APT. L. & CRAIG J.M. (1959) Agamma-globulinaemia. In *Cellular and Humoral Aspects of the Hypersensitive State*, pp. 375–441, ed. H. SHERWOOD LAWRENCE. New York: Hoeber-Harper.

GOOD R.A., BRIDGES R.A. & CONDIE R.N. (1960) Host-parasite relationships in patients with dysproteinaemias. *Bact. Rev.* **24,** 115–32.

GORDON H.A. & WOSTMANN B.S. (1960) Morphological studies on the germ-free albino rat. *Anat. Rec.* **137,** 65–70.

GRÖER F. VON & KASSOWITZ K. (1919) Studien über die normalen diphtherie-immunität des menschen. IV. Die normale diphtherieimmunität in kindesalter. *Z. Immun.-Forsch., Orig.* **28,** 327–67.

GRUBB R. (1959) Hereditary gamma globulin groups in man. In Ciba Foundation Symposium, *Biochemistry of Human Genetics*, pp. 264–73. London: J. & A. Churchill.

GUSTAFSSON B.E. & LAURELL C.G. (1959) Gamma globulin production in germ-free rats after bacterial contamination. *J. exp. Med.* **110,** 675–84.

HEIMBECK J. (1936) Tuberculosis in hospital nurses. *Tubercle* **18,** 97–9.

HORSTMANN D.M., MCCOLLUM R.W. & MASCOLA A.D. (1955) The incidence of infection among contacts of poliomyelitis cases. *J. clin. Invest.* **34,** 1573–80.

HYGE T.V. (1947) An epidemic of tuberculosis in a state school, with an observation period of about three years. *Acta tuber. scand.* **21,** 1–57.

KRAUSE A.K. (1926) Studies on tuberculous infection. XII. The dissemination of tubercle bacilli in the immune guinea-pig, with a discussion of probable factors involved in tuberculo-immunity. *Amer. Rev. Tuberc.* **14,** 211–36.

LAURENCE D.R. & WEBSTER R.A. (1963) Pathologic Physiology, Pharmacology and Therapeutics of Tetanus. *Clin. Pharm. Therap.* **4,** 36–72.

LENNARTZ H. & VALENCIANO L. (1961) Virologische Untersuchungen zur oralen Schutzimpfung gegen Poliomyelitis in Westberlin 1960. *Dtsch. med. Wschr.* **86,** 1497–1503.

LINNET-JEPSEN P., GALATIUS-JENSEN F. & HAUGE M. (1958) On the inheritance of the Gm serum group. *Acta genet. (Basel)* **8,** 164–96.

LURIE M.B. (1933) A correlation between the histological changes and the fate of living tubercle bacilli in the organs of re-infected rabbits. *J. exp. Med.* **57,** 181–201.

LURIE M.B. (1950) Native and acquired resistance to tuberculosis. *Amer. J. Med.* **9,** 591–610.

MCCARTHY K., DOWNE A.W. & BRADLEY W.H. (1958) The antibody response in man following infection with viruses of the pox group. II. Antibody following vaccination. *J. Hyg. (Lond.)* **56,** 466–78.

MCCUTCHEON M., STRUMIA M., MUDD S., MUDD E.B. & LUCKÉ B. (1929) On the mechanism of opsonin and bacteriotropin action: the development and effect of the antibodies found in experimental tuberculosis of rabbits. *J. exp. Med.* **49,** 815–31.

MACKANESS G.B. (1954) The growth of tubercle bacilli in monocytes from normal and vaccinated rabbits. *Amer. Rev. Tuberc.* **69,** 495–504.

MARKS J. & COOMBS R.R.A. (1957) The conglutination phenomenon. XI. Immuno-conglutination in human sera. *J. Hyg. (Lond.)* **55,** 81–90.

MEDICAL RESEARCH COUNCIL (1961) Trial of living attenuated poliovirus vaccine. Report of the Public Health Laboratory Service. *Brit. med. J.* **ii,** 1037–44.

MILES A.A. (1955) Early tissue reactions to tubercle bacilli and their products. In Ciba Foundation Symposium, *Experimental Tuberculosis*, pp. 87–89. London: J. & A. Churchill.

MORGAN H.R. (1945) Active immunization with purified somatic antigens of *Eberthella typhosa, Salmonella paratyphi* and *Salmonella schotmuelleri*. *Amer. J. publ. Hlth.* **35,** 614–20.

NICOLLE C. & LEBAILLY C. (1919) Les infections expérimentalles inapparantes: exemples tirés de l'étude du typhus exanthématique. *C.R. Acad. Sci. (Paris)* **168,** 800–1.

NOCARD E. (1897) Sur la sérothérapie du tétanos chez des animaux; essais de traitement préventif. *Bull. Acad. Med. (Paris)*, 3rd ser. **38,** 109–21.

PANUM P.L. (1847) Observations made during the epidemic of measles on the Faroe Islands in the year 1846. Reprinted in *Medical Classics* (1939) **3**, 802–86.

PORGES R.F. (1956) A method for demonstrating secondary agammaglobulinaemia in patients with multiple myelomatosis using paper electrophoresis. *J. Lab. clin. Med.* **47**, 960–8.

Prophit Tuberculosis Survey (1948) *Tuberculosis in Young Adults*. London: H. K. Lewis & Co. Ltd.

Public Health Laboratory Service (1958) Report on examination of home contacts of poliomyelitis. *Mth. Bull. Minist. Hlth Lab. Serv.* **17**, 58–64.

RAFFEL S. (1955) The mechanism involved in acquired immunity to tuberculosis. In Ciba Foundation Symposium. *Experimental Tuberculosis*, pp. 261–79. London: J. & A. Churchill.

REIMANN H.A. (1960) Spectrums of infectious disease. *Arch. intern. Med.* **105**, 779–815.

ROSEN L. & THOORIS G.C. (1953) Poliomyelitis in French Oceania: epidemiologic observations on outbreak with notes on incidence of paralysis following intramuscular injections. *Amer. J. Hyg.* **57**, 237–52.

SHAW R.K., SZWED C., BOGGS D.R., FAILEY J.L., FREI E., MORRISON E. & UTZ J.P. (1960) Infection and immunity in chronic lymphatic leukaemia. *Arch. intern. Med.* **106**, 467–78.

SQUIRE J.R. (1960) Hypogammaglobulinaemia in Great Britain. *Acta haemat.* (*Basel*) **24**, 99–104.

STOCKS P. (1930) The mechanism of a measles epidemic. *Lancet* **i**, 796–802.

SUTER E. (1954) Correspondence. *Amer. Rev. Tuberc.* **69**, 1060–2.

TURNER T.B., HOLLANDER D.H., BUCKLEY S., KOKKO U.P. & WINSOR C.P. (1950) Age incidence and seasonal development of neutralizing antibodies to Lansing poliomyelitis virus. *Amer. J. Hyg.* **52**, 323–47.

WAGNER M. (1959) Serologic aspects of germ-free life. *Ann. N.Y. Acad. Sci.* **78**, 261–71.

WILLIS H.S. (1925) Studies on tuberculosis infection. X. The early dissemination of tubercle bacilli after intracutaneous inoculation of guinea-pigs at first infection. XI. The early dissemination of tubercle bacilli after intracutaneous inoculation of immune guinea-pigs on reinfection. *Amer. Rev. Tuberc.* **11**, 427–38 and 439–49.

WOSTMANN B.S. & GORDON H.A. (1960) Electrophoretic studies on the serum protein pattern of the germ-free rat and its changes upon exposure to a conventional bacterial flora. *J. Immunol.* **84**, 27–31.

WRIGHT G., PAYLING & WRIGHT H. PAYLING (1942) The influence of social conditions upon diphtheria, measles, tuberculosis and whooping-cough in early childhood in London. *J. Hyg.* (*Camb.*) **42**, 451–73.

ZAK S.J. & GOOD R.A. (1959) Immunochemical studies of human serum gamma-globulins. *J. clin. Invest.* **38**, 579–86.

CHAPTER 17

ALLERGIC RESPONSE AND TRANSPLANTATION

THE HOMOGRAFT REACTION

M.F.A. WOODRUFF

THE HOMOGRAFT REACTION

The fate of an *autograft*, i.e. piece of tissue or a whole organ transplanted from one place to another in the same organism, depends on whether or not it is provided with, or is able to acquire, an adequate supply of nutrient material,

adequate facilities for removal of waste products, and, in a few special cases, appropriate nervous connections. If conditions are favourable in these respects the graft becomes established and survives indefinitely. Under similar conditions a *homograft*, i.e. a piece of tissue or a whole organ transplanted from one individual to another of the same species, behaves initially like an autograft but sooner or later, as a general rule, lymphocytes and plasma cells begin to accumulate in the graft, the circulation (assuming this has become re-established) slows and is finally arrested, the cells of the graft all die, and the graft itself is either cast off or gradually replaced by tissue derived from the recipient.

This local reaction is typically accompanied by enlargement of the regional lymph nodes associated with the appearance of large cells with pale-staining nuclei, and sometimes by systemic manifestations of various kinds such as enlargement of the spleen.

EXCEPTIONS TO THE RULE THAT HOMOGRAFTS ARE RAPIDLY DESTROYED

It sometimes happens that a homograft appears to survive when it is in fact destroyed because it provides a scaffolding for regenerating recipient tissue and undergoes a process known as *creeping replacement*. This occurs characteristically with massive bone grafts and with grafted segments of blood vessels, but may also occur with various other tissues transplanted *orthotopically*, i.e. to an anatomically natural environment.

True prolonged survival, which may in some cases persist throughout the normal life span of the recipient, also occurs under certain conditions. The factors responsible may be grouped under the following headings:

1. Compatibility of donor and host.
2. Special properties of certain tissues.
3. Special properties of certain sites.
4. Constitutional abnormalities of the recipient.
5. Experimental and therapeutic procedures.

Compatibility of Donor and Host

Many experiments have been performed in which there was some clearly defined genetic difference between the donor and the recipient of a homograft, and as a result it has become possible to distinguish a special class of dominant genes, named by Snell (1948) *histocompatibility genes*, which determine the fate of typical homografts. The necessary and sufficient condition that a homograft of any tissue from a given donor D will be treated like an autograft by a recipient R which has not been subjected to any special treatment, is that D should not possess any histocompatibility genes which are absent from R.

This condition is satisfied when D and R are identical twins, and advantage

has been taken of this fact clinically in treating irreversible renal failure by transplantation of a whole kidney when the patient happened to be lucky enough to have a healthy identical twin willing to serve as donor.

The condition may also be virtually satisfied experimentally when D and R are members of a strain of animals which has been maintained by brother-sister mating for many generations, or when R is the F_1 hybrid of two such strains and D is a member of one of them, provided also that D and R are of the same sex, or that D is female and R male. The necessity for the qualification about the sex of D and R, which was first recognized by Eichwald & Silmser (1955), arises from the fact that, at any rate in mice, some histocompatibility genes are located on the Y chromosome.

In the special case when donor and host have the same genetic constitution the graft is described as *isogeneic*; all other homografts are termed *allogeneic.*

Histocompatibility genes have been studied most extensively in mice, and several histocompatibility loci have been identified, of which the most important is that known as the H2 locus. The histocompatibility genes of the H2 system all appear to be strong genes, in the sense that if the donor possesses even one of these which the recipient lacks the graft is likely to be destroyed rapidly; most if not all of the histocompatibility genes relating to other loci, on the other hand, are weak, and differences in respect of them may have only a slight effect on homograft survival.

Little is known definitely about histocompatibility genes in man, but Simonsen (1965) has suggested that the strong antigens are determined by a single genetic locus with a small number (possibly three) of alleles.

Various tests are used clinically to assess the degree of histocompatibility between patients needing organ transplants and available donors; these will be considered later.

Special Properties of Certain Tissues

Homografts of avascular tissues like cornea and cartilage may survive indefinitely provided that they remain avascular.

A homograft of ovarian tissue, for some reason which has not yet been elucidated, is likely to survive considerably longer than a skin graft from the same donor to the same recipient. Moreover, as Linder (1962a, b) has shown, the survival of a skin homograft may be prolonged (and in weak histocompatibility situations prolonged indefinitely) in a recipient which has previously received an ovarian homograft from the same donor strain. Homografts of testis, and of various endocrine tissues, sometimes also survive for a surprisingly long time, especially if the recipient is deficient in respect of the endocrine in question.

Tissue from embryos sometimes survives longer than the corresponding tissue from a normal adult donor, but this is by no means always the case.

Tumour homografts often behave very much like homografts of normal

tissues though the histological reaction they evoke is more varied (see Gorer 1960). Sometimes, they grow progressively and kill the recipient even when the genetic disparity is such that homografts of normal tissues from the same donor would be rapidly rejected.

Special Properties of Certain Sites

Homografts of various tissues may survive for a long time, or even indefinitely, in the white matter of the brain and in the anterior chamber of the eye. Sometimes this happens because the graft has failed to develop a blood supply, but it may also happen, at any rate in the anterior chamber, with well vascularized grafts. Possible reasons for this phenomenon have been discussed by Woodruff & Woodruff (1950).

The mammalian foetus *in utero*, which is a form of homograft, may also be considered in this category. Although many factors contribute to its survival the necessary and sufficient condition appears to be isolation of the foetus from maternal circulating cells by the placental barrier (Woodruff 1957).

Constitutional Abnormalities of the Recipient

It has been reported that in man homografts may survive abnormally long if the recipient is suffering from chronic uraemia (Dammin, Couch & Murray 1957), Hodgkin's disease (Kelly *et al* 1960), or agammaglobulinaemia (Good & Varco 1955, and see Chapter 19). In experimental animals homograft survival has been found to be prolonged when the recipient is pregnant (Heslop, Krohn & Sparrow 1954).

Experimental and Therapeutic Procedures

These may be subdivided into two main groups: *non-specific procedures*, which may reduce an individual's capacity to reject homografts in general, and *specific procedures* which reduce the capacity of an individual to reject homografts from a particular donor. Some of these will be considered in detail later.

The Mechanism by which Homografts are Destroyed

A decisive step towards an understanding of the mechanism by which homografts are destroyed was provided by the observation of Gibson & Medawar (1943) that a second skin homograft from the same (*human*) donor to the same recipient was destroyed more quickly than the first. This *second-set phenomenon*, as it is called, has since been demonstrated in many mammalian species, and also in fish and amphibia, and with a wide variety of tissues. It has been shown further that a first graft of one kind of tissue may provoke accelerated rejection

of a second graft of a different kind provided that both come from the same donor. The first graft may take the form of a whole organ transplanted by vascular anastomosis, a free graft of a small piece of tissue, or an injection of a suspension of living cells. The rate at which the state of increased reactivity develops depends among other things on the type and magnitude of the graft, and its location in the recipient.

These observations suggested that the rejection of a homograft was an immunological phenomenon stimulated by antigens liberated from the graft, and further, that different tissues from the same donor had at least some antigens in common.

Direct proof that homografts may be antigenic is provided by the demonstration of humoral antibodies in homograft recipients. In rodents, as was first shown by Lumsden (1938) and Gorer (1937, 1938), haemagglutinins may appear in the recipient's blood during or immediately after regression of a tumour graft. Further analysis, in which Gorer has played a leading part (see e.g. Gorer 1956, 1960), has shown that in mice haemagglutinins regularly appear following homotransplantation of tumours, and probably also of normal tissues, when the donor possesses histocompatibility genes of the H2 system which are lacking in the recipient. Haemagglutinins have also been demonstrated following homografting of skin in other species including man, but the precise conditions under which they appear have not been defined. Cytotoxic antibodies, demonstrable by a variety of *in vitro* or combined *in vivo* and *in vitro* tests (for review see Woodruff, 1960), may also appear in the serum of homograft recipients.

Despite these observations there is much evidence to suggest that the role of humoral antibodies in the rejection of homografts of normal tissues, and probably also of many tumours, is at most a subsidiary one.

It the first place, it has been shown that increased resistance to homografts from a particular donor can be transferred from the recipient to another animal of the same inbred strain by means of cells obtained from lymph nodes draining the graft, but not as a rule by serum or erythrocytes. This was first shown by Mitchison (1954) working with a transplantable mouse tumour, and confirmed by Billingham, Brent & Medawar (1954) with skin homografts in mice. More recently Lawrence *et al* (1960) have succeeded in transferring increased resistance to homografts in man with leucocyte extracts.

Secondly, as Algire and his colleagues have shown (Algire, Weaver & Prehn 1957), homografts enclosed in 'diffusion chambers', the walls of which are made of a membrane permeable to large molecules but not to cells, will survive indefinitely provided that their metabolic needs can be adequately met. Further analysis has shown that such grafts do not invoke immunity nor are they destroyed in pre-immunized recipients; it thus seems likely that circulating recipient cells play a crucial role on the afferent as well as on the efferent side of the homograft reaction, since non-cellular antigenic material would be

expected to escape through the membrane and enter lymphatics in the vicinity. This hypothesis, and other evidence in support of it, is discussed in a recent review by Gowans & McGregor (1965).

Experiments with diffusion chambers suffer from the disadvantage that even with very small grafts nutritional exchange may be inadequate. This objection does not apply to experiments with the mammalian foetus which, as we have seen, is a form of homograft in a specially privileged site. It has been shown, however (Woodruff 1957), that the foetus does not ordinarily immunize the mother, nor does it suffer if the mother has been pre-immunized with a homograft from the father, the effect of which must have been to immunize the mother against foetal antigens determined by histocompatibility genes inherited from the father. These results are not due to some peculiarity of foetal tissue, because grafts of such tissue in extra-uterine sites in the mother do induce a state of immunity and are rejected like other types of homograft.

A third piece of evidence of the dominant role of recipient cells in the homograft reaction comes from the observation of Brent, Brown & Medawar (1958) that delayed-type hypersensitivity reactions occur when a guinea-pig which has rejected a homograft is injected intradermally with donor lymphoid cells, or even with antigenic material prepared from such cells, and also when cells from the regional lymph nodes of the recipient are injected intradermally into the donor.

It is possible that in some circumstances humoral antibodies and cellular mechanisms act synergistically in destroying homografts, and in the special case of the transplantable mouse leukaemias it may well be, as Gorer has suggested, that humoral antibodies play the dominant role, since immunity to these tumours can be transferred passively with serum (Gorer & Amos 1956). On the other hand, as we shall see when considering the phenomenon of enhancement, humoral antibodies may also have the opposite effect, and help to protect a graft against cellular attack.

THE ANTIGENS CONCERNED IN THE HOMOGRAFT REACTION

Much work has been done with the object of isolating and determining the chemical nature of the antigens concerned in the homograft reaction. A few years ago it was widely believed that, at any rate in mice, there were two sets of antigens: 'T antigens' which elicit transplantation immunity as manifested for example by the second-set response, and 'H antigens' which elicit the formation of haemagglutinating antibodies. Billingham, Brent & Medawar (1956b) reported that the T antigens were present in the nuclei of cells, and succeeded in extracting antigenic material by ultrasonic distintegration of cell nuclei and centrifugation at 25,000 ***g***. The solubility properties of the material, and the fact

that it was inactivated by DNA-ase but not by RNA-ase suggested that the antigens were in fact DNA proteins, but further investigations (Billingham, Brent & Medawar 1958) showed that DNA was not a necessary constituent of antigenically active preparations, and suggested that the antigens were probably amino-acid polysaccharide complexes chemically related to the human blood group substances. More recent investigations have cast doubt on the validity of the distinction between T and H antigens (Brent, Medawar & Ruszkiewicz 1961, 1962), and have established that in mice, while a small amount of antigenic activity may be present within cells, most of it is located in a fraction derived from cell membranes which is predominantly lipoprotein but is not entirely devoid of carbohydrate (Kandutsch 1961; Castermans 1962; Davies 1962, 1964 1966; and others). Antigens determined by the H2 locus have been most studied, but the H2 active lipoproteins contain also antigens determined by other loci.

Insoluble or 'semi-soluble' antigenic material may be prepared from lymphoid cells and tumour cells by breaking up the cells mechanically or lysing them in hypotonic solutions, or more simply, as Davies (1966a) has shown, by washing the cells with hypotonic salt solution up to a point short of lysis. Similar material may also be prepared from ascitic fluid (Davies 1962). The antigenic activity of these preparations has been demonstrated *in vivo* by their capacity to sensitize mice of a different strain to homografts from mice of the strain from which the antigen was prepared and to evoke the formation of cytotoxic isoantibodies, and *in vitro* by their capacity to inhibit the cytotoxic effect of isoimmune antisera.

It was shown by Medawar (1963) that intravenous injection of semi-soluble antigen may induce tolerance of homografts (*v. infra*) in weak histocompatibility systems, and it seems likely from the work of Dresser (1962a, b) that tolerance might be induced in stronger histocompatibility systems if the antigens were available in soluble form. Solubilization is also the key, as Davies (1966a) has pointed out, to further elucidation of the chemical structure of the transplantation antigens. Kandutsch and his colleagues (Kandutsch & Stimpfling 1963; Kandutsch, Jurgeleit & Stimpfling 1965) attacked this problem by using a non-ionic detergent, Triton, but material solubilized in this way became insoluble when the detergent was removed. Further treatment with phospholipase yielded a water soluble preparation consisting of lipoprotein with a small amount of carbohydrate but in this form the H2 antigen was polydisperse on ultracentrifugation at physiological pH. Davies (1966b; see also Nathenson & Davies 1966a, b) has developed two methods of solubilization which open up new and exciting prospects for future work. One is based on the autolytic incubation of lipoprotein derived from fresh mouse lymphoid cells in Tris-HCl buffer at 37°C; the other on the treatment of lipoprotein from freeze-dried tumour cells with ficin (a mixture of enzymes, mainly proteolytic, from the latex of certain figs).

NON-SPECIFIC INHIBITION OF THE HOMOGRAFT REACTION

The capacity of an animal or person to react to a variety of antigenic stimuli including that provided by a homograft may be reduced by exposure to whole body X-irradiation, thymectomy in early life, depletion of lymphocytes by drainage through a thoracic duct fistula, and administration of immunosuppressive drugs or antilymphocyte serum. The immunosuppressive drugs include corticosteroids, alkylating agents such as nitrogen mustard, and various antimetabolites such as 6-mercaptopurine and azathioprine.

Whole Body Irradiation

It was shown many years ago by Murphy that the resistance of rats to tumour heterografts was diminished by exposing the animals to X-rays (see Murphy 1926). Much more recently Dempster, Lennox & Boag (1950) showed that the survival of skin homografts in rabbits was increased up to about three-fold by exposing the recipients to approximately 250 rads whole body irradiation the day before grafting. On the other hand irradiation in similar dosage has no effect on survival time of kidney homografts in dogs.

In man irradiation in a dosage of about 400–600 rads has been found to promote the survival of kidney homografts; only in exceptional circumstances however is it likely to result in very long survival (see Hamburger *et al* 1959; Merrill *et al* 1960; Küss *et al* 1961; Tubiana, Lalanne & Surmont 1961), and the risk of death from overwhelming infection is prohibitive (Woodruff *et al* 1962).

The possibility of using very much higher doses of irradiation became apparent when it was shown that mice and guinea-pigs could recover after irradiation which would otherwise be lethal provided that they received transplants of haemopoietic tissue either from an animal of the same inbred strain or, under certain conditions, from an animal of a different strain (Lorenz, Congdon & Uphoff 1952). The same phenomenon has subsequently been demonstrated in other species.

There has been much discussion of the nature of the protective effect of transplanting haemopoietic tissue, but it has now been proved beyond doubt that, if the dose of irradiation is sufficient, cells derived from the transplanted tissue may persist indefinitely in the recipient (Ford *et al* 1956; Porter 1957). Animals in which this occurs are termed *irradiation chimaeras*.

Beyond a certain dose level of irradiation, which varies with the species, transplantation of haemopoietic tissue is unable to promote recovery because death results from damage to intestinal epithelium. The safe dose of irradiation may be increased considerably by prior administration of certain sulphydril compounds such as AET (S,2-aminoethylisothiouronium. Br.HBr). Mice protected by this drug show less inhibition of their immunological reactivity,

including their capacity to reject homografts, than unprotected mice given the same dose of irradiation: Raffel (1962) has shown, however, that this effect is relatively slight as compared with the protection afforded to the gut, so that it is possible to achieve a greater degree of inhibition of immunological reactivity without killing the animal by using the drug in combination with irradiation than by irradiation alone.

Irradiation is much less effective if given after transplantation of a homograft. There is some suggestive evidence, however, that the rejection of a kidney homograft in man may be retarded by post-operative irradiation of the patient generally, or of the size of the graft, or both (Merrill *et al* 1960; Küss *et al* 1961), and in mice whole body irradiation may reduce the immunizing effect of an intravenous injection of spleen cells from an animal of a different strain (Woodruff 1961).

Thymectomy

It has been established, largely by the work of Miller & Good, and their respective colleagues (see e.g. Miller 1965), that thymectomy of newborn mice and various other animals results in marked impairment of the capacity to reject homografts whereas thymectomy of adult animals without other treatment has no such effect. Mice which have been thymectomized as adults are, however, slower to recover their immunological responsiveness after whole body irradiation than non-thymectomized mice.

Thoracic Duct Fistula

It was shown by McGregor & Gowans (1963) that a specific depletion of small lymphocytes in rats can be produced by chronic drainage of cells from a thoracic duct fistula. This depletion is associated with impaired immunological responsiveness including impairment of the homograft reaction (McGregor & Gowans 1964; Woodruff & Anderson 1963, 1964).

Immunosuppressive Drugs

The drugs which have been shown to have an inhibitory effect on the homograft reaction fall into three main categories: corticosteroids, alkylating agents and anti-metabolites. The anti-metabolites include 6-mercaptopurine and the related compound azathioprine, which have become widely used in the treatment of human renal transplant recipients since their capacity to inhibit the homograft reaction was first demonstrated experimentally by Calne & Murray (Calne 1960, 1961; Calne & Murray 1961).

The investigations of Berenbaum and others (for review see Berenbaum 1965) have thrown much light on the mode of action of these agents, and the optimal time of administration in relation to the time of grafting.

Anti-Lymphocyte Serum (ALS)

The demonstration by Chew & Lawrence (1937) that serum prepared by immunizing an animal of one species with lymphoid tissue from another had a powerful anti-lymphocytic effect *in vivo* suggested that serum prepared in this way might have immunosuppressive properties. Initial attempts to confirm this failed (Woodruff 1960), apparently because the serum was of low potency, but subsequently Waksman, Arbouys & Arnason (1961) succeeded in producing marked depression of the tuberculin and contact allergic reactions and slight prolongation of homograft survival in guinea-pigs treated with rabbit anti-guinea-pig lymphocyte serum, and Woodruff & Anderson (1963, 1964) obtained striking prolongation of skin homograft survival in rats treated with rabbit anti-rat lymphocyte serum.

Similarly spectacular results have now been reported with skin homografts in mice (Gray, Monaco & Russell 1964; Monaco, Wood & Russell 1965; Levey & Medawar 1966a) and with kidney and liver homografts in dogs treated with horse anti-dog lymphocyte serum (Abaza *et al* 1966; Starzl *et al* 1966). It has been shown moreover by Levey & Medawar (op. cit.) that a pre-existing state of immunity to skin of a particular donor strain may be weakened or even abolished by treatment with ALS in a dosage which is well tolerated, a finding which distinguishes ALS from all other known immunosuppressive agents.

ALS (horse anti-human lymphocyte serum) has been used in several human renal transplant recipients by Starzl (Starzl *et al* 1966) and in one such case by Woodruff (1966b).

The mode of action of ALS is still the subject of speculation. For a discussion of current theories the reader is referred to a recent review by Levey & Medawar (1966b).

SPECIFIC INHIBITION OF THE HOMOGRAFT REACTION

In this section we shall discuss a group of conditions in which the capacity of an organism to react to homografts from a particular donor is reduced or abolished by previous exposure to antigenic material derived from the graft donor or another animal of the same genetic constitution.

Specific Immunological Tolerance

It was predicted by Burnet & Fenner (1949) that if an animal was exposed while it was still sufficiently immature, to a particular antigen, it would fail to react if challenged later in life with the same antigen.

In fact, an example of the phenomenon predicted occurs in nature when dizygotic twins become blood chimaeras as a result of the persistence of haemo-

poietic tissue exchanged during intrauterine life. The true explanation of this condition, which is common in cattle, was established as long ago as 1945 by Owen, but the first experimental confirmation of the Burnet-Fenner prediction came from the discovery of Billingham, Brent & Medawar (1953, 1955, 1956a) that CBA mice injected on the 15th to 16th day of intra-uterine life with cells obtained by chopping up the kidney, spleen and testis of an A-strain donor, would subsequently in postnatal life accept permanently grafts of A-strain skin. Soon afterwards it was shown that non-reactivity to skin homografts could be induced by injecting donor (or donor strain) cells to newborn rats (Woodruff & Simpson 1955) and mice (Billingham & Brent 1957a), and also that the non-reactivity extended to donor tissues other than skin, including *inter alia* thyroid (Woodruff & Sparrow 1958) and adrenal (Woodruff & Sparrow 1958; Medawar & Russell 1958).

Billingham *et al* found however that the non-reactivity did not extend to skin grafts from mice of a third strain, and they therefore named the phenomenon *specific immunological tolerance*. They showed further that tolerance could be abolished by re-equipping the tolerant animal with lymphoid cells from a normal member of the recipient strain, or more quickly with cells from the regional lymph nodes of an animal of the recipient strain which had previously been immunized against donor strain skin. They concluded in consequence that tolerance was the result of a specific *central* failure of the mechanism of immunological response.

It was concluded from all this work that there are three stages in immunological development. During the first stage, which ends at about the time of birth or soon afterwards, exposure to antigen results in the development of specific tolerance. During the second stage, which is of short duration and is sometimes known as the *null period*, exposure to antigen evokes neither tolerance nor immunity, while during the third and final stage the organism exhibits full immunological maturity.

It is now apparent that this scheme is a gross over-simplification.

In the first place, tolerance is not an all-or-nothing phenomenon. An animal may become partially tolerant, in which case its capacity to react to the antigen in question is impaired but not abolished. In the case of skin homotransplants this may lead to the curious situation (Weber, Cannon & Longmire 1954; Woodruff & Simpson 1955) in which a first transplant may be permanently accepted while the second one from the same donor is sooner or later rejected. Secondly, the result of exposure to antigen in the form of allogeneic cells depends not only on the age of the recipient but on the species and strain of donor and host, the number of cells injected, and the route of injection. Indeed it may be possible at the same time to induce tolerance to one antigen (or a set of antigens) and immunity to another, a condition which is known as *split tolerance* (Billingham & Brent 1959; Brent & Courtenay 1961).

Thirdly, it has been shown that a state of unresponsiveness to skin homografts which is at least partly specific may be induced in adult mice by a variety of procedures including irradiation in lethal dosage, and resuscitation by the injection of haemopoietic tissue from the prospective skin donor or an animal of the same genetic constitution (Main & Prehn 1955; Trentin 1957; Barnes & Loutit 1959); sub-lethal irradiation followed by the injection of donor strain spleen cells (Main & Prehn 1957; Michie & Woodruff 1962); parabiosis (Rubin 1959; Martinez *et al* 1960); repeated intravenous injection of donor strain spleen cells over several weeks (Shapiro *et al* 1961); intravenous injection of donor epidermoid cells in rabbits (Billingham 1957), and injection of antigenic extracts, with or without treatment in the form of whole body irradiation or the administration of cytotoxic drugs (Medawar 1963).

It is not suggested that the mechanism is the same in all these cases. After lethal irradiation and resuscitation with haemopoietic tissue, for example, the recipient's immunological functions may be taken over completely by cells of donor origin, but in some circumstances, as in the experiments of Michie & Woodruff (op. cit.) the non-reactivity has been shown to be due to specific central inhibition akin to that described by Billingham, Brent and Medawar in their original experiments on tolerance induction in the foetus.

Fourthly, Howard & Michie (1962) have adduced evidence that newborn mice become immune, not tolerant, if injected with a sufficiently *small* dose of allogeneic cells.

A fuller discussion of tolerance and its significance from the point of view of immunological theory will be found in a recent review by Woodruff (1965).

Enhancement

The term *enhancement* was applied originally to prolonged survival of tumour homografts following the injection or transplantation of tissue from the same tumour which had been killed by heating, freezing and thawing, or freeze drying, or by injection of tumour tissue extracts. It was discovered by Kaliss and his colleagues (Kaliss 1957) that this phenomenon depends in some way on the presence of antibody in the serum of the recipient, and the term enhancement is now applied to all forms of specific non-reactivity mediated in this way, including non-reactivity to homografts of normal tissues, although this is admittedly not very appropriate.

Enhancement may be quite dramatic in the case of tumours, but with homografts of normal tissues the most that has been achieved so far is to prolong the period of survival by a few days or occasionally to double it (Billingham, Brent & Medawar 1956c).

It appears from the work of Möller (1963) that enhancing antibody may inhibit both the uptake of antigen from homografts and the destruction of homografts by cellular immune mechanisms.

REACTION OF GRAFT AGAINST HOST

Sometimes when living cells are transplanted the recipient suffers injury, which may be serious and even fatal, due to some of the grafted cells reacting immunologically against the tissues of the recipient.

For this to occur the following conditions must be fulfilled:

1. The recipient must possess at least one antigen which is not present in the cell donor.

2. The recipient must be either completely unable to reject the grafted cells, or only able to reject them relatively slowly. This may occur for example if the recipient is immature, or possesses all the antigens which are present in the donor, or has been subjected to some procedure such as whole body irradiation which has reduced its capacity for immunological response.

3. The injected cells must include a sufficient number which are *immunologically competent*, i.e. capable of mounting an immunological reaction when exposed to antigenic stimulation.

Immunologically competent cells are present in large numbers in the spleen and lymph nodes, and in thoracic duct lymph. They occur also, but in smaller numbers, in bone marrow, and in the peripheral blood. Since thoracic duct lymph contains only two kinds of cell, namely large and small lymphocytes, it follows that one or both of these cell types must be immunologically competent, and it appears from the work of Gowans *et al* (1961) that this property belongs to the small but not to the large lymphocyte.

Disease due to graft-versus-host reaction, which may appropriately be designated G *v* H disease, takes various forms. It was first recognized by Billingham & Brent (1957b) in mice following intravenous injection of allogeneic spleen cells immediately after birth, and by Simonsen (1957) in chickens injected with allogeneic spleen cells or buffy coat leucocytes 3 days before hatching. In mice the main features described by Billingham & Brent were wasting, diarrhoea and hypoplasia of lymphoid tissue, and they named the condition *runt disease*. In chicks Simonsen observed severe haemolytic anaemia, associated with the appearance of numerous plasma cells in the spleen, bone marrow and thymus. A condition closely resembling runt disease in mice has since been described in rats injected at birth with allogeneic spleen cells (Woodruff & Sparrow 1957) or thoracic duct lymphocytes (Anderson, Delorme & Woodruff, 1960).

Another condition which appears to be due largely to a graft-versus-host reaction is that known as *secondary disease*, which sometimes develops in heavily irradiated animals which have recovered temporarily following injection of allogeneic spleen or bone marrow cells. This was first observed in mice by Barnes *et al* (1956) and has since been reported to occur in many other species including man (Mathé *et al* 1960).

G *v* H disease has also been observed in mice following irradiation in a dose

of 350 to 500 rads and intravenous injection of about 1000 million allogeneic spleen cells (Michie & Woodruff 1962), but in this case the term secondary disease is not appropriate since the condition was not preceded by primary illness and temporary recovery.

Yet another form of G *v* H disease occurs when spleen cells from an animal of a strain which has been inbred sufficiently to become virtually uniform and homozygous in respect of all histocompatibility genes, are injected in appropriate dosage into a normal adult hybrid of this and another inbred strain. The situation, simplified by treating each strain as if it possessed only a single histocompatibility gene, may be schematically represented thus:

$$(AA) \times (BB)$$

$$\Big\downarrow$$

(AA) spleen cells $\rightarrow$ $(AB)F_1$ = Cells accepted but react against antigens in recipient determined by gene B.

Finally, the 'intoxication' which often develops in animals connected in parabiosis is probably due, at least in part, to each partner reacting immunologically against the other; it may therefore be regarded as a form of mutual G *v* H disease.

It must be emphasized that the severity of G *v* H disease depends on many factors, including the degree of histoincompatibility of cell donor and recipient, the source of the injected cells, and the number of cells injected. Moreover, animals developing G *v* H disease of mild to moderate severity may subsequently recover more or less completely, either because the grafted cells disappear or because they become immunologically tolerant of the recipient (Simonsen 1960; Michie, Woodruff & Zeiss 1961).

One of the characteristic features of graft-versus-host reactions is enlargement of the recipient's spleen. Simonsen & Jensen (1959) have taken advantage of this fact in developing a method known as the discriminant spleen assay for analyzing a mixed population of immunologically competent cells. A slightly modified version of their procedure is illustrated in Fig. 17.1.

This type of assay has been applied to the analysis of animals injected with allogeneic spleen cells in the neonatal period (Michie, Woodruff & Zeiss 1961), or in adult life after sublethal (Michie & Woodruff 1962) or lethal (van Bekkum 1962) irradiation. One curious conclusion which has emerged is that most of the immunologically competent cells in the spleens of mice showing marked symptoms and signs of G *v* H disease are of recipient origin. Parallel investigations, using donors possessing an easily recognizable chromosomal translocation, have shown that the same is true of most of the proliferating cells in the recipients' spleens, irrespective of whether or not they are immunologically competent.

It seems likely, therefore, that the manifestations of G *v* H disease are the result of damage caused by the foreign cells within a short time of their being injected. Evidence in support of this conclusion is provided by experiments

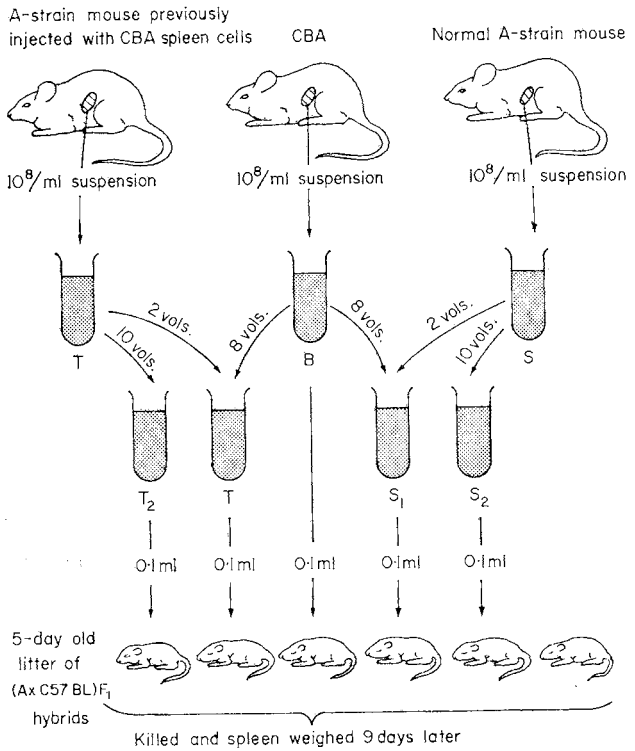


FIG. 17.1. Form of discriminant spleen assay for determining the relative number of immunologically competent cells of recipient origin in a suspected A-CBA chimaera as compared with a normal A-strain mouse. The 5-day-old hybrids are mature enough to reject CBA cells but they accept A cells and these react against antigens inherited from the C57BL parent and cause splenomegaly. The logarithm of the relative spleen weight (i.e. ratio of weight of spleen to weight of mouse) about 9 days after injection has been shown by Michie and Simonsen to be a linear function of the logarithm of the number of immunologically competent A-strain cells injected. An assay of the donor component of the immunologically competent cells in the spleen of the suspected chimaera is performed similarly in 5-day-old (CBA × C57BL)F_1 hybrids (after Michie and Woodruff).

in which it has been shown that the symptoms and signs of G *v* H disease following the injection of allogeneic spleen cells to newborn or irradiated mice may be reduced in severity, or even suppressed entirely, by treating the recipient with an anti-metabolite, A-methopterin (Uphoff 1958; Russell 1962), ro

alternatively by injecting normal recipient strain spleen cells (Russell 1960), provided that the treatment is given within a few days of injection of the foreign cells. Spleen cells from an animal of the recipient strain which has been immunized against donor strain tissue are effective as a prophylactic agent for a few days beyond the time at which normal recipient strain spleen cells cease to be effective, but once overt manifestations of G *v* H disease have developed no treatment so far tried appears to alter the course of events. For a more detailed discussion of the natural history of G *v* H reactions and their use as tools of research the reader is referred to a review by Simonsen (1962).

CLINICAL IMPLICATIONS OF THE HOMOGRAFT REACTION

Some types of homograft are clinically useful even though they, or at any rate their constituent cells, do not survive very long. Thus, for example, homografts of skin may provide valuable temporary cover in patients with severe burns, and homografts of bone provide a scaffolding which guides the regeneration of host tissue. Homografts of segments of arteries, which were widely used in vascular surgery before the development of woven arterial prostheses, provide a mass of inert material, some of which persists and is gradually incorporated into the structure of the host, and more recently homografts of heart valves have been shown to behave in a similar way (Ross 1966).

Most homografts, however, remain useful only in so far as they continue to survive and function, and except in the case of homografts of privileged tissues like cornea and cartilege, they will sooner or later cease to do this unless either the donor is histocompatible with the recipient or immunological rejection is prevented by one of the procedures already described.

It is doubtful if complete compatibility will ever be obtained except when the donor and recipient are identical twins; in practice however it will suffice if the degree of compatibility is such that rejection can be prevented indefinitely with the methods currently available.

The clinical demand for homografts embraces many types of tissue in addition to those already mentioned, including bone marrow and endocrine tissue, and whole organs such as the kidney, liver, lungs and heart.

The first organ to be transplanted in man by vascular anastomosis was the kidney, and even now transplantation of other organs in this way is rarely attempted on account of the formidable technical difficulties. Initially no form of immunosuppression was used other than small doses of cortisone or ACTH in some cases. Then came the era of whole body irradiation in which some grafts functioned for a few months or even for a year or more, but many patients died within a few weeks from septicaemia. Today it is customary to use a combination of immunosuppressive drugs, usually prednisone and

azathioprine routinely with the addition of actinomycin C if acute rejection is threatened. Some hundreds of patients have been treated in this way (see e.g. Calne, 1963; Starzl 1964; Murray, Gleason & Bartholomay 1965; Dunea *et al* 1965; Hamburger, Crosnier & Dormont 1965; Woodruff 1966a) and it appears that with good pre-operative and post-operative care, including haemodialysis or peritoneal dialysis if required before operation and during the first week or two thereafter, one can expect a mean graft survival time of between 1 and 2 years even with unrelated donors selected on the basis of red cell grouping only. The patient survival time should be much longer if adequate dialysis facilities are available because when the transplant is becoming ineffective it can be removed and replaced by another one after a period of rehabilitation by twice weekly dialysis.

It seems likely that the prognosis will be greatly improved in the near future as the result of improved methods of matching donor and recipient. Various tests have been proposed, including the normal lymphocyte transfer test (Brent & Medawar 1963; see also Gray & Russell 1965), the mixed leucocyte culture test (Bain, Vas & Lowenstein 1964; Bach & Hirschhorn 1964), the third party skin graft test (Matsukura *et al* 1963; Rapaport *et al* 1962; Murray *et al* 1963), and leucocyte grouping of donor and recipient. Of these, leucocyte grouping, which has been developed largely by Dausset (1961, 1963), van Rood (van Rood & van Leeuwen, 1965), and Ceppellini (Ceppellini, Mattius & Curtoni 1965) in Europe, and Terasaki (Terasaki, Marchioro & Starzl 1965) in the United States, seems to be the most promising as judged by the results of retrospective typing of available living patients and their corresponding donors (see e.g. Terasaki *et al*, op. cit.; Woodruff 1966a).

The results are also likely to be improved by the introduction of better or additional immunosuppressive agents, including anti-lymphocyte serum. Finally there is the exciting possibility of being able to induce specific immunological tolerance to the tissues of a particular donor, or multivalent tolerance to human homografts in general, by the injection of the transplantation antigens in a soluble form.

The homograft reaction has other clinical implications which go beyond the surgery of replacement. In the first place the study of homograft rejection has contributed greatly to our understanding of auto-allergic diseases and their treatment. Secondly, it has reawakened interest in tumour immunology, and has stimulated research which may one day culminate in the development of effective immunological methods for the treatment of cancer.

REFERENCES

ABAZA H.M., NOLAN B., WATT J.G. & WOODRUFF M.F.A. (1966) Effect of antilymphocytic serum on the survival of renal homotransplants in dogs. *Transplantation* **4**, 618.

ALGIRE G.H., WEAVER J.M. & PREHN R.T. (1957) Studies on tissue homotransplantation in mice, using diffusion-chamber methods. *Ann. N.Y. Acad. Sci.* **64,** 1009.

ANDERSON N.F., DELORME E.J. & WOODRUFF M.F.A. (1960) Induction of runt disease in rats by injection of thoracic duct lymphocytes at birth. *Transplant Bull.* **7,** 93.

BACH F. & HIRSCHHORN K. (1964) Lymphocyte interaction: A potential histocompatibility test *in vitro*. *Science* **143,** 813.

BAIN B., VAS M.R. & LOWENSTEIN L. (1964) The development of large immature mononuclear cells in mixed leucocyte cultures. *Blood* **23,** 108.

BARNES D.W.H., CORP M.J., LOUTIT J.F. & NEAL F.E. (1956) Treatment of murine leukaemia with X-rays and homologous bone marrow. Preliminary communication. *Brit. med. J.* **ii,** 626.

BARNES D.W.H. & LOUTIT J.F. (1959) Immunological status and longevity of radiation-chimaeras. *Proc. roy. Soc. B.* **150,** 131.

VAN BEKKUM D.W. (1962) Tolerance of donor cells towards the host in radiation chimaeras. In *Symposium on Mechanisms of Immunological Tolerance.* pp. 385–395. Prague: Czechoslovak Academy of Sciences.

BERENBAUM M.C. (1965) Immunosuppressive agents. *Brit. med. Bull.* **21,** 140.

BILLINGHAM R.E. (1957) Studies on epidermal cell suspensions, with particular reference to problems of transplantation immunity. *Ann. N.Y. Acad. Sci.* **64,** 799.

BILLINGHAM R.E. & BRENT L. (1957a) Acquired tolerance of foreign cells in newborn animals. *Proc. roy. Soc. B.* **146,** 78.

BILLINGHAM R.E. & BRENT L. (1957b) A simple method for inducing tolerance of skin homografts in mice. *Transplant. Bull.* **4,** 67.

BILLINGHAM R.E. & BRENT L. (1959) Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in newborn mice on the phenomenon of runt disease. *Phil. Trans. B.* **242,** 439.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1953) Actively acquired tolerance of foreign cells. *Nature (Lond.)* **172,** 603.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1954) Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc. roy. Soc. B.* **143,** 58.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1955) Acquired tolerance of tissue homografts. *Ann. N.Y. Acad. Sci.* **59,** 409.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1956a) Quantitative studies on tissue transplantation immunity. *Phil. Trans. B.* **239,** 357.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1956b) The antigenic stimulus in transplantation immunity. *Nature (Lond.)* **178,** 514.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1956c) Enhancement in normal homografts with a note on the possible mechanism. *Transplant. Bull.* **3,** 84.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1958) Extraction of antigens causing transplantation immunity. *Transplant. Bull.* **5,** 377.

BRENT L., BROWN J.B. & MEDAWAR P.B. (1958) Skin transplantation immunity in relation to hypersensitivity. *Lancet* **ii,** 561.

BRENT L. & COURTENAY T.H. (1961) In *Symposium on Mechanisms of Immunological Tolerance,* p. 113. Prague: Czechoslovak Academy of Sciences.

BRENT L. & MEDAWAR P.B. (1963) Tissue transplantation: a new approach to the 'typing problem'. *Brit. med. J.* **ii,** 269.

BRENT L., MEDAWAR P.B. & RUSZKIEWICZ M. (1961) Serological methods in the study of transplantation antigens. *Brit. J. exp. Path.* **42,** 464.

BRENT L., MEDAWAR P.B. & RUSZKIEWICZ M. (1962) Studies on transplantation antigens. In *Transplantation*, p. 6. Ciba Foundation Symposium. London: J. & A. Churchill.

BURNET F.M. & FENNER F. (1949) *The Production of Antibodies*. 2nd ed. Melbourne: Macmillan.

CALNE R.Y. (1960) The rejection of renal homografts. Preliminary communication. *Lancet* **i**, 417.

CALNE R.Y. (1961) Inhibition of the rejection of renal homografts in dogs by purine analogues. *Transplant. Bull.* **28**, 445.

CALNE R.Y. (1963) *Renal Transplantation*. E. Arnold (Publishers) Ltd., London.

CALNE R.Y. & MURRAY J.E. (1961) Inhibition of the rejection of renal homografts in dogs by Burroughs Wellcome 57–322. *Surg. Forum* **12**, 118.

CASTERMANS A. (1962) *Étude des Antigènes de Transplantation présents dans les Cellules spléniques et thymiques*. Editions Arscia S.A., Bruxelles.

CEPPELLINI R., MATTIUZ P.L. & CURTONI M. (1965) Characteristics and evolution of leukoagglutinins developed in a patient receiving multiple transfusions from selected donors. In *Histocompatibility Testing*, Part 1, p. 71, ed. RUSSELL P.S. & WINN H.J. National Academy of Sciences Publication No. 1229.

CHEW W.B. & LAWRENCE J.S. (1937) Antilymphocytic serum. *J. Immunol.* **33**, 271.

DAMMIN G.J., COUCH N.P. & MURRAY J.E. (1957) Prolonged survival of skin homografts in uremic patients. *Ann. N.Y. Acad. Sci.* **64**, 967.

DAUSSET J. (1961) Iso-anticorps anti-leucocytaires et greffe. *Rev. franc. Etud. clin. biol.* **6**, 993.

DAUSSET J. (1963) Leucocyte-agglutination. *Bibl. haemat. Basel* **14**, 31.

DAVIES D.A.L. (1962) The isolation of mouse antigens carrying H-2 histocompatibility specificity: some preliminary studies. *Biochem. J.* **84**, 307.

DAVIES D.A.L. (1964) Some properties of mouse histocompatibility antigens: H-2, non H-2, X (leukaemic) and Y (sex-linked). *Ann. N.Y. Acad. Sci.* **120**, 230.

DAVIES D.A.L. (1966a) Mouse histocompatibility isoantigens derived from normal and from tumour cells. *Immunology* **11**, 115.

DAVIES D.A.L. (1967) Soluble mouse H-2 isoantigens. *Transplantation* **5**, 31.

DEMPSTER W.J., LENNOX B. & BOAG J.W. (1950) Prolongation of survival of skin homotransplants in the rabbit by irradiation of the host. *Brit. J. exp. Path.* **31**, 670.

DRESSER D.W. (1962a) Specific inhibition of antibody production. I. Protein-overloading paralysis. *Immunology* **5**, 161.

DRESSER D.W. (1962b) Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. *Immunology* **5**, 378.

DUNEA G., NAKAMOTO S., STRAFFON R.A. FIGUEROA J.E., VERSACI A.A., SHIBAGAKI M. & KOLFF W.J. (1965) Renal homotransplantation in 24 patients. *Brit. med. J.* **i**, 7.

EICHWALD E.J. & SILMSER C.R. (1955) Communication. *Transplant. Bull.* **2**, 148.

FORD C.E., HAMERTON J.L., BARNES D.W.H. & LOUTIT J.F. (1956) Cytological identification of radiation chimaeras. *Nature (Lond.)* **177**, 452.

GIBSON T. & MEDAWAR P.B. (1943) The fate of skin homografts in man. *J. Anat. (Lond.)* **77**, 299.

GOOD R.A. & VARCO R.L. (1955) A clinical and experimental study of agammaglobulinaemia. *J. Lancet* **75**, 245.

GORER P.A. (1937) The genetic and antigenic basis of tumour transplantation. *J. Path. Bact.* **44**, 691.

GORER P.A. (1938) The antigenic basis of tumour transplantation. *J. Path. Bact.* **47**, 231.

GORER P.A. (1956) Some recent work on tumour immunity. In *Advances in Cancer Research*. vol. 4, p. 149. New York: Academic Press.

GORER P.A. (1960) Interactions between sessile and humoral antibodies in homograft reactions. In *Cellular Aspects of Immunity*, p. 330, Ciba Foundation Symposium. London: J. & A. Churchill.

GORER P.A. & AMOS D.B. (1956) Passive immunity against C57BL leukosis E.L.4 by means of iso-immune serum. *Cancer Res.* **16,** 338.

GOWANS J.L., GESNER B.L. & MCGREGOR D.D. (1961) The immunological activity of lymphocytes. In *Biological Activity of the Leucocyte*, p. 32. Ciba Foundation Study Group No. 10. London: J. & A. Churchill.

GOWANS J.L. & MCGREGOR D.D. (1965) The immunological activities of lymphocytes. In *Progress in Allergy*, vol. 9, p. 1. S. Karger, Basel (Switzerland), New York.

GRAY J.G., MONACO A.P. & RUSSELL P.S. (1964) Heterologous mouse anti-lymphocyte serum to prolong skin homografts. *Surg. Forum* **15,** 142.

GRAY J.G. & RUSSELL P.S. (1965) The lymphocyte transfer test in man. In *Histocompatibility Testing*, Part 1, p. 105, ed. RUSSELL P.S. & WINN H.J. National Academy of Sciences Publication No. 1229.

HAMBURGER J., CROSNIER J. & DORMONT J. (1965) Experience with 45 renal homotransplantations in man. *Lancet* **i,** 985.

HAMBURGER J., VAYSSE J., CROSNIER J., TUBIANA M., LALANNE C.M., ANTOINE B., AUVERT J., SOULIER J.-P., DORMONT J., SALMON C., MAISONNET M. & AMIEL J.-L. (1959) Transplantation d'un rein entre jumeaux non monozygotes après irradition du receveur. *Presse. méd.* **67,** 1771.

HESLOP R.W., KROHN P.L. & SPARROW E.M. (1954) The effect of pregnancy on the survival of skin homografts in rabbits. *J. Endocrin.* **10,** 325.

HOWARD J.G. & MICHIE D. (1962) Induction of transplantation immunity in the newborn mouse. *Transplant. Bull.* **29,** 91/1.

KALISS N. (1957) The survival of homografts in mice pretreated with antisera to mouse tissue. *Ann. N.Y. Acad. Sci.* **64,** 977.

KANDUTSCH A.A. (1961) The chemistry of transplantation antigens. *Transpl. Bull.* **27,** 135.

KANDUTSCH A.A., JURGELEIT H.C. & STIMPFLING J.H. (1965) The action of snake venom on an isoantigenic lipoprotein from a mouse sarcoma. *Transplantation* **3,** 748.

KANDUTSCH A.A. & STIMPFLING J.H. (1963) Partial purification of tissue isoantigens from a mouse sarcoma. *Transplantation* **1,** 201.

KELLY W.D., LAMB D.L., VARCO R.L. & GOOD R.A. (1960) An investigation of Hodgkin's disease with respect to the problem of homotransplantation. *Ann. N.Y. Acad. Sci.* **87,** 187.

KÜSS R., LEGRAIN M., CAMEY M., DESARMENIEN J., MATHE G., NEDEY R. & VOURC'H C. (1961) Homotransplantation rénale chez l'homme. *Mém. Acad. Chir.* **87,** 183.

LAWRENCE H.S. RAPAPORT F.T., CONVERSE J.M. & TILLETT W.S. (1960) Transfer of delayed hypersensitivity to skin homografts with leucocyte extracts in man. *J. clin. Invest.* **39,** 185.

LEVEY R.H. & MEDAWAR P.B. (1966a) Some experiments on the action of antilymphoid antisera. *Ann. N.Y. Acad. Sci.* In press.

LEVEY R.H. & MEDAWAR P.B. (1966b) Nature and mode of action of antilymphocytic antiserum. *Proc. nat. Acad. Sci.* **56,** 1130.

LINDER O.E.A. (1962a) Further studies on the state of unresponsiveness against skin homografts, induced in adult mice of certain genotypes by a previous ovarian homograft. *Immunology* **5,** 195.

LINDER O.E.A. (1962b) Modification of the homograft response after pretreatment with ovarian grafts. *Ann. N.Y. Acad. Sci.* **99,** 680.

LORENZ E., CONGDON C. & UPHOFF D. (1952) Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections. *Radiology* **58,** 863.

LUMSDEN T. (1938) Agglutination tests in study of tumour immunity, natural and acquired. *Amer. J. Cancer* **32,** 395.

MCGREGOR D.D. & GOWANS J.L. (1963) The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct. *J. exp. Med.* **117,** 303.

MCGREGOR D.D. & GOWANS J.L. (1964) Survival of homografts of skin in rats depleted of lymphocytes by chronic drainage from the thoracic duct. *Lancet* **1,** 629.

MAIN J.M. & PREHN R.T. (1955) Successful skin homografts after the administration of high dosage X irradiation and homologous bone marrow. *J. nat. Cancer Inst.* **15,** 1023.

MAIN J.H. & PREHN R.T. (1957) Fate of skin homografts in X-irradiated mice treated with homologous marrow. *J. nat. Cancer Inst.* **19,** 1053.

MARTINEZ C., SHAPIRO F., KELMAN H., ONSTAD T. & GOOD R.A. (1960) Tolerance of F_1 hybrid skin homografts in the parent strain induced by parabiosis. *Proc. Soc. exp. Biol. Med.* **103,** 266.

MATHE G., BERNARD J., DE VRIES M.J., SCHWARZENBERG L., LARRIEU M.J., LALANNE C.M., DUTREIX A., AMIEL J.L. & SURMONT J. (1960) Nouveaux essais de greffe de moelle osseuse homologue après irradiation totale chez des enfants atteints de leucémie aiguë en remission. Le problème du syndrome secondaire chez l'homme. *Rev. Hémat.* **15,** 115.

MATSUKURA M., MERY A.M., AMIEL J.L. & MATHE G. (1963) Investigation on a test of histocompatibility for allogeneic grafts. II. A study on rabbits. *Transplantation* **1,** 61.

MEDAWAR P.B. (1963) The use of antigenic tissue extracts to weaken the immunological reaction against skin homografts in mice. *Transplantation* **1,** 21.

MEDAWAR P.B. & RUSSELL P.S. (1958) Adrenal homografts in mice, with special reference to 'immunological adrenalectomy'. *Immunology* **1,** 1.

MERRILL J.P., MURRAY J.E., HARRISON J.H., FRIEDMAN E.A., DEALY J.B. & DAMMIN G.J. (1960) Successful homotransplantation of the kidney between non-identical twins. *New Engl. J. Med.* **262,** 1251.

MICHIE D. & WOODRUFF M.F.A. (1962) Induction of specific immunological tolerance of homografts in adult mice by sublethal irradiation and injection of donor type spleen cells in high dosage. *Proc. roy. Soc. B.* **156,** 280.

MICHIE D., WOODRUFF M.F.A. & ZEISS I.M. (1961) An investigation of immunological tolerance based on chimaera analysis. *Immunology* **4,** 413.

MILLER J.F.A.P. (1965) The thymus and transplantation immunity. *Brit. med. Bull.* **21,** 111.

MITCHISON N.A. (1954) Passive transfer of transplantation immunity. *Proc. roy. Soc. B.* **142,** 72.

MÖLLER G. (1963) *On the Role of Isoimmune Reactions in Tissue Transplantation.* Monograph: Stockholm: Tryckeri Balder Ab.

MONACO A.P., WOOD M.L. & RUSSELL P.S. (1965) Adult thymectomy: Effect on recovery from immunologic depression in mice. *Science* **149,** 432.

MURPHY J.B. (1926) The lymphocyte in relation to tissue grafting, malignant disease and tuberculous infection. *Monographs of Rockefeller Institute for Medical Research.* No. 21.

MURRAY J.E., GLEASON R. & BARTHOLOMAY A. (1965) Fourth report of the human kidney transplant registry: 16th September 1964 to 15th March 1965. *Transplantation* **3,** 684.

MURRAY J.E., MERRILL J.P., HARRISON J.H., WILSON R.E. & DAMMIN G.J. (1963) Prolonged survival of human kidney homografts by immuno-suppressive drug therapy. *New Eng. J. Med.* **268,** 1315.

NATHENSON S. & DAVIES D. (1966a) Transplantation antigens. Studies of the mouse model system. Solubilization and partial purification of H-2 isoantigens. *Ann N.Y. Acad. Sci.* **129,** 6.

NATHENSON S. & DAVIES D. (1966b) Solubilization and partial purification of mouse histocompatibility antigens from a membranous lipoprotein fraction. *Proc. Nat. Acad. Sci.* **56,** 476.

OWEN R.D. (1945) Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* **102,** 400.

PORTER K.A. (1957) Effect of homologous bone marrow injections in X-irradiated rabbits. *Brit. J. exp. Path.* **38,** 401.

RAFFEL S. (1962) Personal communication.

RAPAPORT F.T., THOMAS L., LAWRENCE H.S., CONVERSE J.M., TILLETT W.S. & MULHOLLAND J.H. (1962) Skin homograft sensitivity cross reactions in man. *Ann. N.Y. Acad. Sci.* **99,** 564.

VAN ROOD J.J. & VAN LEEUWEN A. (1965) Defined leukocyte antigenic groups in man. In *Histocompatibility Testing*, Part 1, p. 21, ed. RUSSELL P.S. & WINN H.J. National Academy of Sciences Publication No. 1229.

ROSS D.N. (1966) Aortic valve surgery. *Ann. roy. Coll. Surg. Eng.* **39,** 192.

RUBIN B.A. (1959) Tolerance to skin homografts of adult mice after parabiosis. *Nature* **184,** 205.

RUSSELL P.S. (1960) The weight-gain assay for runt disease in mice. *Ann. N.Y. Acad. Sci.* **87,** 445.

RUSSELL P.S. (1962) The modification of runt disease in mice by various means. In *Transplantation*, p. 350, Ciba Foundation Symposium. London: J. & A. Churchill.

SHAPIRO F., MARTINEZ C., SMTIH J.M. & GOOD R.A. (1961) Tolerance of skin homografts induced in adult mice by multiple injections of homologous spleen cells. *Proc. Soc. exp. Biol. Med.* **106,** 472.

SIMONSEN M. (1957) The impact on the developing embryo and newborn animal of adult homologous cells. *Acta path. microbiol. scand.* **40,** 480.

SIMONSEN M. (1960) On the acquisition of tolerance by adult cells. *Ann. N.Y. Acad. Sci.* **87,** 382.

SIMONSEN M. (1962) Graft versus host reactions. Their natural history and applicability as tools of research. In *Progress in Allergy*, vol. 6, p. 349. S. Karger, Basel (Switzerland), New York.

SIMONSEN M. (1965) Strong transplantation antigens in man. *Lancet* **1,** 415.

SIMONSEN M. & JENSEN E. (1959) The graft-versus-host assay in transplantation chimaeras. In *Biological Problems of Grafting*, p. 214. University of Liège Symposium. Oxford: Blackwell Scientific Publications.

SNELL G.D. (1948) Methods for the study of histocompatibility genes. *J. Genet.* **49,** 87.

STARZL T.E. (1964) *Experience in Renal Transplantation.* W.B. Saunders Company: Philadelphia, London.

STARZL T.E., MARCHIORO T.L., PORTER K.A., IWASAKI Y. & CERILLI G.J. (1967) The use of heterologous antilymphoid agents in canine renal and liver homotransplantation, and in human renal homotransplantation. *Surg. Gynec. Obstet.* **124,** 301.

TERASAKI P.I., MARCHIORO T.L. & STARZL T.E. (1965) Sero-typing of human lymphocyte antigens: Preliminary trials on long-term kidney homograft survivors. In *Histocompatibility Testing.* Part 1, p. 83, ed. RUSSELL P.S. & WINN H.J. National Aacdemy of Sciences Publication No. 1229.

TRENTIN J.J. (1957) The immunological basis for induced tolerance to skin homografts in irradiated mice receiving bone marrow transfusions. *Transpl. Bull.* **4,** 74.

TUBIANA M., LALANNE C.-M. & SURMONT J. (1961) Total body irradiation for organ transplantation. *Proc. roy. Soc. Med.* **54,** 1143.

UPHOFF D.E. (1958) Alteration of homograft reaction by A-methopterin in lethally irradiated mice treated with homologous marrow. *Proc. Soc. exp. Biol. (N.Y.)* **99,** 651.

WAKSMAN B.H., ARBOUYS S. & ARNASON B.G. (1961) The use of specific 'lymphocyte' antisera to inhibit hypersensitive reactions of the 'delayed' type. *J. exp. Med.* **114,** 997.

WEBER R.A., CANNON J.A. & LONGMIRE W.P. (1954) Observations on the regrafting of successful homografts in chickens. *Ann. Surg.* **139,** 473.

WOODRUFF M.F.A. (1957) Transplantation immunity and the immunological problem of pregnancy. *Proc. roy. Soc. B.* **148,** 68.

WOODRUFF M.F.A. (1960) *The Transplantation of Tissues and Organs.* Springfield, Ill.: Charles C. Thomas.

WOODRUFF M.F.A. (1961) Unsuccessful attempts to eliminate selectively clones of immunologically competent cells. *Transpl. Bull.* **27,** 447.

WOODRUFF M.F.A. (1965) Specific immunological tolerance. *Lect. Sci. Basis Med.*, p. 120. London: Athlone Press.

WOODRUFF M.F.A. (1966a) Experience with transplantation of the kidney in man. *Ann. roy. Coll. Surg. Eng.* **39,** 178.

WOODRUFF M.F.A. (1966b) Antilymphocyte serum as an immunosuppressive agent. In *Transplantation von Organen un Geweben.* Eds. K.E. Seiffert and R. Geissendörfer. pp. 93–97. Stuttgart. George Thieme Verlag.

WOODRUFF M.F.A. & ANDERSON N.F. (1963) Effect of lymphocyte depletion by thoracic duct fistula and administration of antilymphocytic serum on the survival of skin homografts in rats. *Nature* **200,** 702.

WOODRUFF M.F.A. & ANDERSON N.F. (1964) The effect of lymphocyte depletion by thoracic duct fistula and administration of antilymphocytic serum on the survival of skin homografts in rats. *Ann. N.Y. Acad. Sci.* **120,** 119.

WOODRUFF M.F.A., ROBSON J.S., MCWHIRTER R., NOLAN B., WILSON T.I., LAMBIE A.T., MCWILLIAM J.M. & MACDONALD M. (1962) Transplantation of a kidney from a brother to sister. *Brit. J. Urol.* **34,** 3.

WOODRUFF M.F.A. & SIMPSON L.O. (1955) Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth. *Brit. J. exp. Path.* **36,** 494.

WOODRUFF M.F.A. & SPARROW M. (1957) Further observations on the induction of tolerance of skin homografts in rats. *Transpl. Bull.* **4,** 157.

WOODRUFF M.F.A. & SPARROW M. (1958) Induction of tolerance to homografts of thyroid and adrenal in rats. *Quart. J. exp. Physiol.* **43,** 91.

WOODRUFF M.F.A. & WOODRUFF H.G. (1950) The transplantation of normal tissue: with special reference to auto- and homotransplants of thyroid and spleen in the anterior chamber of the eye, and subcutaneously, in guinea-pigs. *Phil. Trans. B.* **234,** 559.

CHAPTER 18

THE ALLERGIC RESPONSE IN MALIGNANT DISEASE

P. ALEXANDER & G. HAMILTON FAIRLEY

INTRODUCTION

There are two distinct immunological problems in malignant disease. First, there is the effect that malignant disease may have on the normal immunity or allergic mechanisms, and this has been studied extensively in man. Secondly, there is the possibility of an allergic or immune reaction by the host directed against his own tumour, which in turn leads to the question of whether the

behaviour of malignant disease can be modified by immunological procedures. Because of the difficulties of research into this problem in man, most of the experimental work has been done in animals.

The possibility that immunological factors influence the growth of tumours has been considered for over half a century (cf. Ehrlich 1909), but it is only in the past decade that real evidence has accumulated to support this view. There are two reasons for this. First, much of the early work was concerned with attempts to demonstrate circulating antibodies against malignant tissue, and the difficulty here is to prove that an antiserum is specific for a particular tumour. Secondly, many of the earlier transplantation experiments were carried out in genetically dissimilar animals and the tumours were simply rejected like any other homograft.

It was only when transplantation experiments with tumours were carried out in genetically identical (syngeneic) animals that the presence of tumour-specific antigens could be proved and immunological methods for modifying the growth of tumours could be investigated.

There is now definite evidence in animals and to a much lesser extent in man that there are tumour-specific antigens of a transplantation type, that there is a reaction of the host against its own tumour, that this reaction is in some cases immunological, mediated by lymphocytes, and similar to the delayed hypersensitivity reaction and homograft rejection. It is necessary to consider the properties of homograft tumours and their modification by immunological means, before those of experimentally induced tumours in animals and of spontaneous tumours in man.

STUDIES IN EXPERIMENTAL ANIMALS

Properties of Homograft Tumours

The majority of primary tumours show the same transplantation behaviour as ordinary tissues and will not grow as homografts unless special conditions prevail such as pretreatment of the host with immuno-suppressive agents or the induction of specific tolerance. The existence of sites, such as the anterior chamber of the eye, the cheek pouch of the hamster or the brain where for a variety of reasons (see Chapter 17) homograft reactivity is impaired can be easily demonstrated with tumour transplants.

Very occasionally a primary tumour will grow in genetically different recipients. Such tumours initially require large inocula and their transplantation behaviour is erratic. After prolonged serial passage stable tumour lines may be obtained which give successful (i.e. eventually lethal) grafts in allogeneic hosts every time. An important reason for the growth of such tumours is that cells capable of very rapid proliferation have been selected, so that by the time the

host has developed the capacity to react immunologically (i.e. about 4 to 7 days after immunization) the size of the tumour is beyond control by host reactions. The appearance and selection of cells that are less antigenic, i.e. have lost some of the transplantation antigens, and therefore immunize less effectively also contribute to the growth of tumour homografts in some cases.

In no instance, however, is the loss of antigenicity absolute and such tumours always retain the essential characteristics of a homograft as shown by the fact that animals can be immunized against them. This is best demonstrated by removing the homograft surgically and then challenging the animal with a second graft of the same tumour. Rejection of the graft demonstrates 'immunity'. The machinery for the elimination of the tumour cells is already in existence in the immunized animals and as a result the graft is destroyed before it has had an opportunity to attain a size which overwhelms the host response. Biologically, this process is closely analogous to the accelerated rejection (i.e. the second set response) of a skin homograft by an immunized animal, and as with normal tissues, tumour homograft immunity can be transferred by lymphoid cells from an immune animal.

Role of Lymphocytes

As with skin, the process of tumour graft rejection is accomplished, largely if not wholly, by lymphocytes (probably small) which have the capacity of either killing or preventing the multiplication of the 'target cells', but the nature of this process has not yet been elucidated. Specific cytotoxicity of immune lymphocytes (actively allergized lymphocytes, see Chapter 15) can be demonstrated *in vitro* when it is necessary that the two cell types be brought into close proximity with one another and this raises the possibility that cell-bound antibody on the surface of the lymphocytes binds them to the target cells which are then destroyed by the localized release of enzyme from the lymphocytes.

It is not however at all certain whether the *in vitro* experiments constitute a valid model for the *in vivo* situation. What is clear is that more than one type of lymphocyte is involved (cf. Gowans *et al* 1965) and Fig. 18.1 gives a tentative scheme for three cell types. The process is initiated by an immunologically competent cell—morphologically a small lymphocyte—which has been committed to a specific course of action by immunization (allergization) with the foreign tissue; this may be called the '*primed lymphocytes*'. The process is completed by another lymphocyte—the '*cytotoxic lymphocyte*'—and rather limited experimental evidence suggests that morphologically this again would be classified as a small lymphocyte. In between these two there are the morphologically very characteristic 'pyroninophilic blast cells'. These rapidly dividing cells leave the node by the efferent lymphatics some 50 hours or so after stimulation and being highly motile migrate throughout the lymphoid tissue of the body (see Plate 18.1). The electron microscope shows that they are quite distinct from plasma cells

as they have very little endoplasmic reticulum, but they are extremely rich in polysomes. By cannulating the efferent lymphatic of a locally stimulated node in sheep, Hall *et al* (1966) were able to demonstrate that removal of these cells prevents the formation of antibody yet these cells themselves contained relatively little antibody. They are capable of transferring immunity and are clearly an important intermediary in the dissemination of the immune response, though they contain no actual antigen. Hall *et al* (1966) have referred to them as

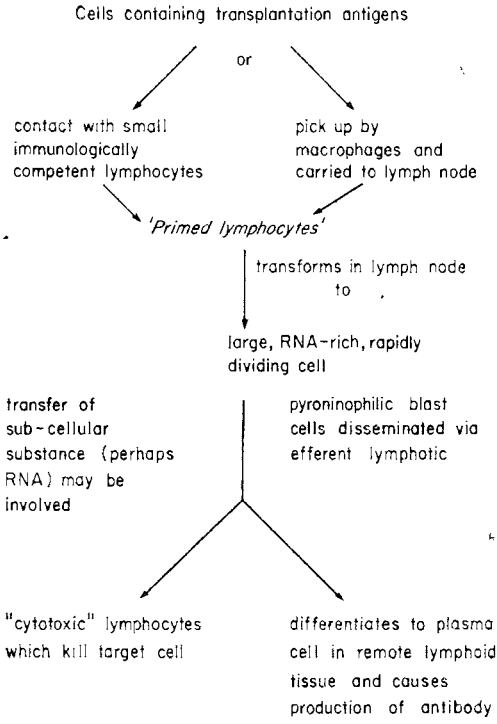


FIG. 18.1. Different lymphocytes involved in homograft reaction.

messenger cells and there is evidence for their transformation into plasma cells (Birbeck & Hall 1967), but in addition they may also act by transferring a subcellular component possibly containing RNA (see later).

The first stage of the immunization may occur by lymphocytes travelling to the tumour graft; the existence of this so-called 'peripheral sensitization' has been experimentally demonstrated by Strober & Gowans (1965). Another and possibly more important step is the transport of antigens by macrophages to the

local node and evidence for this has been obtained by draining the afferent lymphatics of a suitably stimulated node.

Role of Circulating Antibodies

Gorer (1961) showed quite clearly that homograft tumours also evoke the formation of circulating antibodies which, *in vitro*, cause certain tumour cells to lyse as long as complement is present. Also *in vitro* antibody has been shown to act as an opsonin causing specific phagocytosis of tumour cells (Old *et al* 1963). The extent to which this antibody contributes in bringing about the rejection of allogeneic tumour grafts *in vivo* is still uncertain, but in general it appears to play only a minor role possibly because the 19S antibodies which are most cytotoxic cannot gain access to the interior of tumours. Injection of antiserum has in some systems (see later) caused ascites tumours to regress, but with solid tumours this procedure is either without any effect or causes the tumour to grow more rapidly. This phenomenon is called enhancement (see Chapter 17) and there are at least two ways by which this effect is brought about. The injected antiserum may combine with the tumour antigens and interferes with their stimulation of the allergic mechanism of the host. This will reduce the magnitude of the effective (i.e. cell mediated) anti-tumour response of the host. Possibly of greater biological importance is the finding—so far confined to guinea-pigs and mice—of a class of γ-globulin in animals immunized with foreign tissue which are not cytotoxic even in the presence of complement, but which coat the target cells and render them resistant to destruction by the cytotoxic lymphocytes. Enhancement may therefore be a manifestation of a physiological process protecting homografts. The rate of graft rejection (or indeed its survival) will be determined by the interplay between 'protective' antibody and the 'cytotoxic' lymphocytes. The production of 'protective' antibodies may be one of the factors allowing the genesis of primary tumours containing transplantation antigens. It would be interesting to learn whether such antibodies contribute to the protection of the trophoblast.

The Immunology of Experimentally Induced Primary Tumours

There is a vast literature on the use of serological methods and related procedures, such as the induction of anaphylaxis to detect differences between cancer and normal cells. By preparing heterologous antiserum, tumours can be shown to have suffered both antigenic loss and antigenic gain when compared with tissues from their cells of origin (*cf.* Haddow 1965). The loss of organ specific antigens by tumours has been frequently recorded and may be of relevance to the biochemical nature of the malignant transformation. The significance of new antigens detected serologically is difficult to determine; there is always the possibility of infection particularly by passenger viruses. Differentiation of tumour cells

may also cause the appearance of antigenic substances not present in the normal tissue but having no direct relevance to cancer. For example, in chemically induced hepatomata a so-called tumour antigen proved to be foetal serum albumin—in the adult animal this material was indeed confined to the hepatoma, but in no sense is it an antigen peculiar to tumours (Abelev 1965). Another example where an antigen present only in embryonal life reappears in the adult when a neoplastic process has occurred has been reported in human colon carcinomas (Gold & Freedman 1965). Of great importance is the relatively recent discovery (see reviews by Old & Boyse 1964, 1966; Klein & Klein 1962) of antigens on the surface of primary tumour cells which have many of the properties of transplantation antigens and which are: (1) truly specific to tumours, and (2) capable of evoking a host response which is cytotoxic to the tumour cells

TABLE 18.1

Protection of mice against syngeneic lymphoma cells by prior immunization with irradiated tumour cells (Alexander *et al* 1966)

Number of L5178 Y cells injected	Number of animals with tumours
1. Non-immunized DBA/2 mice	
1	35%
10	90%
10^3	100%
2. Immunized with 10^7 L5178 Y cells irradiated *in vitro* with 3000 rads of X-rays	
10^3	0%
10^4	0%
10^5	20%
10^6	80%

only and which does not affect the normal cells of the tumour-bearing animal. Such antigens were first recognized by transplantation and their identification depends on transplantation tests between syngeneic (i.e. genetically identical) animals as found in highly inbred lines of mice, rats and guinea-pigs.

INDUCED TUMOURS

Tumours that have been induced by carcinogen (chemical, viral or physical) in one member of a pure inbred line of animals are almost always readily transplantable to other animals of the same strain and in this respect resemble skin grafts which invariably take between such animals. But while no method of immunization can prevent skin grafts from being accepted between pure line animals this does not apply to the grafting of tumours. Syngeneic tumours can be prevented from growing in the strain of origin by immunization. Immuniza-

tion is usually achieved either by removing, surgically or by ligation, a tumour transplant that had grown in a syngeneic recipient or by injecting cells that have been rendered incapable of indefinite growth by exposure to X-rays or chemicals. Immunization is most effective if such cells are 'physiologically alive' in that normal metabolism continues and that they are impermeable to dyes. Such immunized animals fail to develop tumours when challenged with an inoculum of cells that causes tumours in unimmunized animals (see Table 18.1 and Fig. 18.2).

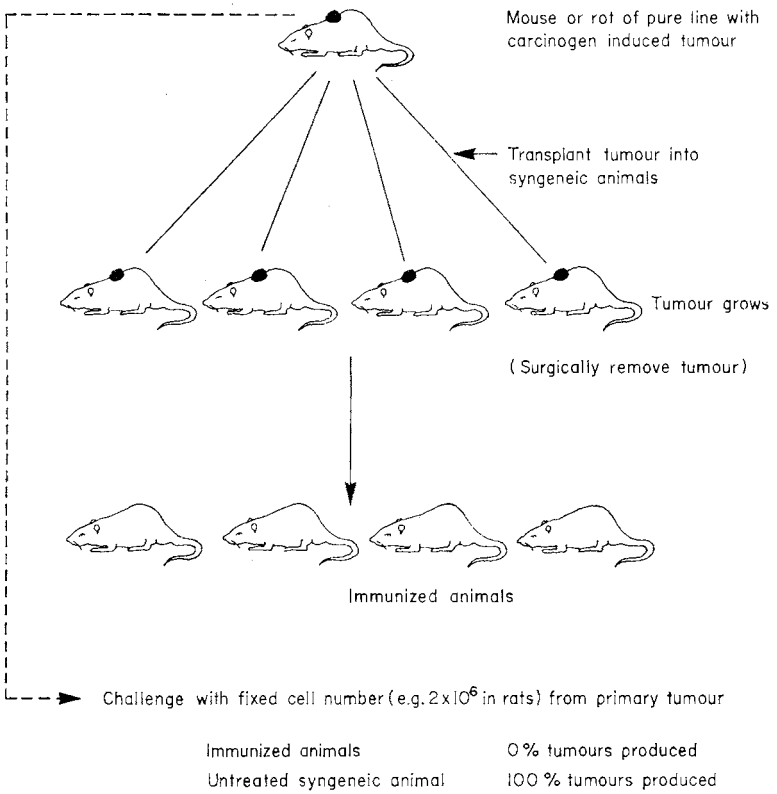


FIG. 18.2. Method of inducing immunity to syngeneic tumours.

In the initial experiments of Foley (1953) and of Prehn & Main (1957) immunity could only be achieved against some sarcomata and the presence of tumour specific antigens in these tumours was not therefore thought to be of universal occurrence. The failure to find them in all of the tumours was due to the fact that tumour specific antigens are sometimes very weak so that the degree of immunity that is produced is only sufficient to cause the rejection of relatively few cells, while a trocar implant of tumour will grow because it

overwhelms the response (see Table 18.2). If the procedure of challenging with minimal cell dose is used then many chemically and almost every virally induced tumour and leukaemia that has been studied has been found to be antigenic though the need for pure line animals has limited these studies to rats

TABLE 18.2

Immunization by an isogenic tumour which is removed surgically is only able to cause the rejection of a second graft of the same chemically induced fibrosarcomata if this challenge contains relatively few cells (Old *et al* 1962)

(*a*) Weakly antigenic tumour

Challenge with	Controls untreated*	Immunized by tumour transplant killed by ligation
Trocar piece	5/5	5/5
400,000 cells	5/5	5/5
50,000 cells	4/5	0/5
5000 cells	3/5	0/5

* Animals with progressive tumour growth/animals challenged.

(*b*) Strongly antigenic tumour

Challenge with	Controls untreated*	Immunized by tumour transplant killed by ligation
Trocar piece	6/6	6/6
400,000 cells	6/7	1/7
93,000 cells	4/7	2/7
11,000 cells	1/7	0/7
1000 cells	3/7	0/7
500 cells	1/7	
100 cells	2/7	

* Animals with progressive tumour growth/animals challenged.

and mice. In these species a very wide spectrum of tumour types (e.g. sarcoma, carcinoma, hepatoma, lymphoma, etc.) has now been investigated and the carcinogens used for induction include most of the well-known chemical carcinogens as well as oncogenic viruses and physical agents such as inert plastic films and ultraviolet radiation.

Spontaneous Tumours

Tumours which arose in mice without exposure to any specific carcinogen were thought to be non-antigenic on the basis of a trocar challenge but more refined tests have shown that this is no longer true. The so-called spontaneous mammary carcinoma and leukaemias which arise with a very high incidence in certain specific strains of mice (e.g. C3H mice for mammary tumours and AK mice for leukaemia) are due to a virus, the infective agent being transmitted vertically from mother to progeny. Such tumours are readily transplantable to animals that have not yet developed a spontaneous neoplasm and syngeneic animals cannot be immunized against such tumour grafts. This however is not—as was once believed—due to the absence of tumour specific antigens. As a result of

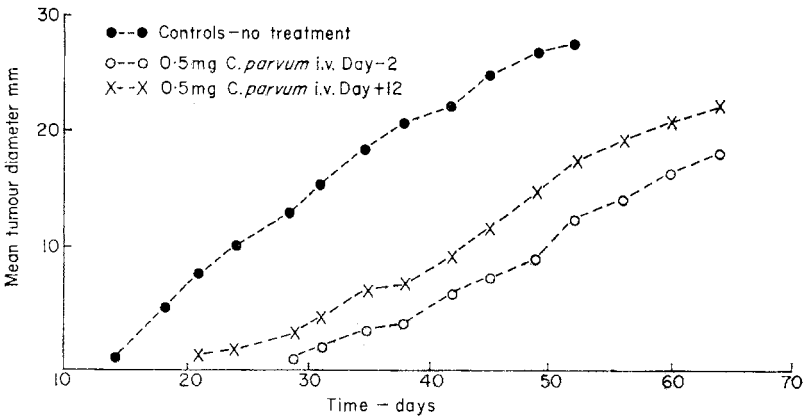


Fig. 18.3. Effect of non-specific stimulation of RE system with Corynebacterium parvum on the rate of growth of syngeneic murine mammary carcinoma (Woodruff *et al* 1966).

neonatal contact with the virus all animals in the strain are tolerant to the tumour specific antigen and hence cannot be immunized (Volkert & Spärck 1965). Hybrids in which the father is of the high tumour strain (e.g. C3H) and the mother of another strain (e.g. C57) will accept, in accordance with the normal laws of transplantation, both C3H and C57 cells, but they are not tolerant to the tumour specific antigens because there has been no neonatal exposure to the antigenic virus. Such F_1 hybrids can be immunized against the 'spontaneous' tumours of the paternal strain by standard methods like those illustrated in Fig. 18.2. (Morton 1965.)

The position with regard to sporadically occurring spontaneous tumours in mice and rats is confusing; a degree of immunity can be obtained by immunization against some of them, indicating the presence of weak tumour

specific antigens (Riggins & Pilch 1964; Baldwin 1966) but with others even the most sensitive tests have failed to reveal any host resistance following immunization. Negative results of this type cannot, of course, exclude completely the existence on such tumours of specific transplantation-type antigens, but they should be borne in mind before extrapolating too readily from the near universality of tumour specific antigens in tumours induced by viruses or chemical carcinogens to neoplasms of other aetiology. In this connection it may be relevant to point out that sarcomata induced by ionizing radiations have also not, so far, been shown to be immunogenic.

Properties of the Tumour-Specific Antigens

The specific transplantation-type antigens of tumours induced by chemical carcinogens or by plastic films are very diverse and a cross-resistance has been encountered only very occasionally, if at all (Klein & Klein 1962). In general, every tumour seems to have a unique antigen. If, say, twenty different primary sarcomata of identical morphology are induced in the same strain of mice by the same chemical agent, virtually all will be antigenic yet none show cross-resistance. Globerson & Feldman (1964) produced two sarcomata at different sites in the same mouse as the result of two separate injections of 3:4-benzpyrene. Both tumours were antigenic by the usual transplantation procedures, but they did not cross-react. The number of tumour specific antigens is unlikely to be infinite and cases of true cross-resistance may well be discovered, but there is every reason to believe that they will be rare.

For virus-induced tumours the situation is the exact opposite (cf. Old & Boyse 1965) and all the tumours induced by a given oncogenic virus have the same tumour specific antigens so that one tumour can be used to immunize against transplants of all other tumours induced by this virus. Each oncogenic virus, however, causes the formation of a different set of new antigens. Cross-reaction extends to tumours induced in different strains and even different species.

Viruses need not be present in the tissue used to induce immunity to tumour transplants. While RNA viruses usually persist in the tumour cells even after prolonged transplantation this is not the case for DNA viruses which, in general, cannot be found in the cells of the tumour they have induced. The specific transplantation antigen in DNA virus induced tumours is not that of the protein of the virus. The virus as part of the carcinogenic process causes the cells that are transformed to make characteristic new antigens some of which are situated on the cell surface and it is these which elicit a cytotoxic action of the homograft type. With some oncogenic viruses there is also serological evidence for the presence of tumour specific antigens which however are not of the transplantation type (see Table 18.3).

Under some conditions exposure of animals to the virus is sufficient to render them resistant to a subsequent graft of an isogenic tumour which had originally

been induced by this particular virus. The reason for this can be that the virus used for immunization transforms some cells—without however causing a tumour—so that they contain the particular tumour specific antigen and the animals thereby acquire resistance to cells containing such antigens. This

TABLE 18.3

Specific antigens in experimentally induced tumours

Nature of antigen	Cell mediated immunity	Activity of serum from immunized syngeneic animals
(*a*) *in chemically induced sarcomata*		
Transplantation type on cell surface	+	−
(*b*) *in virus-transformed* cells*		
Transplantation type on cell surface (caused by infection with RNA and DNA viruses)	+	Fluorescent antibodies binding to tumour cells, cytotoxic (in presence of complement) only to lymphoid tumour cells.
Internal—often nuclear (caused by infection with RNA and DNA viruses)	−	Complement fixation and immunofluorescence
Components of the virus (in general only found with RNA viruses)	+ (?)†	Complement fixation and immunofluorescence

* Cells may be transformed *in vivo* without the production of a tumour; the reasons for this may be that they are eliminated by host reactions or that there are several stages of transformation and the induction of the capacity to make new antibodies is not sufficient to render cells malignant.

† RNA viruses may be incorporated in the membrane of the cell and may be responsible for the cytotoxic host response, mediated by lymphocytes. The intracellular antigens in RNA virus induced tumours may also be the same as those in the protein coat of the virus. As yet there is no compelling reason for postulating that RNA viruses induce the formation of new proteins other than viral protein. In tumours induced by DNA viruses, at least two new proteins are present, one on the membrane and the other inside the cell.

phenomenon illustrates some of the complexities in the relationship between the appearance of the tumour specific antigen and the transformation to malignancy. Immunity to tumour grafts has also been produced with killed RNA viruses and this suggests a close relationship between the tumour antigen d an the viral protein.

The chemical nature of the tumour specific antigens is not known and they may be very unstable or susceptible to inactivation by enzymes since activity is completely or partially lost when the cells are disrupted or homogenized and a method of preparing an effective cell-free extract capable of immunizing against syngeneic tumours has not yet been described. Improved methods for the isolation of plasma membranes (cf. Davies 1966) have yielded cell-free preparations of normal transplantation antigens which immunize effectively against the strong H2 transplantation barrier and such procedures may be applicable also to tumour specific antigens. Homogenates of tumour cells induced by RNA viruses are active not because the tumour specific antigen is preserved, but because a virus is released which, on injection, leads to immunity (see above).

The antigenicity of virus-induced tumours remains unaltered after long periods of transplantation but the fate of the specific antigens in chemically induced tumours on successive transplantation is less clear. Instances have been reported where the antigenicity decreases though a complete loss of antigenicity was not observed.

Cell-Mediated Host Reaction

Cells from tumours—whether these were originally induced by viruses or by chemical carcinogens—can be prevented from growing in suitable recipients if, prior to grafting, they are mixed *in vitro* with lymphoid cells from syngeneic animals that had been immunized (e.g. by exposure to irradiated tumour cells) against the specific tumour, Klein *et al* (1960) used spleen cells whereas Old *et al* (1963) used cells from a peritoneal exudate. The specificity of the lymphoid cells follows that of the tumour specific antigens as demonstrated by immunity to transplantation (i.e. no cross resistance with chemically induced tumours, but a common determinant with virus-induced tumours). Lymphocytes from syngeneic animals immunized with tumour have also proved effective against established tumours (e.g. primary fibrosarcoma, Delorme & Alexander 1964, and ascites lymphoma, Alexander *et al* 1966).

In tissue culture, allogeneic immune lymphocytes (i.e. directed against H2 transplantation barriers) have been shown to arrest the growth of tumour cells to which they have been claimed to adhere (e.g. Taylor *et al* 1963). A similar action of syngeneic lymphocytes directed against only the tumour specific antigen has been observed (Delorme *et al* 1966; Rosenau *et al* 1966; Alexander *et al* 1966) but the effect is small and it may be questioned whether this *in vitro* test constitutes a valid model for the *in vivo* action of immune syngeneic lymphocytes when they appear to be much more effective.

By suitable absorption techniques, animals immunized with an allogeneic tumour could be shown to make cytotoxic lymphocytes directed both against the normal and the tumour specific transplantation antigens (see later).

Role of Circulating Antibodies

The production of tumour specific circulating antibodies by animals that have been immunized against syngeneic tumours—as judged by graft rejection—is complex (see Table 18.3) and there would appear to be at least four situations:

1. No activity can be detected in the serum from immune animals by either cytotoxic, immunofluorescent or agar diffusion techniques but their lymphocytes inhibit tumour growth. This appears to be the case for all chemically induced sarcomata (Klein & Klein 1962) and the only indication for the presence of a circulating factor comes from *in vitro* studies of phagocytosis suggesting the presence of a specific opsonin (Old *et al* 1963).

2. For many leukaemias and lymphomas syngeneic serum is cytotoxic (i.e. damages the cell membrane and allows dyes to penetrate) in the presence of complement *in vitro* and no activity can be demonstrated *in vivo*.

3. Some other lymphomas (Gorer & Amos 1956; Alexander *et al* 1966) give rise in allogeneic animals to an antiserum which after extensive absorption with normal tissue also inhibits tumour growth *in vivo* presumably by acting against the tumour specific antigens. The surprising feature is that the *in vivo* acting antibody is not apparently produced in syngeneic animals.

4. The serum from animals immunised against most virus-induced tumours whether they be sarcomata or lymphomas contain both complement fixing antibodies and antibodies which by immunofluorescence can be shown to be absorbed by the tumour cells. They are only cytotoxic *in vitro* to lymphomas and then only if complement is present (see Table 18.3).

Even in those cases where circulating antibody has been demonstrated its role in the immune process *in vivo* is obscure and there is no evidence that it plays an active part in the rejection of isografts. Specific anti-tumour serum has been used in experimental studies to characterize antigens and to follow changes in antigenicity. In man, where it is not possible to use transplantation tests to detect tumour specific antigens, absorption of fluorescent antibody by cells has been one of the few techniques available for this purpose. A positive finding using autologous serum indicates their presence but from a negative result little can be deduced.

Escape Mechanisms which Permit the Growth of Antigenic Tumours

The nature of the mechanisms which make it possible for malignant cells containing transplantation-type antigens to develop into tumours constitute a key problem of cancer immunology. The continued growth of a palpable tumour mass in the face of host resistance is probably a reflection of the fact that the production of new tumour cells is greater than the host's capacity for their elimination, but this cannot be the case in the early stages of tumour development when there are relatively few cancer cells.

Immune Paralysis

Generalized depression of the immune or allergic response may be one factor in the sharp rise of cancer incidence with age which is such a characteristic feature of the majority of human neoplasias. In experimental animals the magnitude of the response to a primary antigenic stimulus has been shown to fall towards the end of the normal life-span. Some carcinogenic agents, notably the polycyclic hydrocarbons, will in relatively high doses reduce the capacity of animals to mount a primary immune response (Prehn 1963; Stjernswärd 1965). It is doubtful however if immune suppression is a requirement for chemical carcinogenesis since tumours develop in hosts which are fully competent to react immunologically (see below).

Immune Isolation

Inert films of plastic, metal or glass, when implanted subcutaneously induce sarcomata which contain transplantation antigens. The initial reaction to such implants is the formation of a pocket surrounded by an acellular dense connective tissue pocket on the inside of which the tumours originate. This pocket is isolated from normal defence reactions (Alexander & Horning 1959; Bates & Prehn 1965) and it is conceivable that antigenic tumour cells may proliferate here relatively unopposed.

Specific Tolerance

Specific tolerance to the tumour antigens has been shown to exist in mice of strains having a high incidence of mammary carcinoma or leukaemia (see earlier). The reason for the high 'spontaneous' rate in such strains is associated with the vertical transmission of a virus and it is reasonable to suppose that exposure of the foetus to the virus carried by the mother is responsible for the state of tolerance. In general, viruses are most effective in inducing tumours when the mice are infected in the first days of life; while it is tempting to interpret this in terms of tolerance to virus induced antigens, such tolerance is certainly not complete and animals can in later life be immunized against the transplantation antigens induced by the oncogenic virus with which they were injected at birth. The role of tolerance seems to vary from virus to virus. Animals, carrying primary tumours induced by relatively small innocula of polyoma virus seem to be tolerant but this is not the case with SV 40 or the oncogenic adeno viruses. Factors other than immunosuppression must contribute to allowing virus transformed cells to grow into tumours.

Experiments using homografts suggest that prolonged exposure to antigen may produce limited immunological paralysis—or specific tolerance—in the primary host. With chemically induced sarcoma—unlike those induced by polyoma—this would not appear to be the case. Klein *et al* (1960), and Mikulska, Smith & Alexander (1966) showed that animals react actively against these

tumours once they have become large even without any attempts at further immunization. Primary chemically induced sarcomata in rats were removed surgically and their cells injected back into the primary host. The viability of the cell suspensions was tested by its ability to grow in normal syngeneic rats. While the cells from different tumours invariably grew as syngeneic grafts the autografts were frequently rejected. Autografts were only found to grow if a substantial portion of the primary tumour had been left *in situ*. An interpretation of these results is that the primary host was actively reacting against the tumour and that in the absence of the primary tumour the immune capacity was sufficient to reject an autograft. When the primary tumour was present it exhausted the host response and hence the autograft grew.

This interpretation was strengthened by the demonstration that spleen cells taken from a rat in which a primary sarcoma had been induced were cytotoxic to the tumour cells (i.e. spleen cells from the rat in which the primary tumour had occurred were capable of preventing the transplantation of cells from this tumour in a syngeneic recipient). Lymphoid cells from a tumour bearing animal behaved in this respect in exactly the same way as lymphoid cells from a syngeneic animal immunized against the tumour (see earlier). The autochthonous spleen cells, however, were only cytotoxic if they were taken from animals in which the tumour had been surgically removed three weeks earlier. When the spleen and tumour were removed at the same time then the spleen cells had no appreciable effect on the growth of the autochthonous tumour on transplantation. The actively growing primary tumour appears to exhaust the supply of anti-tumour lymphocytes that are produced by the host, but after the tumour is removed the concentration of the cytotoxic lymphocytes builds up and the animal can reject an autograft.

Tumour Growth Versus Rate of Immunization

The existence of escape under conditions when there is no possibility of supression of the immune response is evident from the growth of grafted tumours. Small inocula—occasionally one cell is sufficient—of tumour cells grow and eventually kill a normal host that after suitable immunization develops a degree of immunity which is capable of rejecting a challenge of somewhere between 10^4 and 10^7 tumour cells—depending on the strength of the particular antigen (see Tables 18.1 and 18.2). One reason is that after inoculation tumour cells remain initially at the site of injection and grow to give a local lesion without immunizing the host. Old *et al* (1962) found that some highly antigenic sarcoma cells would grow from an inoculum containing either a few cells or very many cells, but an intermediate number of cells was rejected after they had grown to minute nodules. The interpretation is that few cells did not immunize because they remained localized until they had grown to a tumour which has passed the critical size for host control. With a graft of several thousand cells, some of these migrated and provided an antigenic stimulus causing an allergic response or

immunity sufficient to reject small growths. With the large inoculum tumour growth was so rapid that it overwhelmed the host response. These experiments illustrate one mechanism by which an antigenic tumour can arise even if the host's immune or allergic response is normal and that suppression of the immune response is not necessary for chemical carcinogenesis. The observation of Prehn (1963) that the most rapidly growing primary tumours are often the most antigenic is not as paradoxical as it may appear.

Similar considerations might explain the greater susceptibility of newborn animals to oncogenic viruses. The period of relative immunological immaturity after inoculation tips the balance in the race between the multiplication of tumour cells to produce a mass too great for immunological rejection and the establishment of maximum host response. With chemically induced tumours the release of antigenic cells may be the critical factor in determining whether a malignant focus develops into a tumour.

Protective Antibody

Reference has already been made in relation to the phenomenon of enhancement (see earlier and Chapter 17) to the existence of antibodies which coat foreign cells and thereby render them resistant to cytotoxic lymphocytes. If such antibodies were also formed in response to tumour specific antigens in the primary host they might protect the tumour in the early stages until it has passed its critical size. The paradoxical finding of Mathé *et al* (1964) that those choriocarcinoma patients with the highest titre of anti-husband leukocyte agglutinating serum showed the slowest rejection rate of the husband's skin might be interpreted in terms of the presence of 'protecting' antibody.

The Role of Host-Reactions in Conventional Therapy

In animal experiments homograft tumours such as the Walker carcinoma, Sarcoma 180 and Ehrlich ascites cells can be readily cured by a large number of chemotherapeutic agents. These successful results are not the result of eradicating the last cancer cell. Far from it—these tumours grow in the face of a host resistance which is capable of eliminating millions of cells, but not the vast tumour mass which has formed by the time the host response has been established. A procedure which kills most of the tumour cells without at the same time abolishing immunity will therefore in conjunction with the host reaction bring about a 'cure'. While most of the cytotoxic agents used in cancer chemotherapy damage the lymphoid organs, immunosuppressive action against an established immune response is relatively small. This is clearly shown by the fact that animals that have had their homograft tumours 'cured' in this way are immune and will reject a subsequent challenge with the same tumour.

The effectiveness of cytotoxic substances against primary or syngeneic

transplanted tumours is usually much less than against homograft tumours. The same phenomenon is seen with local irradiation by X-rays; the homograft Walker tumour is eradicated by 1200 rads, whereas primary rat tumours require, in general, four to five times this dose. Until the discovery of the tumour specific antigens it was widely held that to cure a primary tumour or a syngeneic graft by surgery, radio- or chemo-therapy, the last cell had to be destroyed. It would certainly be difficult to explain the effectiveness of methotrexate against the syngeneic murine lymphoma L 1210 in this way. The view has been expressed that the last cell of a clinically evident cancer cannot be destroyed by any of the available therapeutic procedures and that a cure of any established tumours can only be achieved with the co-operation of a host reaction. The difference between homograft and primary tumour is then only a quantitative one in that the host response can deal with a much smaller residium of primary tumour than of homograft tumour. If the therapist cannot destroy the last tumour cell he must ensure that the host reaction is not impaired by treatment. This poses difficult and as yet unanswered problems; such as should the nodes draining the tumour be left intact? Is it conceivable that 'prophylactic' chemotherapy given, for example, after a radical mastectomy is harmful?

Experimental Approaches to Immunotherapy

The proof of the existence of antigens which are specific to tumours and which are themselves capable of inducing a selectively cytotoxic host reaction have justifiably led to greatly renewed interest in the role which immunological procedures may play in cancer therapy.

Non-Specific Stimulation of the Reticulo-Endothelial System

Almost half a century ago Murphy (see review 1924) showed that a number of non-specific methods of stimulating lymphopoiesis—such as treatment with oleic acid—had a pronounced growth retarding effect on autografts of spontaneous mammary tumours in mice. This approach was completely neglected until recently, when a range of microbes such as BCG (Old *et al* 1961) Corynebacterium parvum (Woodruff *et al* 1966) and microbial products such as zymosan, polysaccharides of diverse origin and especially an ethanolic extract of BCG (Weiss *et al* 1961), all of which stimulated phagocytic activity, were found to inhibit the growth of a number of transplanted tumours in mice and rats. While the most pronounced effects were seen with homograft tumours these procedures also retarded the growth of grafted syngeneic mammary carcinoma (see Fig. 18.3) but had little or no action on grafts of chemically induced tumours. The failure to act against the latter is surprising, since their antigenic activity as judged by transplantation immunity is greater than that of mouse mammary tumours.

Active Immunization

Active immunization with cells prepared from the tumour to be treated might be expected to enhance the host's immune reaction because the actively growing tumour may not release sufficient antigen to induce the maximum reaction. Also, since the tumour specific antigens are very labile it may be that necrotizing tumour tissue is not very antigenic and presentation of the antigens as an autograft is more immunogenic. This approach has proved successful with the papilloma induced in rabbits by the Shope virus which is of great immunological interest because spontaneous and complete regressions occur in an appreciable proportion of the animals, in striking contrast to chemically induced tumours in rodents which never regress spontaneously. Grafts consisting of intact papilloma cells whether of autologous or allogeneic origin, but not of homogenates or of cells disrupted by freezing, or of non-tumour tissue, increased the rate of complete regression from 20 to about 60% (Evans *et al* 1962). The addition of an adjuvant of *Bordetella pertussis* cells did not improve the effectiveness of the 'vaccine'. This neoplasm being virus induced probably has the same specific antigens in all tumours (see earlier) and immunization with tumour tissue from other animals should therefore be as effective as injection of autologous tumour.

With other tumours this approach has not been so successful. To prevent an autograft from growing, while maintaining the activity of the tumour specific antigen, it has usually been exposed to X-rays before being used as a 'vaccine'. Such irradiated autologous tumour tissue produced no detectable effect on the growth rate of primary chemically induced sarcoma in mice (Matsuyama *et al* 1963) or rats (Haddow & Alexander 1964). Haddow (1965) found that very occasionally autografting of viable tumour tissue obtained by biopsy caused a temporary regression of primary rat sarcomata, but this occurred too infrequently to be amenable to study. The failure of an irradiated autograft to influence the growth of primary tumours in the absence of other treatments is not unexpected; even if the host was not optimally immunized against the tumour the induction of the maximum response possible by immunization would, on the basis of the experience gained from transplantation experiments lead to the rejection of only a small fraction of the total tumour mass. Immunization with an autograft is only likely to show an effect once the tumour mass had been greatly reduced by other means and Haddow & Alexander (1964) found that the response of fibrosarcomata in the rat to local irradiation with X-rays was increased by implanting autochthonous tumour biopsy material that had been rendered non-viable. Although there is no direct proof that the irradiated autograft increases the host response mediated by the tumour specific antigens this seems to provide the most plausible explanation for its radio-sensitizing action. The fact that only autologous tumour tissue was effective is to be expected from the individuality of the specific antigens of chemically induced tumours.

Active immunization has been very effective as a prophylactic measure in virus induced tumours (see review by Hilleman 1966). Action here is possible both before and after exposure to the virus. Killed virus vaccines have been shown to prevent infection by stimulating the formation of neutralizing antibody, but protection has also been obtained by giving live virus during the latent period of carcinogenesis. The observation was made independently in the U.S.S.R. (Deichman & Kluchareva 1964) and in the U.S.A. (Eddy *et al* 1964) that the incidence of sarcoma induced by injecting the DNA virus SV 40 into 1-day-old hamsters was greatly reduced by a further injection of virus later in life. This effect does not require complete virus since injection of cells from an SV 40 induced tumour at 1 month of age also reduces the carcinogenicity of exposure to virus on the day of birth (Goldner *et al* 1964). The cells of a tumour that has been induced by a DNA virus do not contain complete virus and the

TABLE 18.4

Effect of a second exposure to oncogenic virus SV 40 on development of tumours induced by infection of hamsters with same virus on day of birth (Deichman & Kluchareva 1966)

Treatment	Progressively growing tumours	Palpable nodules that had regressed	No detectable tumours
Infection on day of birth only	82%	6%	12%
Immunized during latent period with SV 40	9%	74%	17%

protection must be attributed to immunization with the tumour specific antigens.

Quite recently Deichman *et al* (1966), by delaying the second treatment with virus until an actual tumour had become palpable, showed that this procedure is not only prophylactic but brings about genuine regressions (see Table 18.4). The mechanism of this effect has yet to be elucidated; the simple explanation that the second exposure to virus (or tumour tissue) heightens the level of immunity is difficult to reconcile with the observation that resistance (measured as the minimum number of cells to give a tumour) to SV 40 transformed cells was not increased by a further exposure to virus in adult life. From the point of view of immunotherapy this is a dramatic experiment, as it constitutes the first 'cure' of primary malignant tumours by active immunization and further clinical trials with 'vaccines' from autologous tumour seem to be called for in situations where the disease has not progressed too far.

Anti-Sera

Where there are histo-incompatibility differences between the tumour and the host (i.e. in homograft-type tumours) passively transferred humoral antibodies inhibit the growth of some tumours, enhance that of others, while leaving many unaffected (*cf.* Gorer 1961). In the presence of complement such antisera are cytotoxic *in vitro*, but there are indications for differences in mechanisms between the *in vivo* and *in vitro* systems (*cf.* Hellström & Möller 1965).

Immune sera prepared by immunizing either syngeneic, allogeneic or heterogeneic animals with tumour have not so far been shown to have any effect on the growth of established primary or syngeneic-grafted solid tumours. The only syngeneic tumours that respond to treatment with antiserum *in vivo* are ascitic lymphomas. Gorer & Amos (1956) immunized CBA mice with a leukaemia of C57/B1 origin and showed that the serum from the CBA mice not only protected C57/B1 mice against a subsequent challenger with tumour, but even retarded the growth of a tumour that had been established by injection of cells prior to the administration of the antiserum. The 'curative' effect of both allogeneic and heterogeneic antiserum—the latter having been rendered non-toxic by absorption against mouse tissue—was demonstrated by Alexander *et al* (1966) for another murine lymphoma transplanted in its host of origin. Suitable absorption tests showed that the action was directed against the tumour specific antigen and that antibody directed against the normal transplantation antigen played no part in spite of the fact that the antiserum was obtained from allogeneic hosts that had been immunized with the tumour. No explanation can be offered why the serum from syngeneic animals immunized with irradiated tumour—and rendered resistant to challenge with tumour—was apparently ineffective. Levi (1963) found antiserum against syngeneic murine lymphoma prepared in rabbits rendered tolerant to mouse was effective *in vivo*. Recently Old *et al* (1967) have been able to induce in rats an antiserum directed against the leukaemia specific antigen associated with tumours induced by the Gross virus. This serum has proved to be highly effective in irradicating in mice leukaemias carrying this antigen.

Specifically Immune (Allergized) Lymphocytes

The use of lymphocytes from donors that have been immunized against the tumour to be treated has proved to be one of the most promising immunotherapeutic procedures against primary or syngeneic grafted tumours.

Lymphocytes specifically immunized (allergized) against tumour specific antigens have been shown to kill tumour cells by immediate contact, and injection of such cells might therefore be expected to exert an anti-tumour action by interacting directly with the tumour. Such a process which can be described as 'passive cellular immunity' has been demonstrated *in vivo*, but is probably not the most promising way of utilizing lymphocytes for therapy. A number of experiments suggest that there is another class of lymphocytes which while not

cytotoxic themselves confer on the host the capacity to mount an immune reaction (see Fig. 18.1). The growth of several syngeneic murine lymphomas growing as ascites cells was arrested (see Table 18.5) by injection of spleen cells from donors immunized against the tumour (Alexander *et al* 1966). The donors could be either syngeneic—in which case they were immunized with irradiated cells—or allogeneic, but heterogeneic spleen cells were without effect. To prevent the tumour from growing—i.e. to 'cure' the animals—at least 200 immune spleen cells had to be injected for every tumour cell present in the animal. A single immunization with tumour cells was sufficient to produce anti-tumour spleen cells but to obtain an active antiserum hyperimmunization was necessary.

A number of tests demonstrated clearly that the anti-tumour activity of spleen cells from allogeneic animals depended on an immune response directed against the tumour specific antigen and not against the normal transplantation

TABLE 18.5

Effect of treatment with immune spleen cells on growth of ascites tumour established two days earlier by injecting 10^3 lymphoma cells (L5178Y) into their strain of origin (DBA/2 mice) (Alexander *et al* 1966).

Source of spleen cells used	Ratio of injected spleen cells to L5178Y cells present at time of treatment	% animals free of tumour
None	—	0
Syngeneic (i.e. DBA/2) mice hyperimmunized with irradiated tumour	400:1	60
	900:1	80
Non-immune allogeneic mice	400:1	4
Allogeneic mice immunized once with tumour	400:1	96
	200:1	80
	100:1	40
Allogeneic mice hyperimmunized with normal DBA/2 tissue	800:1	7
	6000:1	75
Rats hyperimmunized with tumour	2000:1	0

barriers. The lymphoma used grows readily in tissue culture and the spleen cells were growth inhibitory in this system. *In vitro*, unlike the situation *in vivo*, both spleen cells directed against the tumour specific and against the normal transplantation barriers were cytotoxic.

Immune lymphocytes were found effective in retarding the growth, frequently causing temporary regression and very occasionally permanent disappearance of primary fibrosarcoma induced in rats by pellets of the carcinogen 3:4-benzpyrene. These tumours are extremely malignant and no case of a spontaneous regression has been observed. A piece of the primary tumour was removed by biopsy and used to immunize: (1) syngeneic rats—in this case the

piece used for immunization had to be irradiated, (2) allogeneic rats, (3) sheep and goats. The immune lymphocytes were obtained from rats by cannulating the thoracic duct (Delorme & Alexander 1964) and from sheep by cannulating the efferent lymphatic ganglion of the node stimulated by the tumour (Alexander, Delorme & Hall 1966). To be effective 10^8 to 10^9 lymphocytes were injected intravenously, usually over a time interval of from 5–8 days after immunization.

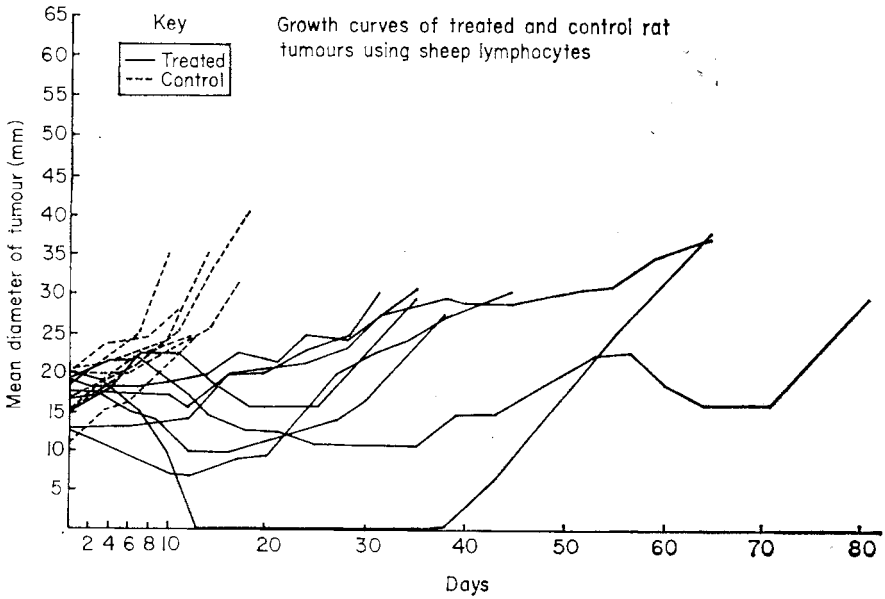


FIG. 18.4. Growth curves of primary rat sarcomata after treatment with sheep lymphocytes.
Lymphocytes obtained from sheep immunized with the tumour under test were used in the treated group. In the control group, lymphocytes obtained from sheep immunized with another tumour were used.

A significant feature of this therapy was the absence of wasting or lymphoid atrophy, suggesting that the foreign lymphocytes provoked no adverse reactions in healthy animals. Typical graft versus host disease symptoms were seen in animals that had been exposed to whole body irradiation with X-rays prior to receiving the foreign lymphocytes. The action seems to be wholly directed against the tumour specific antigens since the lymphocytes were only effective against the particular tumour that had been used for immunization. This was shown clearly (see Fig. 18.4) when sheep were used since sufficient lymphocytes could then be obtained to treat two tumours, the biopsy from one being used for

immunization, whereas the biopsy from the other was rejected. This specificity is to be expected if the activity relies on the tumour specific antigens since for chemically induced tumours these are different for each tumour. The surprising aspect of this interpretation is that a heterologous donor should recognize a minor antigenic variant.

There are indications that the active cells are the 'messenger' lymphoblasts (see Fig. 18.1) released by stimulated nodes. Autoradiography as well as other tests (see Alexander *et al* 1966) shows that the injected lymphocytes do not act directly on the tumour and the anti-tumour action of lymphocytes *in vivo* would appear to be different from that seen in the *in vitro* systems. The life-span of the injected cells—particularly of heterologous origin—is fairly short and they may initiate a process which is carried to completion in the host's lymphoid organs. The possibility that transfer of a subcellular component carrying a message from the injected lymphocytes to the tumour bearing host is suggested by the finding (Alexander *et al* 1967) that RNA extracted from the immune lymphocytes also has a growth inhibitory effect which shows the same degree of tumour specificity as that of the intact cells.

It would appear that there are several and distinct mechanisms by which lymphocytes directed against the tumour specific antigens can retard the growth of primary and syngeneic grafted tumours. These different processes may involve different populations of lymphocytes.

NON-SPECIFIC FOREIGN LYMPHOCYTES

Unsensitized foreign lymphocytes can interfere with the growth of tumour cells in several ways if the tumour-bearing animal is suitably pretreated (e.g. by exposure of the whole body to X-rays), the injected cells take over the haemopoietic system and effect a general graft versus host reaction which will be inimical to the growth of the tumour. This procedure has been exploited by Woodruff *et al* (1962) against syngeneic mammary carcinoma and by Mathé *et al* (1965) in both murine and human leukaemias.

Snell & Stevens (1961) found that certain tumours grew less well in F_1 hybrids than in the parental strain and since then many examples of what is called hybrid resistance have been observed and the phenomenon may be basically similar to that of 'allergic death' described by Gorer & Boyse (1959). *In vitro* experiments by Holm *et al* (1964, 1965) showed that tumour cells can be killed by being brought into close contact (i.e. by agglutination) with a very large number of immunologically competent cells so long as these are genetically dissimilar (i.e. are *not* histocompatible). This phenomenon which has been called 'callogeneic inhibition' has been claimed by Hellström & Möller (1965) to explain hybrid resistance *in vivo* but at the present time this hypothesis should be treated with caution as it fails to account for many of the salient features of the phenomenon (cf. Oth and Burg 1967) and Cudkowicz (1965) has provided genetic

evidence to show that F_1 hybrids react against certain parental antigens thereby making it unnecessary to invoke a non-specific reaction. The *in vitro* property of lymphoid cells to inhibit quite unspecifically allogeneic cells could not so far be exploited for immuno-therapy and the growth of established primary or syngeneic tumours was not slowed down by the injection of non-immune foreign lymphoid cells under conditions where specifically sensitized lymphoid cells were effective.

STUDIES IN MAN

The Effect of Malignant Disease on Immunological Reactions to Foreign Antigens

Patients with malignant disease are more prone to develop infections for one of three reasons:

1. A failure to form adequate amounts of circulating antibody (the antibody deficiency syndrome), occurring particularly in chronic lymphocytic leukaemia.
2. Impairment in cellular immunity (delayed hypersensitivity and graft rejection), particularly in Hodgkin's disease.
3. A failure to form normal granulocytes, particularly in acute leukaemia.

Only the first two are regarded as immunological reactions, and will be considered in detail. Since both the formation of circulating antibody and cellular immunity are mediated by cells of the reticulo-endothelial system it is not surprising that the greatest disruption in the immunological responses occurs in the primary malignant diseases of lympho-reticular tissue.

Circulating Antibodies

The term 'reticulosis' is used to include all primary malignant diseases of lympho-reticular tissue, which as far as antibody formation is concerned may be divided into two groups.

(i) Malignant disease of lymphocytes and plasma cells—Chronic lymphocytic leukaemia; Lymphosarcoma; Myelomatosis.

(ii) Other reticuloses—Hodgkin's disease; Reticulum cell sarcoma; Myeloproliferative diseases: (Acute leukaemia, Chronic granulocytic leukaemia, Myelofibrosis, Polycythaemia rubra vera).

Since the work of Moreschi in 1914 it has been known that antibody formation is impaired in chronic lymphocytic leukaemia, and this has been confirmed many times; a similar immunological incompetence (see Chapter 19) exists in myelomatosis (Howell 1920; Bernstein 1934; Weinstein & Fitz-Hugh 1935; Larson & Tomlinson 1953; Zinnerman & Wendell 1954; Lawson *et al* 1955; Shaw *et al* 1960; Fairley & Akers 1962).

However, with the other reticuloses there have been conflicting reports; in

Hodgkin's disease variable responses have been recorded, some patients forming antibodies normally with others failing to do so (Dubin 1947; Evans 1948; Geller 1953; Larson & Tomlinson 1953; Hoffmann & Rottino 1950; Schier *et al* 1956). In chronic granulocytic leukaemia and acute leukaemia, antibody formation has been found to be impaired, normal and in the case of acute leukaemia sometimes even increased (Weinstein & Fitz-Hugh 1935; Larson & Tomlinson 1953; Silver *et al* 1960). The variable results in these diseases are due to the difference between the response to primary immunization, when the patient is immunized with an antigen which he has never met before, and the secondary response to antigens which the patient has experienced in the past.

Both the primary and secondary antibody responses are impaired in malignant diseases of lymphocytes and plasma cells, whereas in the other reticuloses the secondary response is normal and only the primary response impaired (Greenwood *et al* 1958; Barr & Fairley 1961; Fairley & Akers 1962).

The concentration of the naturally occurring blood group iso-antibodies anti-A and anti-B, is also reduced to a much greater extent in patients with malignant diseases of lymphocytes and plasma cells than in the other reticuloses (Davidsohn 1938; Brem & Morton 1955; Fairley & Akers 1962). Despite this, measuring antibody levels before immunization is not as sensitive a guide to the ability to form antibodies as measuring the response following the injection of an antigen. For example, with influenza antibodies, both the resting levels and the response to immunization are impaired in myelomatosis, but in chronic lymphocytic leukaemia the ability to form new antibody is lost before the level of existing antibody starts to fall (Heath *et al* 1964). This in fact parallels the changes in immunoglobulins in these diseases. Hypogammaglobulinaemia giving rise to the antibody deficiency syndrome (see Chapter 19) is common in chronic lymphocytic leukaemia, the incidence varying between 36 and 68% (Jim 1957; Creyssel *et al* 1958; Ultmann *et al* 1959; Shaw *et al* 1960; Hudson & Wilson 1960; Fairley & Scott 1961). Further, the concentration of immunoglobulin in the serum tends to fall with the passage of time.

A monoclonal increase in the immunoglobulins IgG, IgA, IgD is found in myelomatosis and an increase in IgM in macroglobulinaemia; and frequently in these diseases the production of an abnormal globulin is accompanied by failure to produce normal immunoglobulin, resulting in the antibody deficiency syndrome (Snapper *et al* 1953; Porges 1956; Firkin & Blackburn 1958; Eastham & Yeoman 1960; Hobbs 1966). In malignant diseases of lymphocytes a polyclonal increase in immunoglobulins may occur and a few patients with chronic lymphocytic leukaemia and lymphosarcoma with macroglobulinaemia have been recorded (Mackay *et al* 1957; Glenchur *et al* 1958; Braunsteiner & Sailer 1960; Martin 1960; Onat & Cooper 1960; Prasad & Block 1960).

With the other reticuloses, although abnormalities in immunoglobulins occur they are neither as common nor as dramatic as with malignant diseases of

lymphocytes and plasma cells. In Hodgkin's disease hypergammaglobulinaemia is commoner than hypogammaglobulinaemia, but there is no characteristic pattern in the myeloproliferative syndromes (Neely & Neill 1956; Creyssel *et al* 1957; Teitelbaum *et al* 1959).

Auto-antibodies

When auto-allergic disease occurs in reticuloses it takes the form of auto-allergic haemolytic anaemia, and usually occurs in malignant diseases of lymphocytes (chronic lymphocytic leukaemia, and lymphosarcoma. See Dacie 1962). It would appear that in these diseases the inability to form normal immunoglobulins is accompanied by the formation of an abnormal protein. In chronic

TABLE 18.6

	GROUP 1 Malignant diseases of lymphocytes and plasma cells	GROUP 2 Other reticuloses
Primary response	Impaired	Impaired
Secondary response	Impaired	Normal
Serum immunoglobulin concentrations	Usually abnormal	Usually normal
Iso-agglutinin titres	Usually low	Usually normal

lymphocytic leukaemia gross hypogammaglobulinaemia may be accompanied by an auto-allergic haemolytic anaemia with a positive direct antiglobulin reaction (Pisciotta *et al* 1960) and auto-allergic thrombocytopenic purpura (Ebbe *et al* 1962).

It is also of great interest that the NZB strain of mice which spontaneously develop auto-allergic haemolytic anaemia, also develop both lymphocytic thymomas and malignant proliferation of reticulum cells leading to death from reticulosarcoma (East *et al* 1965; East 1967).

It is therefore possible to divide patients with reticuloses into two groups as far as antibody formation is concerned, as shown in Table 18.6.

Until recently, reports on the formation of circulating antibodies on antigenic challenge in patients with carcinoma have also been confused, but the results of Lytton *et al* (1964) suggest that the situation is similar to Group 2. That is, the

primary response is impaired leaving the secondary response unimpaired. The difference between carcinoma and reticuloses is that the immunological deficit is present early in patients with reticuloses, but only becomes prominent in late cases of carcinoma.

Cellular Responses

Delayed hypersensitivity

Most of the early work in man was done on the tuberculin reaction. There is no doubt that delayed hypersensitivity is suppressed in widespread diseases of lymphoreticular tissue, whether these are benign or malignant, although the mechanism of suppression is not necessarily the same in all these diseases; for example, it is clearly different in sarcoidosis than in the reticuloses. In 50% of the patients with sarcoidosis who fail to respond to tuberculin, the Mantoux reaction becomes positive if cortisone is injected with the tuberculin (Citron & Scadding 1957). This is not just a local effect as systemic cortisone has a similar action (Pyke & Scadding 1952). However, in patients with reticuloses who are insensitive to tuberculin, cortisone has no such effect (Fairley & Matthias 1960). This strongly suggests that the mechanism of suppression of the tuberculin reaction in sarcoidosis is different from that in reticuloses, although the mechanism in the latter is unknown.

In all reticuloses classical delayed hypersensitivity responses to antigens encountered before the onset of the disease, e.g. tuberculin, mumps, etc., is impaired (Dubin 1947; Schier *et al* 1956; Lamb *et al* 1962; Aisenberg 1966); and there is also a reduced capacity to develop a delayed hypersensitivity reaction to a new antigen, such as dinitrofluorobenzene (DNFB) (Rostenberg *et al* 1956; Levin *et al* 1964a). The impairment in delayed hypersensitivity is much greater in Hodgkin's disease than in the other reticuloses and Miller (1962) has stressed the importance of this in rendering these patients more liable to tuberculosis and cryptococcosis.

In carcinoma and sarcoma, delayed hypersensitivity to antigens encountered before the onset of the disease, e.g. tuberculin and mumps, as well as to new antigens such as DNFB may also be impaired (Lamb *et al* 1962; Levin *et al* 1964a; Hughes & Mackay 1965), but most workers have found that the depression is not as marked as in patients with reticuloses, occurs later in the course of the disease, and is related to the extent of the disease (Solowey & Rapaport 1965).

There are, however, some significant differences in the anergic state of patients with reticuloses and carcinomas. For example, it is not possible to transfer delayed hypersensitivity using lymphocytes from a normal allergic donor to anergic patients with Hodgkin's disease (Kelly *et al* 1960; Warwick *et al* 1961), whereas it is possible to do this in anergic patients with carcinomas (Hattler & Amos 1965) and sarcoidosis (Urback *et al* 1952). Levin *et al* (1964b) have shown that the impairment of delayed hypersensitivity in both reticuloses and

carcinomas is not due to general debility, because patients with debilitating non-neoplastic disease show normal delayed hypersensitivity responses. However, the work of Hughes & Mackay (1965) suggests that patients with malignant disease and anergy to tuberculin may have a worse prognosis than those who respond to tuberculin.

Skin homografts

If skin from a patient without malignant disease is grafted on to a patient with cancer the ability to reject this homograft is related to the ability to form a delayed hypersensitivity reaction. The survival of the graft is only prolonged in patients who show a marked suppression in the delayed hypersensitivity response, i.e. in the reticuloses, particularly those with Hodgkin's disease and with carcinomas, particularly the late cases (Kelly *et al* 1960; Grace 1964). The rejection of tumour homografts follows the same pattern and will be discussed later.

However, when skin from patients with malignant disease is grafted either on to other cancer patients or on to recipients without any evidence of neoplastic disease the situation is different. For example, Amos *et al* (1965) grafted skin from four patients with breast cancer, and one with a rhabdomyosarcoma on to 'normal' recipients and found that, in five out of six grafts, rejection was delayed. In the case of the rhabdomyosarcoma, one of the grafts was still present 60 days later when the recipient disappeared. Robinson *et al* (1965) showed that if a cancer patient received a skin graft from a normal control on one arm and skin from another cancer patient on the other, the normal skin was rejected before the skin from the cancer patient.

It is difficult to understand why this should be so. It is very unlikely that the skin of patients with malignant disease will have undergone antigenic loss, whatever antigenic change may have occurred in the tumour itself. It is possible, however, that there may be a delay in the antigens from the skin reaching the lympho-reticular tissue if the recipient of the skin graft was deficient in mobile cells such as lymphocytes and macrophages.

Lymphocyte Transfer Reaction

If lymphocytes from one individual are injected intradermally into a genetically dissimilar recipient an inflammatory response occurs in 24–48 hr, which represents a graft versus host reaction (Brent & Medawar 1964). It therefore depends on the immunological competence of the injected lymphocytes. Robinson & Hochman (1966) took patients with advanced local malignant disease and injected lymphocytes from a normal donor into one arm and lymphocytes from a cancer patient into the other. In fifty-two out of sixty-two such studies the size of the lesion was larger with the normal lymphocytes than with those from the cancer patients; in nine it was less, and in one the reaction was the same. This result is in keeping with the findings of impaired immunity in

advanced malignant disease, already described, and it is interesting that the lymphocyte transformation response to phytohaemagglutinin is impaired in patients with Hodgkin's disease (Hersh & Oppenheim 1965) and chronic lymphocytic leukaemia (Oppenheim *et al* 1965), but in carcinoma the lymphocytes react normally to phytohaemagglutinin but have impaired activity to other cell extracts (Robinson 1966), i.e. the lymphocytes of the carcinoma patient are deficient when tested by the lymphocyte transfer test, but respond normally to phytohaemagglutinin.

Recently it has been shown that the lymphoid cells in the peripheral blood in untreated Hodgkin's disease differ from normal controls in three ways (Crowther *et al* 1967): *First*, there is an increased number of large lymphoid cells which synthesize DNA; *Secondly*, there is an increased number of medium-sized lymphocytes with basophilic cytoplasm; *Thirdly*, occasional plasma cells are seen. These changes are identical with those seen under conditions of known antigenic challenge in man, following immunisation, in infections, and in rheumatoid arthritis and systemic lupus erythematosis (Crowther *et al* 1967). It may be that these cells in Hodgkin's disease represent an immunological response possibly directed against the malignant tissue.

Thus, as expected, the immunological abnormalities arising in malignant diseases are accompanied by an impaired function of cells of the lymphocyte-plasma cell series. However, the significance of impaired immunological responses in relation to the aetiology and prognosis of spontaneous malignant disease in man remains largely unanswered.

One practical result of impaired immune (allergic) reactions is the occurrence of severe and often unusual infection, but the type of infection and its prevention or treatment depends on which defence mechanism is at fault. For example, regular injections of gamma-globulin may prevent infections in patients with chronic lymphocytic leukaemia with the antibody-deficiency syndrome (Shaw *et al* 1960; Fairley & Scott 1961) but are ineffective in acute leukaemia where the basic defect is a failure to form normal granulocytes. In Hodgkin's disease, where the basic defect is a failure in cellular immunity, unexpected infections such as miliary tuberculosis and cryptococcosis may occur, and it is imperative that fever in this disease should not be attributed to the disease until infection has been excluded.

The theoretical interest in impaired immunity in malignant disease lies in its relationship to the reaction of the patient against his own tumour which will now be considered.

Allergic Reactions by the Patient Against his own Malignant Disease

From the work already described it is clear that in animals there is an immunological (i.e. allergic) reaction by the host against many experimental tumours,

but, because of the difficulties of research in man, the situation is not as clear as in animals. There are two reasons for this: first, there is the ethical problem of transplanting malignant tissue in man (see below); and secondly, unlike animals, 'pure-line' (syngeneic) human beings do not exist apart from identical twins. For this reason tumour specific antigens have not as yet been demonstrated in human malignant disease, although the work of Klein (1967) suggests that antigens of the type seen in virus-induced tumours in animals may be present in the Burkitt lymphoma, and of course on theoretical grounds a choriocarcinoma would be expected to contain transplantation antigens not present in the host. However, there is evidence in man that there may be a reaction of the host against his own tumour, that this reaction is cellular rather than humoral and as in animals is mediated by lymphocytes. Its exact nature is not known and there is no proof that the reaction is immunological although the evidence, suggesting that it is, may be summarized as follows:

Tumour Grafts in Man

Homografts

Grafts of malignant tissue into recipients receiving immunosuppressive drugs may be accepted. D.C.Martin *et al* (1965) successfully transplanted a cadaveric kidney from a patient with carcinoma of the bronchus and 4 months later malignant cells were found in the transplanted organ; the recipient died from widespread metastases of the transplanted carcinoma 5 months after the graft. Presumably, in this case the graft of malignant cells took for the same reason that the kidney transplant was successful, i.e. that the immunosuppressive treatment with azathioprine, prednisolone and actinomycin C was so effective.

The subcutaneous transplantation of human cancer cells into normal people almost invariably results in transient growth followed by complete regression during the 3rd and 4th weeks (Southam *et al* 1957). As with other tissues rejected by the homograft reaction, further transplants lead to an accelerated response. Rejection of homografts of malignant cells in patients with advanced cancer is delayed sometimes indefinitely and in one patient with advanced cancer who received a homotransplant of another tumour, the latter actually metastasized to the regional lymph nodes (Southam *et al* 1957). In patients with debilitating non-neoplastic disease the rejection of homografts of malignant cells is the same as in normal controls (Levin *et al* 1964b).

However, as with animals (Delorme & Alexander 1967), malignant cells transplanted into genetically dissimilar normal recipients occasionally take. Scanlon *et al* (1965) took a small biopsy of a melanoma from a woman of 50 and transplanted it into the patient's 80-year-old mother who subsequently died of widespread metastases from this tumour. The fact that 9 months after the implant her Mantoux test was negative, and that she was old, raises the possibility that there may have been some impairment in her immune responses which encouraged this tumour to metastasize. It is also interesting that 5 years before

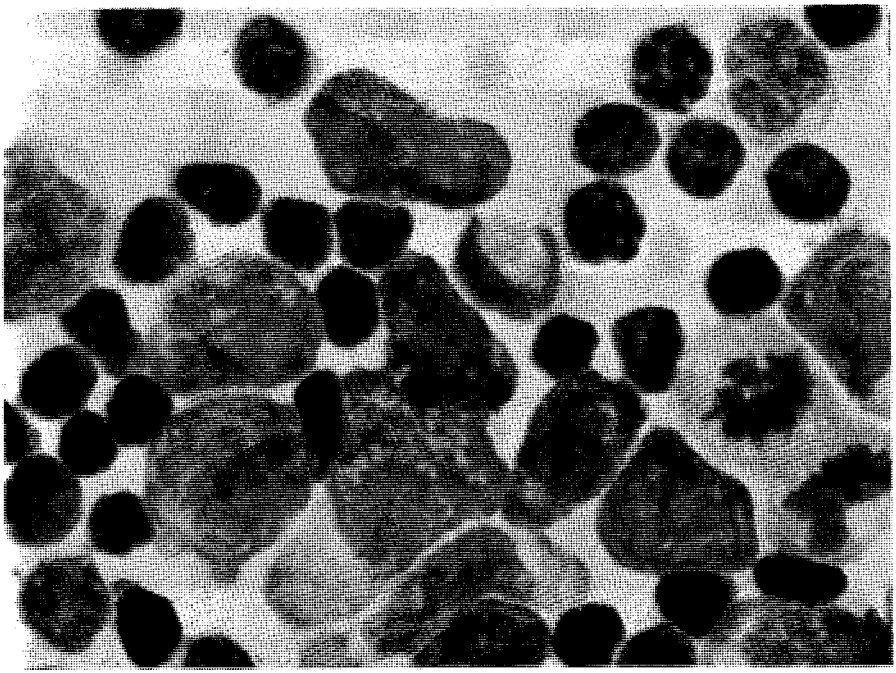


PLATE 18.1. The pyroninophilic lymphoblast:
(*a*) Cells obtained from the efferent duct of a sheep lymph-node draining the site of a foreign tissue graft.

facing p. 528

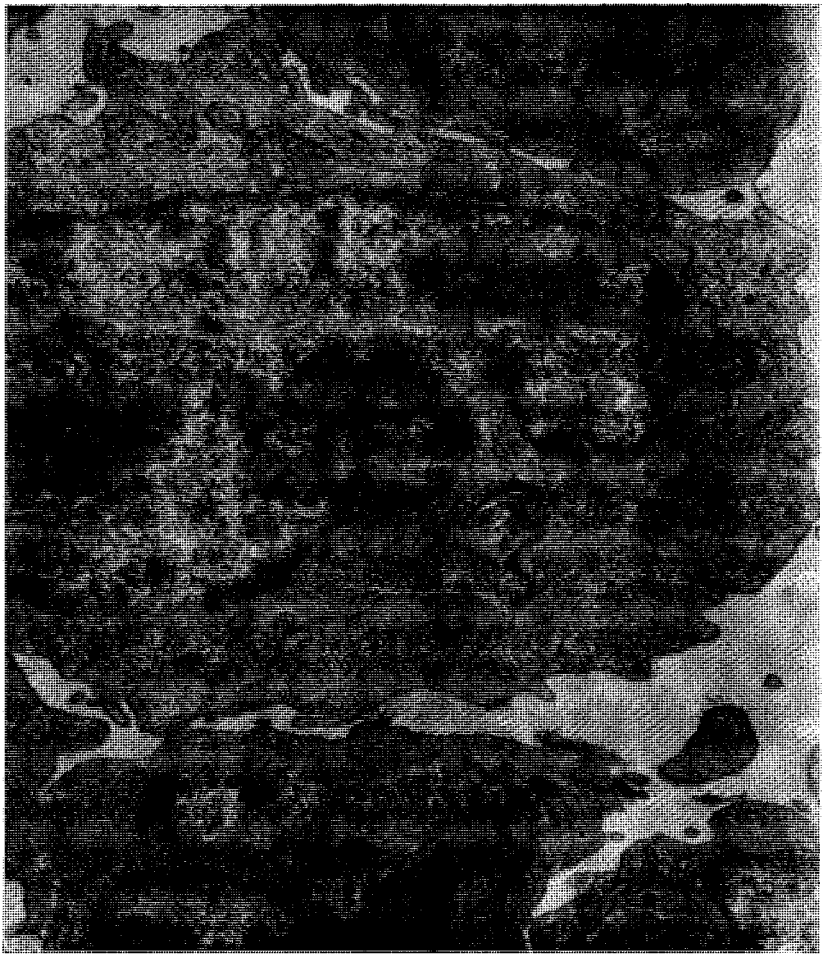


PLATE 18.1b

(*b*) Electron-photomicrograph of large basophil sheep lymph cell from the efferent lymph of a stimulated node. The cytoplasm lacks endoplasmic reticulum but numerous polysomes are present.

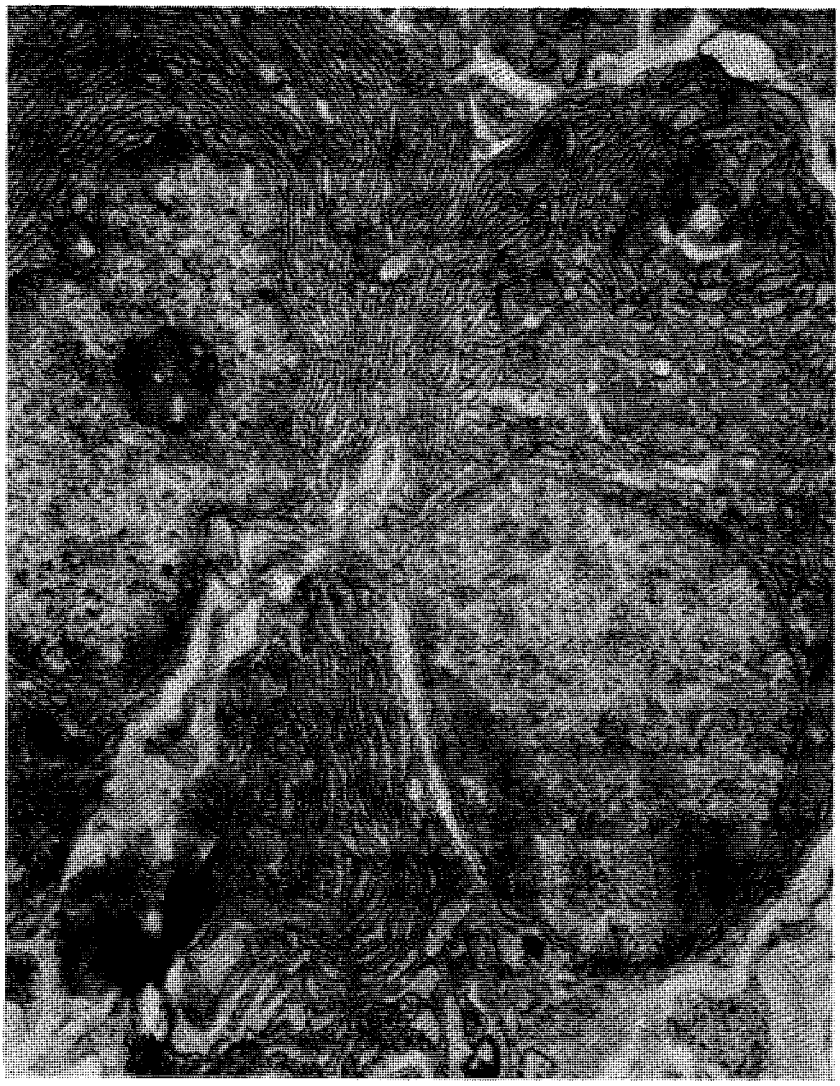


PLATE 18.1C

(*c*) Plasma cells in a sheep lymph node. These cells are full of endoplasmic reticulum and Birbeck and Hall (1967) showed that they can be derived from the basophil cells shown in *a* and *b*.

this, she had a carcinoma of the uterus which was successfully removed. Another explanation could be that because the donor and recipient were so closely related there was a high degree of histocompatibility. In view of this case it is clearly dangerous in some circumstances to transplant human malignant tissue into apparently healthy recipients, particularly if the donor and recipient are related.

Autografts

The subcutaneous implantation of malignant cells or biopsy material from the patient's own tumour may give rise to 'takes' depending on the experimental conditions. For example, Grace (1964) using biopsy material found that only two out of sixteen implants grew, and Nadler & Moore (1965a) only obtained eleven out of eighty-two transplants. Southam (1965) by injecting cell suspensions found that the development of a subcutaneous tumour depended on the number of malignant cells injected; 10^8 cells always produced a nodule, whereas 10^4 cells invariably failed to do so. With 10^5–10^7 cells the number of tumour cells required to produce a successful autograft was related to the ability of the patient to reject a homograft of tumour cells, and this in turn was related to the immunological state of the patient as judged by the tuberculin test, the ability to develop delayed hypersensitivity to DNFB and the macrophage response in inflammatory exudates. The greater the immunological defect, the fewer cells were required to establish a tumour autotransplant.

Patients with carcinomas may respond to the injection of cell free extracts of their own tumours, in about 25% of cases, by a delayed hypersensitivity response and in a small number of cases by an immediate response (Hughes & Lytton 1964).

Histological Evidence

The natural history of malignant disease is related to the morphology of the tumour. Black *et al* (1955, 1956); Black & Speer (1958) showed that the prognosis in carcinoma of the breast and stomach was related to the degree of nuclear differentiation, the amount of lymphoid infiltration in the primary tumour and the presence of sinus histiocytosis of the regional lymph nodes. The greater the degree of differentiation, lymphoid infiltration and sinus histiocytosis, the better the prognosis. A similar situation arises in Hodgkin's disease where lymphocytic depletion is associated with a poor prognosis and widespread disease; lymphocytic and/or histiocytic proliferation is associated with a better prognosis and localized disease (Lukes 1964).

The Phenomenon of Spontaneous Regression

There are now a number of cases of malignant disease in man in which the disease has regressed unexpectedly, although not necessarily without any form of treatment (Everson & Cole 1956; Smithers 1962; Everson 1964).

Everson & Cole (1956) have defined spontaneous regression of cancer as 'the

partial or complete disappearance of a malignant tumour in the absence of all treatment or in the presence of therapy which is considered inadequate to exert a significant influence on neoplastic disease'. Regressions have been recorded in carcinomas of the bladder, breast, kidney, lung, ovary, stomach, uterus and with melanoma and neuroblastoma.

Everson (1964) collected 130 cases in which the evidence of spontaneous regression of malignant disease was beyond all doubt, and of these, 28 had occurred in neuroblastoma, 21 in hypernephroma, 13 in choriocarcinoma and 12 in malignant melanoma. Many of these regressions occurred in metastases after the primary tumour mass was removed, and in these cases it may be that the host reaction against the tumour is being overwhelmed by the mass of malignant tissue, and removal of part of this tissue might enable the host to deal with the remainder. This is a comparable situation to the experiment described under 'Escape mechanisms which permit the growth of antigenic tumours' in the first section of this chapter.

Evidence from Treatment

A total of seventy-one patients with undoubted acute leukaemia who have survived from periods of 5 to 12 years since diagnosis has been collected by Burchenal & Murphy (1965). Thirty-six out of fifty-three children and six out of eighteen adults are alive and well with no evidence of leukaemia more than 5 years after diagnosis. They received a variety of treatments and as yet no common factor has been detected in these patients.

A more dramatic situation has now arisen with the Burkitt African lymphoma. Regressions of tumour masses with freedom from recurrence from up to 5 years have followed the single injection of either methotrexate or cyclophosphamide (Burkitt 1967). It is extremely unlikely that this treatment could have killed all the malignant cells, and this is further evidence that there is host resistance as well, and this could be immunological. For this reason it has been suggested that in certain circumstances it might be disadvantageous to irradiate or remove the drainage lymph nodes with a localized carcinoma, e.g. Crile (1965) has shown that the survival with Stage I carcinoma of the breast is slightly better when a simple mastectomy is performed than after a radical mastectomy. However, with other tumours, e.g. seminoma, the practice of irradiating the pelvic and abdominal lymph nodes has revolutionized the prognosis (Whittle 1957; Hope-Stone *et al* 1963; L.S.J.Martin *et al* 1965). Presumably, the efficacy of irradiating the drainage lymph nodes depends on the sensitivity of the tumour to radiotherapy. With radio-sensitive tumours the treatment may harm the tumour cells more than the lymphocytes, which may be reacting against them. On the other hand with radio-resistant tumours, irradiation may harm the lymphocytes more than the malignant cells.

It is with the background of this evidence that there may be a reaction,

mediated by lymphocytes, by the patient against his own malignant disease, that the problem of immunotherapy in man should be considered.

Immunotherapy

Immunotherapy Using Vaccines and Antisera

In 1922 Kellock *et al* attempted to influence the course of malignant disease by immunizing patients with material from their own tumours without any real benefit. Graham & Graham (1959) prepared a 'vaccine' from the patient's own tumour, injected it together with Freund's adjuvant and concluded that it might exert an effect in potentiating radiotherapy given subsequently—a similar effect has been observed experimentally (see earlier section). However, there are theoretical reasons from the animal work already described why vaccines might even lead to tumour enhancement and Milner *et al* (1964) have drawn attention to this potential danger.

Similarly, antisera produced against human malignant tissue in animals have not been successful in modifying the course of cancer in man with the possible exception of choriocarcinoma, and this form of treatment carries the potential danger of tumour enhancement.

Of all malignant tumours in man, choriocarcinoma should be the most antigenic, for it is derived from the placenta and presumably carries antigens derived equally from each parent. Doniach *et al* (1958) were the first to attempt treatment by immunizing the patient against her husband's cells by skin grafting and by repeated injections of his leucocytes. Cinader *et al* (1961) described a patient in whom chemotherapy had been unsuccessful, and who was subsequently successfully treated with active immunization of her husband's leucocytes and antisera prepared in rabbits against the husbands' seminal fluid. In both cases immunotherapy was used in addition to orthodox treatment and it is difficult to assess the benefits.

Immunotherapy Using Lymphoid Cells

Non-specific reactions

The results of using spleen cells, lymphocytes and peripheral leucocytes have proved more interesting. Woodruff & Nolan (1963) treated eight patients with advanced carcinomatosis by injecting spleen cells from another individual after preparatory treatment of the recipient with a cytotoxic drug designed to delay rejection of the foreign cells. All cases showed some slight improvement. These cells had, of course, not been immunologically activated as the donor had not been immunized with tumour cells.

Schwarzenberg *et al* (1966) have treated twenty-one patients with acute leukaemia with leucocyte transfusions from donors with chronic myeloid leukaemia, and obtained nine remissions although these were only temporary. They concluded that the anti-leukaemic effect was dependent on the immuno-

logically competent cells in the transfused population, as the remissions were related to the onset of a secondary syndrome identical with that complicating marrow grafts in man.

Specific reactions

Following the experience with primary rat tumours (see previous section) Nadler & Moore (1966) took pairs of patients with incurable malignant disease. A biopsy of the malignant tissue was taken from each patient and implanted subcutaneously into the other patient and vice versa. After 10 to 14 days white cells were collected from the recipients of the tumour grafts and injected back into the donor. They used the white cells from 500 ml of blood on each occasion and repeated the transfusion once or twice daily for 3 weeks. Seven out of twenty-six patients derived some benefit from this, and two patients had complete objective remission, one of which had lasted for 2 years at the time of publication.

Southam (1965) found with tumour autotransplants that by mixing the tumour cells with the patient's own lymphocytes there was sometimes an inhibition of growth at the site of injection. Autologous plasma was less inhibitory when mixed with the tumour cells but did have some effect.

Conclusion

From all this evidence it is reasonable to postulate that in some instances there is a host reaction in both man and animals against malignant disease, that this reaction is largely cellular and related to the activity of lymphocytes, and may well be an immunological reaction allied to the delayed hypersensitivity reaction and graft rejection.

The frequency and significance of such reactions, and their relationship to other factors, such as hormonal balance, in human malignant disease remains to be established. As yet immunotherapy in man is still in the experimental stage, but it may well be that in the future it will prove of value, probably being used in conjunction with other forms of treatment. An excellent summary of this may be found in the Report of a W.H.O. Scientific Group (1966) on Immunotherapy of Cancer.

REFERENCES

Studies in Experimental Animals

Abelev G.I. (1965) Antigenic structure of chemically induced hepatomas. *Progr. Exp. Tumor Res.* vol. 7, p. 105, ed. Humburger F. Karger, Basel/New York.

Alexander P., Connell D.I. & Mikulska Z.B. (1966) Treatment of a murine leukemia with spleen cells or sera from allogeneic mice immunized against the tumor. *Cancer Res.* **26**, 1508.

ALEXANDER P., DELORME E.J. & HALL J.G. (1966) The effect of lymphoid cells from the lymph of specifically immunized sheep on the growth of primary sarcomata in rats. *Lancet* **i,** 1186.

ALEXANDER P., DELORME E.J., HAMILTON L.D.G. & HALL J.G. (1967) Effect of nucleic acids from lymphoid cells of specifically immunized donors on the growth of primary sarcomata in rats. *Nature. (Lond.)* **213,** 569.

ALEXANDER P. & HORNING E.S. (1959) Observations on the Oppenheimer method of inducing tumours by subcutaneous implantation of plastic films. In Ciba Symposium on *Carcinogenesis,* pp. 12–22. Churchill.

BALDWIN R.S. (1966) Tumour-specific immunity against spontaneous rat tumours. *Int. J. Cancer* **1,** 257.

BATES D.R. & PREHN R.T. (1965) Role of the fibrous capsule in carcinogenesis by plastic film. *Nature (Lond.)* **205,** 303.

BIRBECK M.S.C. & HALL J.G. (1967) The transformation *in vivo* of basophilic lymph cells into plasma cells: An autoradiographic-electron microscopy study. *Nature. (Lond.)* **214,** 183.

CUDCOVICZ G. (1965) The immunogenetic basis of hybrid resistance to parental marrow grafts. In *Isoantigens and Cell Interactions,* pp. 37–52. The Wistar Inst. Press.

DAVIES D.A.L. (1966) Mouse histocompatibility isoantigens derived from normal and from tumour cells. *Immunology* **11,** 115.

DEICHMAN G. & KLUCHAREVA T. (1964) Prevention of tumour induction in SV 40 infected hamsters. *Nature (Lond.)* **202,** 1126.

DEICHMAN G. & KLUCHAREVA T. (1966) The regression of primary tumours induced by Symian virus-40 in immunized hamsters. *Vestnick Academii Mediziniskih Nauk (U.S.S.R.)* **9,** 12.

DELORME E.J. & ALEXANDER P. (1964) Treatment of primary fibrosarcoma in the rat with immune lymphocytes. *Lancet* **ii,** 117.

DELORME E.J., CONNELL D.I., MIKULSKA Z.B. & ALEXANDER P. (1966) Immunological procedures for treating primary chemically induced fibrosarcoma in the rat and a murine lymphoma. Proceedings of Conference on "Tumour specific antigens" p. 188, by International Union contra Cancer, publ. Munksgaard, Copenhagen

EDDY B.E., GRUBBS S.E. & YOUNG R.D. (1964) Tumour immunity in hamsters infected with adenovirus Type ii or Simian virus 40. *Proc. Soc. exp. biol. Med.* **117,** 575.

EHRLICH P. (1909) In *Collected Papers,* vol. 2, p. 557. 1957. Pergamon Press.

EVANS C.A., GORMAN L.R., ITO Y. & WEISER R.S. (1962a) Antitumor immunity in the Shope papilloma-carcinoma complex of rabbits. I. Papilloma regression induced by homologous and autologous tissue vaccines. *J. Nat. Cancer Inst.* **29,** 277.

FOLEY E.J. (1953) Antigenic properties of methylcholanthrene induced tumours in mice of the strain of origin. *Cancer Res* **13,** 835.

GLOBERSON A. & FELDMAN M. (1964) Antigenic specificity of benzpyrene induced sarcoma. *J. Nat. Cancer Inst.* **32,** 1229.

GOLD P. & FREEDMAN S.O. (1965) Specific carcinoembryonic antigens of the human digestive system. *J. Exp. Med.* **122,** 467.

GOLDNER H., GIRARDI A.S., LARSON V.M. & HILLEMAN M.R. (1964) Interruption of SV 40 virus tumorigenesis using irradiated homologous tumour antigen. *Proc. Soc. Exp. Med.* **117,** 851.

GORER P.A. (1961) The antigenic structure of tumours. *Immunology* **1,** 345.

GORER P.A. & AMOS D.B. (1956) Passive immunity of mice against C57BL leukosis E.L.4 by means of iso-immune serum. *Cancer Res.* **16,** 338.

GORER P.A. & BOYSE E.A. (1959) Pathological changes in F_1 hybrid mice following transplantation of spleen cells from donors of the parental strains. *Immunology (Lond.)* **2,** 182.

GOWANS, J.L. and MCGREGOR, D.D. (1965) The immunological activities of lymphocytes. *Progr. Allergy*, **9,** 1.

HADDOW A. (1965) Immunology of the cancer cell: tumour specific antigens. *Brit. Med. Bull.* **21,** 133.

HADDOW A. & ALEXANDER P. (1964) An immunological method of increasing the sensitivity of primary sarcomas to local irradiation with X-rays. *Lancet* **i,** 452.

HALL J.G., MORRIS B., MORENO J.D. & BESSIS M.C. The ultra structure and function of the cells in lymph following antigenic stimulation. *J. exp. Med.*, **125,** 91.

HELLSTRÖM K.E. & MÖLLER G. (1965) Immunological and immunogenetic aspects of tumour transplantation. *Progress in Allergy* **9,** 158.

HILLEMAN M.R. (1966) In *Malignant Transformations by viruses*, ed. KIRSTEN W. Springer.

HOLM G., PERLMANN P. & WERNER B. (1964) Phytohaemagglutinin-induced cytotoxic action of normal lymphoid cells on cells in tissue culture. *Nature (Lond.)* **203,** 841.

HOLM G. & PERLMANN P. (1965) Phytohaemagglutinin-induced cytotoxic action of unsensitized immunologically competent cells on allogeneic and xenogeneic tissue culture cells. *Nature (Lond.)* **207,** 818.

KLEIN G. & KLEIN E. (1962) Antigenic properties of other experimental tumours. Cold Spring Harbor Symp. on *Quantitative Biology*, vol. 27, p. 463.

KLEIN G., SJÖGREN H.O., KLEIN E. & HELLSTRÖM K.E. (1960) Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res.* **20,** 1561.

LEVI E. (1963) Preparation of an antiserum specific to a spontaneous mouse leukaemia after the induction of artificial immunological tolerance to normal mouse tissue. *Nature (Lond.)* **199,** 501.

MATHÉ G., AMIEL J.L., SCHWARZENBERG L., CATTAN A. & SCHNEIDER M. (1965) Adoptive immunotherapy of acute leukaemia: experimental and clinical results. *Cancer Res.* **25,** 1525.

MATHÉ G., DAUSSET J., HERVET E., AMIEL J.L., COLOMBANI J. & BRULE G. (1964) Immunological studies in patients with placental choriocarcinoma. *J. Nat. Cancer Inst* **33,** 193.

MATSUYAMA M., MAEKAWA A., HORIKAWA H., SUZUMORI K., SOGA K. & IWAI K. (1963) Ineffectiveness of autotransplantation on growth of the methylcholanthrene sarcoma in rats and mice. *Nature (Lond.)* **197,** 805.

MIKULSKA Z.B., SMITH C. & ALEXANDER P. (1966) Evidence for an immunological reaction of the host against its own actively growing primary tumour. *J. Nat. Cancer Inst.* **36,** 29.

MÖLLER G. (1964) Antibody-induced depression of the immune response: a study of the mechanism in various immunological systems. *Transplantation* **2,** 405.

MÖLLER E. & MÖLLER G. (1965) Antagonistic effects of humoral isoantibodies on the *in vitro* cytotoxicity of immune lymphoid cells. *J. exp. Med.* **11,** 122.

MORTON D.L., GOLDMAN L. & WOOD D.A. (1965) Immunological tolerance to spontaneous mammary adenocarcinoma (SMC). *Proc. Amer. Ass. Cancer Res.* **6,** 47.

MURPHY J.B. (1924) Monograph of the Rockefeller Institute No. 21.

OLD L.J., BENACERRAF B., CLARKE D.A., CARSWELL E.A. & STOCKERT E. (1961) The role of the reticuloendothelial system in the host reaction to neoplasia. *Cancer Res.* **21,** 1281.

OLD L.J. & BOYSE E.A. (1964) Immunology of experimental tumors. *Ann. Rev. Med.* **15,** 167.

OLD L.J. & BOYSE E.A. (1965) Antigens of tumours and leukaemias induced by viruses. *Fed. Proc.* **24,** 1009.

OLD L.J., BOYSE E.A., BENNETT B. & LILLY F. (1963) Peritoneal cells as an immune population in transplantation studies. In *Cell-bound Antibodies*. Wistar Inst. Press.

OLD L.J., BOYSE E.A., CLARKE D.A. & CARSWELL E.A. (1962) Antigenic properties of chemically induced tumors. *Ann. N.Y. Acad. Sci.* **101**, 80.

OLD, L.J., STOCKERT, E., BOYSE, E.A. and GEERING, G. (1967) A study of passive immunization against a transplanted G + Leukaemia with specific antiserum. *Proc. Soc. exp. Biol. Med.* **124**, 63.

OTH, D. and BURG, C. (1967) Persistence of syngeneic preference given by a methylcholanthrene induced sarcoma after four consecutive passages in F_1 hybrids. *Nature (Lond.)* **214**, 418.

PREHN R.T. (1963) Tumour specific immunity to non-viral tumours. *Canadian Cancer Conference*, vol. 5, p. 387. New York: Academic Press.

PREHN R.T. (1963) Function of depressed immunologic reactivity during carcinogenesis. *J. Nat. Cancer Inst.* **31**, 791.

PREHN R.T. & MAIN J.M. (1957) Immunity to methylcholanthrene induced sarcomas. *J. Nat. Cancer Inst.* **18**, 769.

RIGGINS R.S. & PILCH Y.N. (1964) Immunity to spontaneous and methylcholanthrene induced tumours in inbred mice. *Cancer Res.* **27**, 1994.

ROSENAU W. & MORTON D.L. (1966) Tumour-specific inhibition on growth of methylcholanthrene induced sarcomas *in vivo* and *in vitro* by sensitized isologous lymphocytes. *J. Nat. Cancer Inst.* **36**, 825.

SNELL G.D. & STEVENS L.C. (1961) Histocompatibility genes in mice. *Immunology* **4**, 366.

SPÄRCK J.V. & VOLKERT M. (1965) Effect of adoptive immunity on experimentally induced leukaemia in mice. *Nature (Lond.)* **206**, 578.

STJERNSWÄRD J. (1965) Immunodepressive effect of 3-methylcholanthrene. Antibody formation at the cellular level and reaction against weak antigenic homografts. *J. Nat. Cancer Inst.* **35**, 885.

STROBER S. & GOWANS J.L. (1965) The role of lymphocytes in the sensitization of rats to renal homografts. *J. Exp. Med.* **122**, 347.

TAYLOR H.E. & CULLING C.F.A. (1963) Cytopathic effects *in vitro* of sensitized homologous and heterologous spleen cells on fibroblasts. *Lab. Invest.* **12**, 884–94.

WEISS D.W., BONHAG R.S. & DEOME K.B. (1961) Protective activity of fractions of tubercle bacilli against isologous tumours in mice. *Nature (Lond.)* **190**, 889.

WOODRUFF M.F.A. & BOAK J.L. (1965) Inhibitory effect of pre-immunized CBA spleen cells on transplants of A-strain mouse mammary carcinoma $(CBA \times A)F_1$ hybrid recipients. *Brit. J. Cancer* **19**, 411.

WOODRUFF M.F.A. & BOAK J.L. (1966) Inhibitory effect of injection of *Corynebacterium parvum* on the growth of tumour transplants in isogenic hosts. *Brit. J. Cancer* **20**, 345.

WOODRUFF M.F.A. & SYMES M.O. (1962) The use of immunologically competent cells in the treatment of cancer: experiments with a transplantable mouse tumour. *Brit. J. Cancer* **16**, 707.

STUDIES IN MAN

AISENBERG A.C. (1966) Immunologic status of Hodgkin's disease. *Cancer (Philad.)* **19**, 385.

AMOS D.B., HATTLER B.G. & SHINGLETON W.W. (1965) Prolonged survival of skin-grafts from cancer patients on normal recipients. *Lancet* **1**, 414.

BARR M. & FAIRLEY G.H. (1961) Circulating antibodies in reticuloses. *Lancet* **1**, 1305.

BERNSTEIN A. (1934) The diagnostic importance of the heterophilic antibody test in leukaemia. *J. Clin. Invest.* **13**, 677.

BLACK M.M., OPLER S.R. & SPEER F.D. (1955) Survival in breast cancer cases in relation to the structure of the primary tumour and regional lymph nodes. *Surg. Obstet. Gynae.* **100**, 543.

BLACK M.W., OPLER S.R. & SPEER F.D. (1956) Structural representation of tumour-host relationships in gastric carcinoma. *Surg. Obstet. Gynae.* **102,** 599.

BLACK M.M. & SPEER F.D. (1958) Sinus histiocytosis of lymph nodes in cancer. *Surg. Obstet. Gynae.* **106,** 163.

BRAUNSTEININ VON H. & SAILER S. (1960) Lymphatische leukämie mit makroglobulinäemic Waldenström. *Acta Haemat.* **23,** 306.

BREM T.H. & MORTON M.E. (1955) Defective serum gamma globulin formation. *Ann. Int. Med.* **43,** 465.

BRENT L. & MEDAWAR P.B. (1964) Nature of the normal lymphocyte transfer reaction. *Nature (Lond.)* **204,** 90.

BURCHENAL J.H. & MURPHY M.L. (1965) Long-term survivors in acute leukaemia. *Cancer Res.* **25,** 1491.

BURKITT D. (1967) *Chemotherapy of Burkitt's Tumour.* ed. BURCHENAL J.H. Springer, Heidelberg

CINADER B., HAYLEY M.A., RIDER W.D. & WARWICK O.H. (1961) Immunotherapy of a patient with choriocarcinoma. *Canad. Med. A.J.* **84,** 306.

CITRON K.M. & SCADDING J.G. (1957) The effect of cortisone upon the reaction of the skin to tuberculin in tuberculosis and in sarcoidosis. *Quart. J. Med.* (New Series) **26,** 277.

CREYSSEL R., MOREL P., VIEUX R., PICHAT M., CROIZAT P. & REVOL L. (1957) L'electrophorese du serum au cours des hemopathies malignes. *Le Sang.* **28,** 34.

CREYSSEL R., MOREL P., PELLET M., MEDARD J., REVOL L. & CROIZAT P. (1958) Deficit en gamma-globulines et complications infectieuses des leucemies lymphoides chroniques. *Le Sang.* **29,** 383.

CRILE G. (1965) Rationale of simple mastectomy without radiation for clinical stage I cancer of the breast. *Surgery Gynae. Obstet.* **120,** 975.

CROWTHER, D., FAIRLEY, G.H. and SEWELL, R.L. (1967) *Nature (Lond.)* **215,** 1086.

DACIE J. V. (1962) *The Haemolytic Anaemias.* Part 2. *The Auto-Immune Haemolytic Anaemias.* London: Churchill.

DAVIDSOHN I. (1938) Isoagglutinin titres in serum disease, in leukaemias, in infectious mononucleosis and after blood transfusion. *A. J. Clin. Path.* **8,** 179.

DELORME E.F. & ALEXANDER P. (1967) In press.

DONIACH I., CROOKSTON J.H. & COPE T.I. (1958) Attempted treatment of a patient with choriocarcinoma by immunization with her husband's cells. *J. Obstet. Gynae. of Brit. Empire LXV,* 553.

DUBIN I.N. (1947) The poverty of the immunological mechanism in patients with Hodgkin's disease. *Ann. Int. Med.* **27,** 898.

EAST J., DE SOUSA M.A.B. & PARROTT D.M.V. (1965) Immunopathology of New Zealand Black (NZB) mice. *Transplantation* **3,** 711.

EAST J. (1967) Auto-immunity and malignancy in New Zealand Black Mice. *Proc. Roy. Soc. Med.* **60,** 823.

EASTHAM R.D. & YEOMAN W.B. (1960) Hypogammaglobulinaemia in myeloma. *Acta Med. Scand.* **166,** 241.

EBBE S., WITTELS B. & DAMESHEK W. (1962) Autoimmune thrombocytopenic purpura (ITP type) with chronic lymphocytic leukaemia. *Blood* **19,** 23.

EVANS R.W. (1948) The antibody responses in cases of radiation lymphopenia, and in the reticuloses. *J. Path. and Bact.* **60,** 123.

EVERSON T.C. (1964) Spontaneous regression of cancer. *Ann. N.Y. Acad. Sci.* **114,** 721.

EVERSON T.C. & COLE W.H. (1956) Spontaneous regression of cancer: preliminary report. *Ann. Surg.* **144,** 366.

FAIRLEY G.H. & AKERS R.J. (1962) Antibodies to blood group A and B substances in reticuloses. *Brit. J. Haemat.* **8,** 375.

FAIRLEY G.H. & MATTHIAS J.Q. (1960) Cortisone and skin sensitivity to tuberculin in reticuloses. *Brit. Med. J.* **ii,** 433.

FAIRLEY G.H. & SCOTT R.B. (1961) Hypogammaglobulinaemia in chronic lymphatic leukaemia. *Brit. Med. J.* **ii,** 920.

FIRKIN B.G. & BLACKBURN C.R.B. (1958) Congenital and acquired agammaglobulinaemia. *Quart. J. Med.* **27,** 187.

GELLER W. (1953) A study of antibody formation in patients with malignant lymphomas. *J. Lab. Clin. Med.* **42,** 232.

GLENCHUR H., ZINNERMAN H.H. & BRIGGS D.R. (1958) Macroglobulinaemia: report of two cases. *Ann. Int. Med.* **48,** 1055.

GRACE J.T. (1964) Clinical aspects of immunity in untreated cancer. *Ann. N.Y. Acad. Sci.* **114,** 736.

GRAHAM J.B. & GRAHAM R.M. (1959) The effect of vaccine on cancer patients. *Surg. Obstet. Gynec.* **109,** 131.

GREENWOOD R., SMELLIE H., BARR M. & CUNLIFFE A.C. (1958) Circulating antibodies in sarcoidosis. *Brit. Med. J.* **i,** 1388.

HATTLER B.G. (Jr) & AMOS D.B. (1965) Reactions obtained with transferred lymphocytes in patients with advanced cancer. *J. Nat. Cancer Inst.* **35,** 927.

HEATH R.B., FAIRLEY G.H. & MALPAS J.S. (1964) Production of antibodies against viruses in leukaemia and related disorders. *Brit. J. Haemat.* **10,** 365.

HERSH E.M. & OPPENHEIM J.J. (1965) Impaired *in vitro* lymphocyte transformation in Hodgkin's disease. *New Eng. J. Med.* **273,** 1006.

HOBBS J.R. (1966) Disturbances of the Immunoglobulins. *Scientific Basis of Medicine Annual Reviews,* p. 106. University of London: Athlone Press.

HOFFMAN G.T. & ROTTINO A. (1950) Studies of immunologic reactions of patients with Hodgkin's disease. *Arch. Int. Med.* **86,** 872.

HOPE-STONE H.F., BLANDY J.P. & DAYAN A.D. (1963) 282 testicular tumours seen at the London Hospital during 1926–61. *Brit. Med J.* **i,** 984.

HOWELL K.M. (1920) The failure of antibody formation in leukaemia. *Arch. Int. Med.* **26,** 706.

HUDSON R.P. & WILSON S.J. (1960) Hypogammaglobulinaemia and chronic lymphatic leukaemia. *Cancer* **13,** 200.

HUGHES L.E. & LYTTON B. (1964) Antigenic properties of human tumours: delayed cutaneous hypersensitivity reactions. *Brit. Med. J.* **i,** 209.

HUGHES L.E. & MACKAY W.D. (1965) Suppression of the tuberculin response in malignant disease. *Brit. Med. J.* **ii,** 1346.

JIM R.T.S. (1957) Serum gammaglobulin levels in chronic lymphocytic leukaemia. *Amer. J. Med. Sci.* **234,** 44.

KELLOCK T.H., CHAMBERS H. & RUSS S. (1922) An attempt to procure immunity to malignant disease in man. *Lancet* **i,** 217.

KELLY W.D., LAMB D.L., VARCO R.L. & GOOD R.A. (1960) An investigation of Hodgkin's disease with respect to the problem of homotransplantation. *Ann. N.Y. Acad. Sci.* **87,** 187.

KLEIN G. (1967) *Chemotherapy of Burkitt's Tumour,* ed. BURCHENAL J.H. Springer, Heidelberg.

LAMB D., PILNEY F., KELLY W.D. & GOOD R.A. (1962) A comparative study of the incidence of anergy in patients with carcinoma, leukaemia, Hodgkin's disease and other lymphomas. *J. Imm.* **89,** 555.

LARSON D.L. & TOMLINSON L.J. (1953) Quantitative antibody studies in man. III. Antibody response in leukaemia and other malignant lymphomata. *J. Clin. Invest.* **32,** 317.

LAWSON H.A., STUART C.A., PAULL A.M., PHILLIPS A.M. & PHILLIPS R.W. (1955) Observations on the antibody content of the blood in patients with multiple myeloma. *New Eng. J. Med.* **252,** 13.

LEVIN A.G., MCDONOUGH E.F. (Jr), MILLER D.G. & SOUTHAM C.M. (1964a) Delayed hypersensitivity response to DNFB in sick and healthy persons. *Ann. N.Y. Acad. Sci.* **120,** 400.

LEVIN A.G., CUSTODIO D.B., MANDEL E.E. & SOUTHAM C.M. (1964b) Rejection of cancer homotransplants by patients with debilitating non-neoplastic disease. *Ann. N.Y. Acad. Sci.* **120,** 410.

LUKES R.J. (1964) Prognosis and relationship of histologic features to clinic stage. *J.A.M.A.* **190,** 914.

LYTTON B., HUGHES L.E. & FULTHORPE A.J. (1964) Circulating antibody response in malignant disease. *Lancet* **i,** 69.

MACKAY I.R., TAFT L.I. & WOODS E.F. (1957) Clinical features and pathogenesis of macroglobulinaemia. *Brit. Med. J.* **i,** 561.

MARTIN D.C., RUBINI M. & ROSEN V.J. (1965) Cadaveric renal homotransplantation with inadvertent transplantation of carcinoma. *J.A.M.A.* **192,** 752.

MARTIN L.S.J., WOODRUFF M.W., WEBSTER J.H. & PICKREN J.W. (1965) Testicular seminoma. A review of 179 patients treated over a 50 year period. *Arch. Surg. (Chicago),* **90,** 306.

MARTIN N.H. (1960) Macroglobulinaemia. *Quart. J. Med.* **29,** 179.

MILLER D.G. (1962) Patterns of immunological deficiency in lymphomas and leukaemias. *Ann. Int. Med.* **57,** 703.

MILNER J.E., EVANS C.A. & WEISER R.S. (1964) Immunity and the treatment of cancer. *Lancet* **ii,** 816.

MORESCHI C. VON (1914) Euber antigene und pyrogene Wirkung des Typhusbacillus bei leukämischen Kranken. *Ztschr. f. Imunitätsforsel. u. Exper. Therap.* **21,** 410.

NADLER S.H. & MOORE G.E. (1965) Autotransplantation of human cancer. *J.A.M.A.* **191,** 105.

NADLER S.H. & MOORE G.E. (1966) Clinical immunologic study of malignant disease: response to tumour transplants and transfer of leucocytes. *Ann. Surg.* **164,** 482.

NEELY R.A. & NEILL D.W. (1956) Electrophoretic studies on the serum proteins in neoplastic disease involving the haemopoietic and reticulo-endothelial systems. *Brit. H. Haemat.* **2,** 32.

ONAT A. & COOPER T. (1960) Relation of bone marrow findings to serum proteins changes in lymphosarcoma, chronic lymphocytic leukaemia and Hodgkin's disease. *Blood* **15,** 114.

OPPENHEIM J.J., WHANG J. & FREI E. III. (1965) Immunologic and cytogenetic studies of chronic lymphocytic leukaemia. *Blood* **26,** 121.

PISCIOTTA A.V., JERMAIN L.F. & HINZ J.E. (1960) Chronic lymphocytic leukaemia, hypogammaglobulinaemia and autoimmune haemolytic anaemia—An experiment of nature. *Blood* **15,** 748.

PORGES R.F. (1956) A method for demonstrating secondary agammaglobulinaemia in patients with multiple myeloma, using paper electrophoresis. *J. Lab. Clin. Med.* **47,** 960.

PRASAD A.S. & BLOCK H.S. (1960) Macroglobulinaemia secondary to a lymphosarcoma of the brain. *Arch. Int. Med.* **105,** 316.

PYKE D.A. & SCADDING J.G. (1952) Effect of cortisone upon skin sensitivity to tuberculin in sarcoidosis. *Brit. Med. J.* **ii,** 1126.

ROBINSON E. (1966) Immunology and phytohaemagglutinin in cancer. *Lancet* **ii,** 753.

ROBINSON E., BEN HUR N., SHULMAN J., HOCHMAN A. & NEUMAN Z.J. (1965) Comparative study of skin homografts of normal donors and donors with malignant neoplasia in a host with malignant disease. *J. Nat. Cancer Inst.* **34,** 185.

ROBINSON E. & HOCHMAN A. (1966) Comparative study of the lymphocyte transfer test with lymphocytes from normal donors and cancer patients. *J. Nat. Cancer Inst.* **36,** 819.

ROSTENBERG A. (JR), MCCRANEY H.C. & BLUEFARB S.M. (1956) Immunologic studies in the lymphoblastomas: II. The ability to develop an eczematous sensitization to a simple chemical and the ability to accept passive transfer antibody. *J. Invest. Derm.* **26,** 209.

SCANLON E.F., HAWKINS R.A., FOX W.W. & SMITH W.S. (1965) Fatal homotransplanted melanoma. *Cancer (Philad.)* **18,** 782.

SCHIER W.W., ROTH A., OSTROFF G. & SCHRIFT M.H. (1956) Hodgkin's disease and immunity. *Amer. J. Med.* **20,** 94.

SCHWARZENBERG L. MATHÉ G., SCHNEIDER M., AMIEL J.L., CATTAN A. & SCHLUMBERGER J.R. (1966) Attempted adoptive immunotherapy of acute leukaemia by leucocyte transfusions. *Lancet* **ii,** 365.

SHAW R.K., SZWED C., BOGGS D.R. FAHEY J.L., FREI E., MORRISON E. & UTZ J.P. (1960) Infection and immunity in chronic lymphocytic leukaemia. *Arch. Int. Med.* **106,** 467.

SILVER R.T., UTZ J.P., FAHEY J. & FREI E. (1960) Antibody responses in patients with acute leukaemia. *J. Lab. Clin. Med.* **56,** 634.

SMITHERS D.W. (1962) Spontaneous regression of tumours. *Clin. Radiol.* **13,** 132.

SNAPPER I., TURNER L.B. & MOSCOVITZ H.L. (1953) Multiple Myeloma. Grune and Stratton, New York.

SOLOWEY A.C. & RAPAPORT F.T. (1965) Immunologic responses in cancer patients. *Surg. Gynec. Obstet.* **121,** 756.

SOUTHAM C.M. (1965) Evidence of immunological reactions to autochthonous cancer in man. *Europ. J. Cancer* **1,** 173.

SOUTHAM C.M., MOORE A.E. & RHOADS C.P. (1957) Homotransplantation of human cell lines. *Science* **125,** 158.

TEITELBAUM J.I., WIENER J. & DESFORGES J.F. (1959) A serologic and electrophoretic study of the malignant and proliferative disorders of the haemopoietic and reticuloendothelial systems. *J. Lab. Clin. Med.* **53,** 535.

ULTMANN J.E., FISH W., OSSERMAN E. & GELHORN A. (1959) The clinical implications of hypogammaglobulinaemia in patients with chronic lymphocytic leukaemia and lymphosarcoma. *Ann. Int. Med.* **51,** 501.

URBACK F., SONES M. & ISRAEL H.L. (1952) Passive transfer of tuberculin sensitivity to patients with sarcoidosis. *New Eng. J. Med.* **247,** 794.

WARWICK W.J., ARCHER O., KELLY W.D. & PAGE A.R. (1961) Antigen-antibody reactions. Anergy of delayed allergy in Hodgkin's disease patients. *Fed. Proc.* **20,** 18.

WEINSTEIN G.L. & FITZ-HUGH T. (JR) (1935) The heterophil antibody test in leukaemia and leukaemoid conditions. *Amer. J. Med. Sci.* **190,** 106.

WHITTLE R.J. (1957) Tumours of the testicle. *Brit. J. Radio.* **30,** 7.

WOODRUFF M.F.A. & NOLAN B. (1963) Preliminary observations on treatment of advanced cancer by injection of allogeneic spleen cells. *Lancet* **ii,** 426.

WORLD HEALTH ORGANIZATION (1966) Immunotherapy of Cancer. *Wld. Hlth. Org. Techn. Rep. Ser. No.* 344.

ZINNERMAN H.H. & WENDELL H.H. (1954) Recurrent pneumonia in multiple myeloma and some observations on immunologic response. *Ann. Int. Med.* **41,** 1152.

CHAPTER 19

IMMUNITY DEFICIENCY STATES

J.F. SOOTHILL

INTRODUCTION

Resistance to infection depends on many functions, some of which are listed in Table 19.1 Deficiency of these may lead to overwhelming acute infection, or to

chronic abnormal susceptibility to infection. Both improvement in the medical care of the former and advances in techniques have led to increasing recognition of the latter. Treatment, and, probably more important, adequate nutrition and housing has led to a profound fall in mortality and morbidity to infection over the years, particularly in earlier life, but, in spite of such measures, infection is exceeded only by injuries and congenital abnormalities as a cause of death in the first year of life (*Registrar General's Statistical Review* 1964), besides leading to much chronic illness. Some individuals have such a remarkable incidence of

TABLE 19.1

Some structures and functions relevant to resistance to infection with examples of diseases of each system, both quantitative and functional.

Structure	Example diseases	
	Quantitative deficiency	Functional deficiency
Skin	Trauma	Eczema
Mucous membranes	Trauma	Cystic fibrosis
Polymorphonuclear leucocytes	Neutropenia	Chronic granulomatous disease[1]
Complement	Hypocomplementaemia (C'_2) [2, 3]	—
Interferon	Not established yet, but see [4]	—
Immunoglobulins	Hypogammaglobulinaemia	Antibody deficiency syndrome without hypogammaglobulinaemia
Cellular immunity	Lymphopenia	Sarcoidosis [5]

[1] Holmes *et al* (1966) [2] Silverstein (1960)
[3] Gewurz *et al* (1966) [4] Baron & Isaacs (1962)
[5] Aisenberg (1962).

infection to multiple organisms in multiple sites that the suspicion arises that there may be defects of some of these mechanisms. A number of syndromes of extreme disturbance of one or more has already been recognized and it seems likely that subtler tests of function will reveal a much higher incidence of such defects in patients with chronic infection. Current work has initially concentrated on gross abnormalities, with attempts to correlate clinical syndromes with disturbances of individual functions.

The relative role of specific immunity, the last two functions in Table 19.1, in protection against infection, as against that of the other functions listed, is difficult to assess, but attention to defects of these mechanisms has been greatly increased lately, as a result of the detection of primary defects of the mechanisms involved. It has long been known that patients with abnormal serum proteins, whether due to abnormal loss (e.g. the nephrotic syndrome), or abnormal or deficient production (e.g. myelomatosis or lymphatic leukaemia) may be liable to repeated infection and their serum may be deficient of antibodies following immunization (Larson & Tomlinson 1952, 1953). Such phenomena of secondary deficiency of humoral immunity was paralleled by a similar demonstration

TABLE 19.2

Deficiency states of specific immunity

	Syndrome	Deficient mechanism	Possible basic defect	Example diseases
1	Antibody deficiency syndrome	Humoral immunity	Gastrointestinal lymphoid tissue deficiency, or disturbance of function or loss, etc.	Hypogammaglobulinaemia in boys Dysgammaglobulinaemia Nephrotic syndrome
2	Cellular immunity deficiency syndrome	Cellular immunity	Thymus dependent lymphoid tissue deficiency or disturbance of function	Isolated thymus dysplasia
3	1 and 2	1 and 2	Perhaps 1 and 2 Perhaps a more profound thymus deficiency	Lymphopenic hypogammaglobulinaemia Ataxia telangiectasia
4	1 and 2 and neutropenia	All reticular structures	Reticular dysgenesis	Reticular dysgenesis

of deficiency of humoral immunity (Bruton 1952), and of cellular immunity (Glanzmann & Riniker 1950) arising from no obvious cause. Attention was soon focused on the very characteristic sex-linked form of hypogammaglobulinaemia (Bruton *et al* 1952), and since then a complex of diseases affecting all ages and both sexes, associated with deficiency of both humoral and cellular immune mechanisms have been recognized. This chapter, concerned almost entirely with defects of the specific mechanisms, deals with defects of the other functions only when they are associated with them. It is probable that all combinations of quantitative and qualitative defects of each one of the immunoglobulins and of cellular immunity may exist independently or together with others as a familial phenomenon and as acquired diseases, some

of known cause and some of unknown cause. Techniques for study of the immunoglobulins and of humoral immunity are more advanced than those for cellular immunity, so the quantitative basis of these deficiencies can be established more satisfactorily. Existing evidence suggests that it is unlikely that an absolute deficiency of any one of the immunoglobulins occurs, and one is inclined to speculate that this is also true of defects of cellular immunity.

Table 19.2 indicates a basic classification of the immunity deficiency syndromes. Aetiologic speculations on the existence of independent humoral and cellular immunity and combinations of the two will be discussed later. But the probable role of the thymus in both is one way of tying the phenomena together.

DEVELOPMENT OF IMMUNITY MECHANISMS

A normal child is born with its mother's IgG, at a concentration slightly higher than that in the mother's serum, a little of its own IgG, IgM and very little IgA or IgD. The maternal IgG decays, and the child increases IgM, IgG and IgA production (in that order) to produce the serum levels of each immunoglobulin which are described in Chapter 13. The newborn child is therefore passively protected by maternal IgG antibodies for some weeks after birth, but only to antigens against which the mother has circulating antibodies. The physiological roles of IgG, IgM, and IgA are discussed in Chapter 13, and from these one can speculate on the possible relevance of the concentrations of the different immunoglobulins on the child's immunity to different infections, apart from the question of presence of specific antibody to the infection. The first antibody to be produced in response to primary immunization is IgM (see Chapter 13). This is largely intravascular, so it will provide little protection in interstitial fluid, but IgM antibodies to Gram-negative bacteria are particularly effective in killing the bacteria, and Gitlin, Rosen & Michael (1964) have suggested that this may be relevant to the susceptibility of infants to such organisms as *E. coli*, which are of little pathogenicity in adults. Also, IgA is secreted specifically in mucus (Tomasi *et al* 1965) and this may well have a local protective function in the upper respiratory, gastro-intestinal and urinary tracts so that absence of it soon after birth may well be associated with transient increased susceptibility. In contrast to these limitations of humoral immunity in infancy, cellular immunity—capacity to react to dinitrochlorobenzene and other skin sensitizing antigens—is present soon after birth (Uhr, Dancis & Neumann 1960).

MAIN SYNDROMES OF IMMUNITY DEFICIENCY

The common feature in the clinical presentation of patients with the various immunity deficiency syndromes is the increased incidence of infection. Certain

patterns of infections in different groups of patients are recognized, and it is claimed that they are related to defects of different immune mechanisms.

The best established and most commonly reported syndrome is the antibody deficiency syndrome (Barandun *et al* 1959). This is characterized by predominance of bacterial infections, particularly of Gram-positive cocci involving many systems (Gitlin *et al* 1959). The incidence of infections of various organs of such

TABLE 19.3

Illnesses recorded in eighty-two patients prior to diagnosis classified by age and sex (Note that an average of more than four different kinds of illness per patient was recorded)

	Children (0–14 years)		Adults		Total
	M	F	M	F	
Total patients	41	9	10	22	82
Disease involving:					
Lungs	68	13	21	51	153
Upper respiratory tract (including middle ear)	51	17	17	24	109
Skin (including conjunctivae and external ear)	40	11	11	29	91
Specific infectious disease	35	4	22	21	82
Digestive tract	9	3	5	6	23
Urinary tract	2	0	0	8	10
Female genital tract	—	1	—	6	7
Bones and joints	3	0	1	3	7
Other congenital disease	1	0	0	2	3
Miscellaneous	14	4	4	11	33
Total disease	223	53	81	161	518

(From Squire 1962)

patients is indicated in Table 19.3. Conspicuous is the lack of evidence of abnormal susceptibility to virus disease. Repeated infection in multiple sites is usual. Symptoms are not usually apparent in the first few months of life, presumably because of the passively acquired maternal antibody. But thereafter the severity of infections may vary very greatly, and some patients with a known family history have been shown to have a gross deficiency of all immunoglobulins in spite of little evidence of abnormal incidence of infection. Apart from such a congenital defect, antibody deficiency syndrome may arise at any stage in life, after a previously normal history of immunity (Wollheim *et al* 1964). Hobbs

(1966) has shown the development of hypogammaglobulinaemia in one such case. It seems likely that tissue damage from chronic infection also leads to increased susceptibility to reinfection, and so this will add to the variation of severity of symptoms from patient to patient, but one factor for this variation is undoubtedly the degree of the defect of cellular immunity, which is often associated with the antibody deficiency syndrome.

The second group of patients emerging as a syndrome are those in whom there is a deficiency of cellular immunity. This is demonstrated by a failure of delayed type allergy and the presentation is one of recurrent or progressive virus or monilia infections. In these patients homografts survive abnormally long. Such a defect is usually associated with hypogammaglobulinaemia—the lymphopenic form of hypogammaglobulinaemia (Hitzig & Willi 1961) or the combined immunity deficiency syndrome (see later), but clear descriptions of the deficiency of cellular immunity with normal immunity (Fulginiti *et al* 1966) associated with abnormal susceptibility to virus infection justify the separate recognition of the cellular immunity deficiency syndrome. The association of such a state with hypoparathyroidism in patients with no detectable parathyroids or thymus as a result of failure of third and fourth bronchial arch development provides important confirmation that the thymus does have a function related to cellular immunity in man (Di George *et al* 1968).

These three syndromes have in common the feature of presenting only after some months of life. In a very rare group of patients a very severe form of combined (humoral and cellular) immunity deficiency is associated with gross neutropenia, aplastic anaemia and thrombocytopenia (de Vaal & Seynhaeve 1959); infections start far earlier, and are rapidly fatal, in spite of the maternal IgG.

These three syndromes, which partly overlap, are now fairly clear, in terms of disturbance of function, but the pathogenesis of various patterns of familial disease such as Aldrich's syndrome, and the immunity defects related to ataxia telangiectasia is less clear, though the abnormal susceptibility to infection can largely be explained by deficiencies of humoral and cellular immunity. It should also be stressed that the association of susceptibility to virus infections, for instance, with cellular rather than humoral defects results from a clinical suspicion rather than critical statistical analysis. There is reason to doubt some such assertions—for instance whether immunity to tuberculosis, *pneumocystis carinii*, etc., are related mainly to cellular or humoral defects, and since it seems likely that we are always dealing with quantitative rather than absolute defects, which usually coexist, such correlations will only be clearly substantiated by statistical analysis of incidence of each infection, with quantitative data on the defect of each function.

ANTIBODY DEFICIENCY SYNDROME

HYPOGAMMAGLOBULINAEMIA

Because it is possible to define quantitatively certain aspects of the defect, it is in the field of hypogammaglobulinaemia that such correlations can best be attempted. IgG is the predominant immunoglobulin, so knowledge in this field has first been developed by study of patients with low levels of IgG, but, as described above, the syndrome of antibody deficiency is similar, whether it is related to a quantitative or qualitative deficiency of the immunoglobulins. Initial reports (Bruton *et al* 1952) of failure to detect γ-globulin electrophoreti-

TABLE 19.4

Antibody deficiency syndrome
(Quantitative disturbance of immunoglobulins)

Cause	IgG	IgA	IgM	IgD
1. Physiological	→	→	→	→
2. Transient	↓	↓→	↓→	?
3. Loss	↓	↓→	↑→	?
4. Myelomatosis	$\uparrow^{x}\rightarrow\downarrow$	$\uparrow^{x}\rightarrow\downarrow$	$\uparrow^{x}\rightarrow\downarrow$	$\uparrow^{x}\rightarrow\downarrow$
5. Other secondary diseases of production	↓	↓→	↓→	?
6. Familial				
(*a*) Sex-linked	↓	↓→	$\downarrow\rightarrow\uparrow^{(x)}$	↓→
(*b*) Non sex-linked (Thymus dysplasia)	↓→	↓→	↓→	?
7. Congenital rubella	↓	(↓)→	↑	?
8. Cause unknown	$\downarrow\rightarrow(^{x})$	↓→	$\downarrow\rightarrow^{(x)}\uparrow^{(x)}$	↓→↑

^{x} = immunoglobulin without detectable antibody function
$^{(x)}$ = immunoglobulin sometimes without detectable antibody function
↑→↓ = raised normal or low for age

cally led to the description of agammaglobulinaemia in boys. Soon, however, adult patients (Grant & Wallace 1954; Gitlin & Janeway 1956) and girls (Squire 1960) were described, and the application of immunological techniques to the study of the serum proteins of these patients showed that IgG is probably never completely absent (Kekwick *et al* 1961) and that, in addition to IgG, two other immunoglobulins, IgA and IgM, are sometimes also deficient (Gitlin, Hitzig & Janeway 1956). But the other immunoglobulins, IgA, IgM and IgD are not necessarily low in patients with low IgG; even in the primary form, they may be normal, or (for IgM) high in concentration (Kekwick *et al* 1961; West, Hong & Holland 1962.)

In Table 19.4 the possible combinations of concentrations of immunoglobulins are indicated for different types of antibody deficiency syndrome. First is the physiological type of early life, after decay of maternal IgG, as already outlined. The levels of all immunoglobulins are, of course, normal at this time, but this does not imply that this does not sometimes represent a serious risk to life, and an indication for administration of γ-globulin. The transfer of IgG from mother to baby occurs in late pregnancy, and the immunoglobulin levels may be very low in premature babies; γ-globulin treatment is probably valuable here (Davies & Hobbs 1967). Occasionally there is a marked delay in IgG production by the infant, though subsequently, even after a year or two's delay, normal levels are reached, apparently with normal immunity (Gitlin & Janeway 1956); this transient form of hypogammaglobulinaemia is perhaps the most rewarding to treat, and it is important that permanent tissue damage is avoided. With heavy proteinuria, or protein-losing enteropathy smaller proteins are lost more readily than large, and the serum concentration of slowly metabolized proteins is more affected than that of the quickly metabolized (Squire, Hardwicke & Soothill 1967); so IgG is profoundly affected, whereas IgM is at normal or raised concentration. Infections are common and dangerous in the nephrotic syndrome, and the local effect of oedema may be important here, but it seems likely that the immunity defect is probably less profound than in the hypogammaglobulinaemia associated with production defects, perhaps because the patient can react to an infection by a secondary antibody response, and cellular immunity is presumbly normal.

Hypogammaglobulinaemia due to loss, and the effects of known generalized diseases of the reticulo-epithelial system have been called secondary hypogammaglobulinaemia, but it is doubtful whether this latter term is meaningful as a distinction from 'primary acquired' hypogammaglobulinaemia; the word 'primary' means 'cause unknown' and is becoming progressively less frequently applicable, as more causes are established. Deficiency of humoral immunity has already been mentioned in such diseases as leukaemia (particularly chronic lymphatic leukaemia), but the situation in myelomatosis needs special consideration, as, though the total γ-globulin may be raised, much of it is abnormal material of a single immunoglobulin class, of circumscribed electrophoretic mobility and single light chain type, which apparently has no antibody function (see Chapter 13), and the normal immunoglobulins are usually at low concentration. This is a definite instance of qualitative defective immunoglobulins, in association with hypogammaglobulinaemia; this will be discussed later.

All these diseases associated with 'secondary' hypogammaglobulinaemia have their own specific symptoms as well as the common factor of susceptibility to infection, particularly bacterial. Of the two main groups of familial hypogammaglobulinaemia (see later)—the sex-linked form can only be identified from the other types if there are, in fact, affected male relatives; there are no

objective differences in the effects of the defect. The non-sex-linked form, associated with lymphopenia and thymus dysplasia besides exhibiting the antibody deficiency syndrome, is associated with the effects of cellular immunity deficiency—recurrent progressive virus infections, etc. Probably in both these forms, and in the group of acquired hypogammaglobulinaemia of unknown cause, the other immunoglobulins may be low, normal or (for IgM) high, but one exception is hypogammaglobulinaemia due to congenital rubella, which has a high IgM (Soothill, Hayes & Dudgeon 1966).

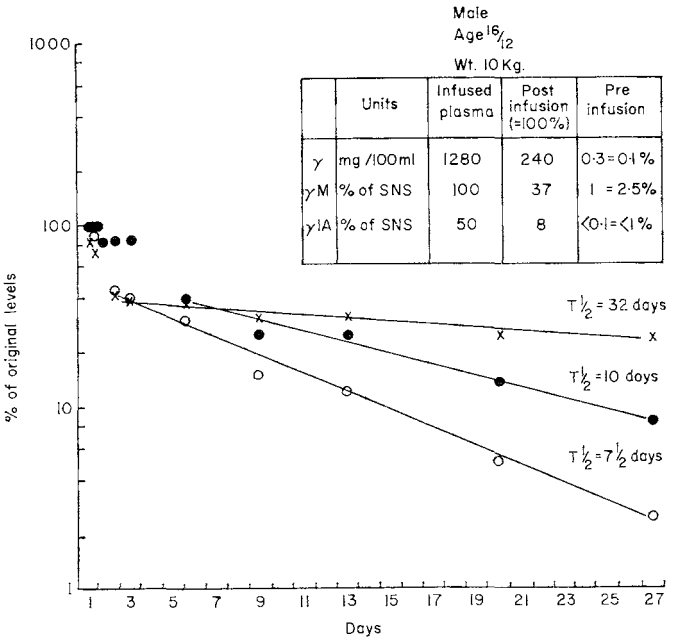


FIG. 19.1. Immunochemically determined decay of IgG, IgM and IgA in a patient with hypogammaglobulinaemia following infusion of fresh whole human plasma (IgG = X, IgA = ○, IgM = ●). (Soothill 1967—reproduced by kind permission of the Athlone Press.)

IMMUNOGLOBULIN METABOLISM IN HYPOGAMMAGLOBULINAEMIA

Apart from hypogammaglobulinaemia due to loss, the defect is one of production of the deficient immunoglobulins. This was shown to be so for IgG by decay of iodine labelled IgG (Gitlin *et al* 1959); the decay time was normal or slow, as is consistent with the more recent observation that the decay of IgG is related to concentration (see Chapter 13). Interpretation of such data is more

difficult in the presence of intestinal disease, which is often associated with hypogammaglobulinaemia; the production defect may be accentuated by loss, when the decay of IgG may be abnormally fast, but so is the decay of albumin (Waldman & Schwab 1965). Comparable data for three immunoglobulins can be obtained in patients with very low levels, by infusion of whole serum followed by serial immunochemical estimation of the serum concentration of

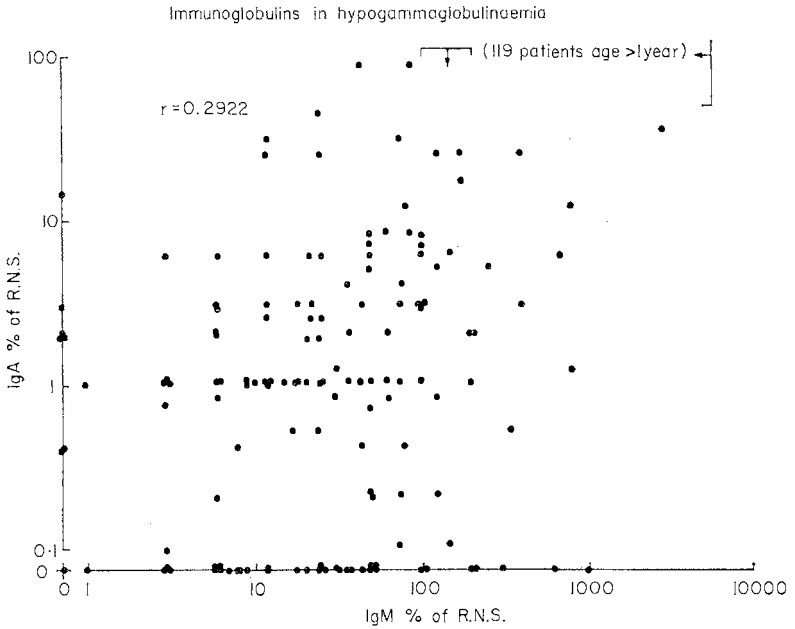


FIG. 19.2. The relationship of IgA to IgM in the sera of 119 patients with hypogammaglobulinaemia (IgG < 200 mg/100 ml) over the age of 1 year, without evidence of protein loss, etc. Mean and ±2 standard deviations of a healthy adult population are indicated by the bracket. Though there is a significant correlation ($P \simeq 0.001$) the scatter is very great.

each. Fig. 19.1 gives such data; the survival of the IgG is abnormally long, but that of IgA and IgM are probably normal.

In 'primary' hypogammaglobulinaemia, arbitrarily defined by IgG level, the serum concentrations of the four immunoglobulins show no correlation with each other. This is illustrated for IgA v. IgM and IgD v. IgG in Figs. 19.2 and 19.3, and applies to all combinations of immunoglobulins, in boys, girls, men and women, and even within families with several affected male relatives (Soothill, Hill & Rowe 1968).

Antibodies in Hypogammaglobulinaemia

Lack of antibodies—the apparent basis of the syndrome—is usually not absolute in these patients. The range of normality of antibody production to any particular stimulus is difficult to define. Antibodies which are present normally in all sera except those of blood group AB are the anti-A or anti-B iso-haemagglutinins. These are easy to detect, and failure to find them during routine cross-matching for blood transfusion has been the means of suspecting the diagnosis (Cooke, Weiner & Shinton 1957), as well as affording a useful means of confirmation. But, as is shown in Fig. 19.4, they may frequently be present, even at

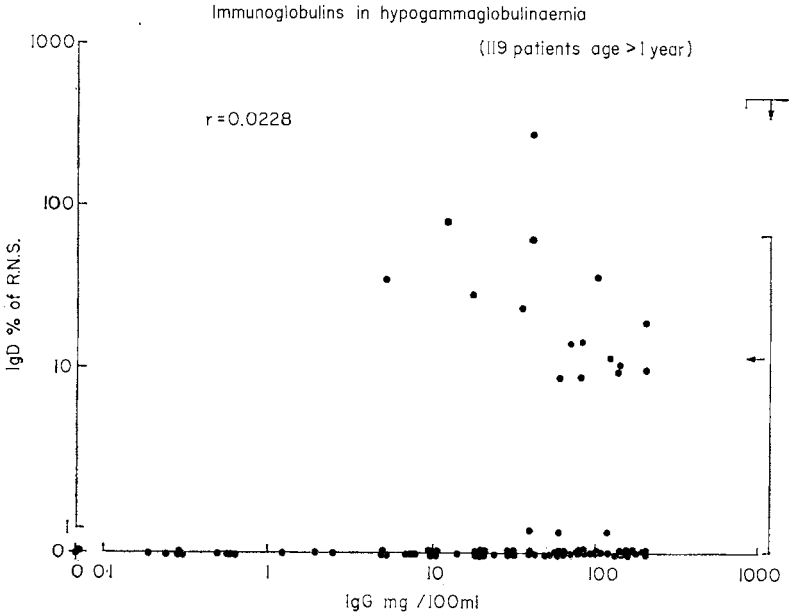


Fig. 19.3. Data for IgD and IgG, similar to Fig. 19.2. There is no significant correlation.

abnormally high titres, in the sera of patients with hypogammaglobulinaemia. They are normally largely IgM and they are present in sera of patients with hypogammaglobulinaemia only when the concentration of IgM is normal or high (Soothill 1962; Cruchaud *et al* 1962). On the other hand, some of the patients represented in Fig. 19.4, who had high IgM concentrations, did not have the expected iso-agglutinin activity, showing that, in patients with hypogammaglobulinaemia, this protein may be functionally deficient. This is presumably a similar situation to the functionally deficient immunoglobulins in patients with antibody deficiency syndrome with normal concentration of IgG (see later).

Antibodies, to bacteria with which the patients are infected, are deficient,

and frequently undetectable by current techniques. Gitlin *et al* (1959) summarize their antibody findings in the serum of twenty-one patients with 'congenital agammaglobulinaemia' after natural or artificial immunization with streptococci, *Staphyloccus aureus*, pneumococcus, diphtheria and tetanus antigens, typhoid and paratyphoid A and B vaccine, and *S. typhimurium*. Though the antibody responses were defective, low titres were occassionally found. Antibodies to bacteria which are IgM, such as anti-typhoid O, may be produced normally by patients with hypogammaglobulinaemia who have normal concentrations of IgM (Cruchaud *et al* 1962).

In spite of the normal clinical course of virus infections, which is the usual

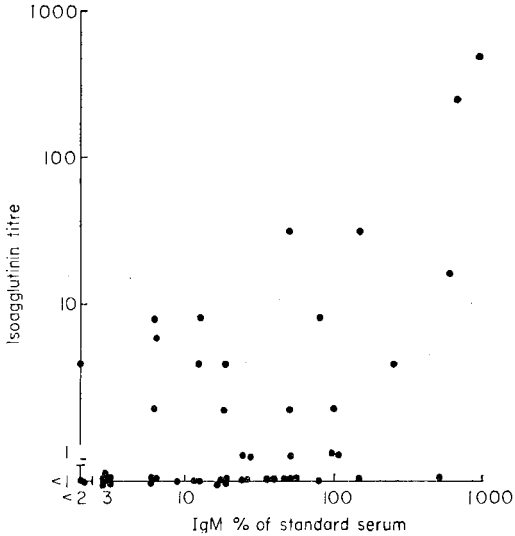


FIG. 19.4. The relationship between the iso-agglutinin and the IgM concentration in the serum of forty-nine patients with hypogammaglobulinaemia. (Reproduced from Soothill 1962a).

experience in patients with hypogammaglobulinaemia, antibodies to viruses are deficient from the sera after natural or artificial stimulus; but here too very sensitive techniques have demonstrated the presence of very small concentrations of anti-virus antibodies in patients with severe hypogammaglobulinaemia (Baron *et al* 1962).

Some patients with hypogammaglobulinaemia recover, partly or wholly, their ability to produce γ-globulin (see later) and they also recover the ability to make antibody (MacCallum 1962) both anti-bacterial and anti-viral. It seems, in general, that, in most patients with hypogammaglobulinaemia, the small amounts of immunoglobulins present in the serum appear to be effective antibody—quantitative rather than qualitative defects—but there are exceptions.

Dysimmunoglobulinaemia

The phenomenon of IgM in hypogammaglobulinaemia serum without iso-agglutinin activity (Fig. 19.4) is an example of an apparent qualitative or functional defect of one immunoglobulin, associated with quantitative defect of another. It is the converse of the syndrome described by Giedion & Scheidegger (1957) of antibody deficiency syndrome with normal concentration of IgG, but deficiency of IgA and/or IgM. This has been shown also to be an acquired condition (Gilbert & Hong 1964). Such defects can be very profound, as was shown by Williams (1966), whose patient, with high level of IgM and near normal levels of IgG, not only had a poor antibody response to various injected antigens, and no iso-agglutinins as measured by standard techniques, but failed to eliminate tracer quantities of labelled incompatible red cells. This was also true of a girl with abnormal susceptibility to infection (she died of infection in spite of antibiotics and γ-globulin treatment) with capacity to make some antibodies, but not others, normal cellular immunity, and normal levels of all the immunoglobulins (Blecher *et al* 1967).

The immunoglobulins, in such patients, are usually normal on conventional qualitative studies, including the presence of both K and L light chains (Blackburn, Chakera & Soothill 1965) but more critical studies are required in this field, because the concept of 'nonsense' γ-globulin, so long debated, is more probable here than elsewhere. But it is impossible to establish that all the immunoglobulin molecules are not antibody to antigens other than those tested for. Such patients are quite typical in their presentation with the antibody deficiency syndrome. This phenomenon seems to be the right meaning for the word dysgammaglobulinaemia (or, perhaps better, dysimmunoglobulinaemia—see later) which has also been applied to patients with low level of IgG but high level of IgM whether the IgM is functionally effective antibody or not, I think erroneously.

Symptomless Immunoglobulin Deficiency

In contrast to this, there is also the phenomenon of gross deficiency of IgA in healthy individuals without evidence of abnormal susceptibility to infection (Rockey *et al* 1964). Whether this is necessarily a partial manifestation of a defect related to the symptomatic ones under discussion is not clear, but Heremans (1960) has reported low levels of IgA in some mothers of patients with hypogammaglobulinaemia. The absence of an obvious excess of upper respiratory infection in such patients, even though their mucous lacks IgA, raises doubts as to the importance of the secretory IgA mechanism. IgD is often undetectable in healthy subjects (Rowe & Fahey 1965), so the occasional detection of igD in hypogammaglobulinaemia is interesting.

CELLULAR IMMUNITY DEFICIENCY SYNDROME

Though first recognized in the combined form with hypogammaglobulinaemia, the syndrome of abnormal susceptibility to virus infection, with normal immunoglobulin concentrations and sometimes with normal antibody production to both bacterial and viral infections, and no abnormal incidence of bacterial infections, has recently been described (Fulginiti *et al* 1966; Di George *et al* 1961). Cellular immunity is deficient, with lymphopenia and immature dysplastic or absent thymus. This does much to confirm the previously made assertions of correlation of bacterial immunity largely with humoral mechanisms, and viral immunity largely with cellular, thymus dependent mechanisms. The combined defect is commoner than the isolated cellular defect, and has been most clearly described as a very severe non-sex-linked familial defect, which has been called the lymphopenic or Swiss form of hypogammaglobulinaemia (Hitzig & Willi 1961; Gitlin & Craig 1963). In these patients, there is abnormal susceptibility to bacterial, viral and Candida infections, and one likely way of presentation is by progressive necrotic vaccinia. Abnormality is usually apparent after the first few weeks of life, and in all typical patients it has been rapidly fatal. The lymphopenia is not absolute, or constant, but lymphocyte counts of less than 1000 per cu. mm. are usual. Homografts are usually accepted which is in contrast to the sex-linked form of hypogammaglobulinaemia.

MISCELLANEOUS FAMILIAL IMMUNITY DEFICIENCY DISEASES

Ataxia Telangiectasia

Another form of combined defect of cellular and humoral immunity is that often, but perhaps not always, associated with the odd syndrome called ataxia telangiectasia. The syndrome, of progressive disturbance of cerebellar function and dementia, with telangiectasia of the conjunctiva and around the eyes, has long been known to be associated with abnormal susceptibility to infection. Varying deficiencies of the immunoglobulins, absence of iso-haemagglutinin, varying deficiency of delayed hypersensitivity, and graft rejection, and, even when the serum concentration of IgG was normal, defective antibody response to bacterioplage have been reported in such patients (Gutman & Lemli 1963; Peterson, Kelly & Good 1964; Fireman, Boesman & Gitlin 1964). Circulating lymphocyte count is often normal, but has been reported to be low (Young, Austen & Moser 1964). Multiple infection is characteristic, but fatal progressive virus infection is not so prominent as in the lymphopenic form described above. There seem therefore to be more than one form of combined defect of humoral and cellular immunity, of differing severity. There is no satisfactory

explanation of the relationship between the immunity defect and the neurological or skin abnormalities.

The most severe combined form is that called reticular dysgenesia by de Vaal and Seynhaeve (1959); (see also Gitlin, Vawter & Craig 1964). Profound deficiency of all immunoglobulins (except for maternal IgG), cellular immunity, and all blood cells is associated with rapid death from infection soon after birth. This familial defect, perhaps sometimes sex-linked, clearly is very rare.

Aldrich's Syndrome

One other familial syndrome of abnormal susceptibility to infection should also be mentioned. Since the description by Aldrich, Steinberg & Campbell (1954) of a family in which boys were suffering from eczema, purpura, diarrhoea and frequent infections, others have confirmed the symptom complex (Krivit & Good 1959) and have shown varying defects of immunity mechanisms, of which the only consistent one is absent iso-haemagglutinins. IgA may be high. There is no satisfactory unifying hypothesis for the syndrome at present.

SECONDARY DEFECTS

Mechanisms of humoral immunity deficiency due both to loss or to leukaemia, myeloma, etc., are probably more complicated than the simple direct effects of immunoglobulin loss or 'crowding out' of producing cells. Immunoglobulin decay studies in these fields have helped to clarify them. But this subject cannot be reviewed here. Mention should be made, however, of 'secondary' defects of cellular immunity in Hodgkin's disease (Aisenberg 1962) and sarcoidosis (Sones & Israel 1954). These are apparently associated with some defect of humoral response also. The former is discussed more fully in Chapter 18. A further complexity has arisen as a result of the study of tuberculin non-converters following BCG immunization (Sutherland, Mitchell & Hart 1965). There are apparently healthy individuals in the population who are incapable of reacting with delayed hypersensitivity, a defect which has something in common with the sarcoidosis, as the Kveim test may be positive.

INHERITANCE

Many family trees, such as that in Fig. 19.5, have been published, showing that a form of hypogammaglobulinaemia may be inherited as a sex-linked recessive characteristic. Sanger & Race (1963) have shown that it is not linked closely with the Xg blood group, in such families. The hypogammaglobulinaemia associated with lymphopenia appears to have a familial incidence suggestive of an autosomal recessive inheritance (Hitzig & Willi 1961) as does the defect associated with

ataxia telangiectasia. The three patients with reticular dysgenesis so far reported were all boys (two were twins) (de Vaal & Seynhaeve 1959; Gitlin *et al* 1964).

Other patients with 'primary' hypogammaglobulinaemia who developed the syndrome of susceptibility to infection in later life, may have a family history, not only of hypogammaglobulinaemia but also of hypergammaglobulinaemia and various abnormalities of the auto-allergic type (Wollheim *et al* 1964; Wolf 1962; Fudenberg, German & Kunkel 1962). These include the presence of LE cells and rheumatoid factor in symptomless individuals, and patients symptomatic with arthritis, DLE and sarcoidosis.

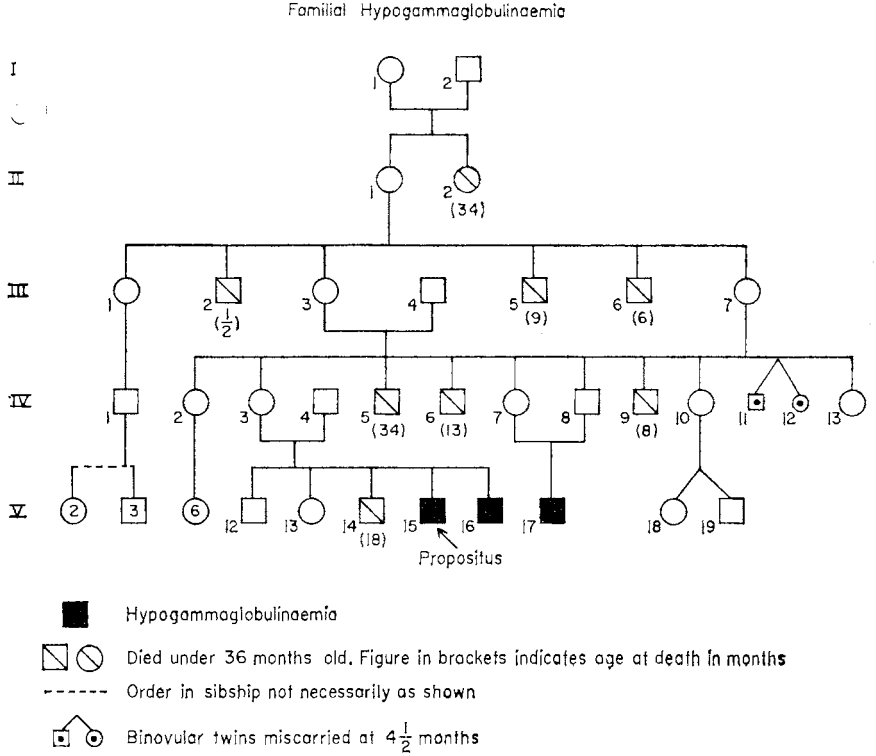


FIG. 19.5. A family tree showing sex-linked inheritance of hypogammaglobulinaemia. Most of the relatives who died in infancy had infective illnesses, and many may also have had the disease, though this was not proved. (From Jamieson & Kerr 1962, by courtesy of the Editors of the *Archives of Disease in Childhood*.)

HAEMATOLOGY IN IMMUNITY DEFICIENCY STATES

Patients with antibody deficiency syndrome frequently have haematological

abnormalities. Such defects listed by Good, Zak, Condie & Bridges (1960) as occurring in patients with hypogammaglobulinaemia include neutropenia (persistent and transient), lymphopenia, hypoplastic anaemia, absence of eosinophils, thrombocytopenia and hypersplenism. Haemolytic and megaloblastic anaemia also occur, the latter responding to B_{12}, and probably independent of associated steatorrhoea (Crowder, Thompson & Kupfer 1959). The neutropenia may be permanent in the short life of patients with reticular dysgenesis (de Vaal & Seynhaeve 1959), or transient—marked early in a severe infection with a profound polymorphonuclear leucocytosis during the recovery period, or terminal, particularly in patients with lymphopenia and hypogammaglobulinaemia. Neutropenia, hypoplastic anaemia and thrombocytopenia, may also be associated with hypogammaglobulinaemia and thymoma (see later), (Ramos 1956; Peterson, Cooper & Good 1965) and splenomegaly may play a part (Citron 1957). Lymphopenia has already been discussed in relation to the non-sex-linked, familial lymphopenic form of hypogammaglobulinaemia (Hitzig & Willi 1961). Auto-allergic haemolytic anaemia occurs in association with hypogammaglobulinaemia (Fudenberg & Solomon 1961).

Besides the association of secondary hypogammaglobulinaemia with lymphatic leukaemia, some patients with 'primary' hypogammaglobulinaemia, whether of the sex-linked type, the acquired type, or, particularly, with ataxia telangiectasia may develop leukaemia or lymphoma (Page, Hansen & Good 1963; Peterson *et al* 1964).

OTHER CLINICAL ASSOCIATIONS

Special mention must be made of the remarkable association of hypogammaglobulinaemia, whether sex-linked, familial with lymphopenia, acquired, or secondary to cytotoxic therapy, with pneumonia due to *pneumocystis carinii* (Hutchison 1955; Marshall, Weston & Bodian 1964). This parasitic infection, common in central Europe, apparently occurs in Britain, U.S.A. etc. only in the face of profound immunity defect. Possibly the organism may be a widespread saprophyte for many animal species, human and others, which becomes pathogenic only in circumstances of failure of host reaction. Diagnosis is important, as it is treatable, and is an important cause of death of patients with hypogammaglobulinaemia (see later). Persistent, antibiotic resistant, diffuse pneumonia, with conspicuous dyspnoeia and cyanosis should raise suspicion in these cases. Diagnostic confirmation currently usually requires lung biopsy.

Diarrhoea, with intestinal malabsorption is a common association with the various form of hypogammaglobulinaemia (Huizlinga *et al* 1966). The cause of this is unclear, though it is attractive to relate it to the diarrhoea and wasting of thymectomized animals (Parrott & East 1964). It may be that bacterial infection

plays a part in this; perhaps lack of IgA antibodies in mucous, or of bacteriocidal IgM antibodies renders chronic infection by normal bacteria flora possible. Mucosal biopsy, though often diagnostic of intestinal disease associated with hypogammaglobulinaemia, owing to the flattened swollen villi with sparse lymphocytes and plasma cells in the submucosa, does not give a lead to the mechanisms. The problem of differentiating hypogammaglobulinaemia due to failure of production, with secondary gut diseases, from the hypogammaglobulinaemia due to protein losing enteropathy (Waldmann & Schab 1965) has already been mentioned.

There have been frequent reports of arthritis of the rheumatoid type occurring in patients with various forms of hypogammaglobulinaemia (Good *et al* 1960). The patients themselves do not have rheumatoid factor in their sera, and it occurs not only in the acquired form in which relatives may have an abnormal incidence of rheumatoid factor, but also in the sex-linked form, in which this does not occur (Fudenberg *et al* 1962). The possibility that it could arise as a result of γ-globulin treatment has been suggested but cases have been reported to have the typical arthritis before γ-globulin treatment, and it has also been suggested that the arthritis may be improved by starting, or increasing the dose of γ-globulin (Rosen & Janeway 1966). It may be important in this context to recall that these patients are usually capable of some antibody production (see above) and so perhaps antigen excess complexes may particularly frequently circulate. Such complexes are probably responsible for Type III allergic reactions, which certainly includes arthritis in experimental situations (see Chapter 20). The possible role of soluble complexes in this arthritis requires further study.

Another interesting association is the fact that amyloidosis may occur in patients with hypogammaglobulinaemia (Teilum 1964). The mechanism of amyloid formation remains in doubt, but clearly high concentrations of immunoglobulins cannot play a major part.

HYPERSENSITIVITY REACTIONS

Though patients with the antibody deficiency syndrome usually fail to respond to antigens by antibody formation, some of the forms of hypersensitivity (see Chapter 20 for classification) may occur. The systematic study of this field has been well reviewed by Gitlin *et al* (1959) and Good *et al* (1960). Available data were obtained mainly from children with the sex-linked form of the disease; results in adults are essentially similar though less severely abnormal, but more work is required on them, and on other forms of the syndrome.

Type I. Anaphylactic Response

Urticaria following penicillin injection, and immediate reaction to skin tests

with allergens have been recorded, including the sex-linked form (Gitlin *et al* 1959) but such reactions are certainly rare in these patients, perhaps rarer than in the normal population. In the remarkable case recorded by Crowder *et al* (1959), a man of 30 years with hypogammaglobulinaemia who had hay fever, asthma and cutaneous hypersensitivity to a very wide range of allergens, reagins were demonstrated in his serum by passive transfer. Reactions of this type have been recorded in a man with isolated symptomless deficiency of IgA (Rockey *et al* 1965).

Type II. Cytotoxic Response

Auto-allergic haemolytic anaemia has been described in hypogammaglobulinaemia (Fudenberg & Solomon 1961). Anti-gastric parietal cell antibodies may also occur (see later).

Type III. Reaction Due to Toxic Complexes

As antibody production occurs, but is quantitatively deficient, it is possible that this might readily lead to the formation of antigen excess soluble complex in these patients. The high incidence of 'rheumatoid arthritis' (which could possibly be included in this group) is therefore interesting. Pepys (1966) has observed Bird Fancier's Lung (probably a disease of this type) in a patient with hypogammaglobulinaemia, whose serum had detectable precipitins to the budgerigar antigen to which she reacted on inhalation, in spite of the very low level of all immunoglobulins.

Type IV. Delayed Response

The heterogeneity of response to tests for this type of allergic reaction are discussed in other sections. It does not seem to be particularly associated with symptomatic illness in these patients.

Auto-Allergic Diseases

The relationship of immunity deficiency syndrome to possible auto-allergic states is discussed in the clinical and aetiological sections. In a striking instance of profound cellular deficiency, with functionally defective immunoglobulins, Schaller *et al* (1966) report both auto-allergic haemolytic anaemia and glomerulonephritis, and give a full review of this field. It seems clear that many forms of immunity deficiency state can be associated with such phenomena.

Reaction to Tissue Grafts

Homografts of skin, lymph nodes, thymus, and other tissue have been undertaken by a number of workers for both investigation and therapeutic purposes. The initial success reported by Good and his colleagues (see Good *et al* 1960) of skin and lymph node grafts in boys with severe hypogammaglobulinaemia

led to optimism. Stimulation of the lymph nodes with antigens either before or after transplant, resulted in the detection of antibody in the serum of the recipient. Some skin grafts survived for 5 years and more, and the cells in the surviving skin grafts from females into male patients were shown to maintain their nuclear sex. But the capacity of the lymph node grafts to react to antigenic stimulation waned after about 2 months, and it was found that only unusual cases of hypogammaglobulinaemia would take grafts so readily. Adults and children with transient hypogammaglobulinaemia rejected grafts normally, or after a few weeks, often with considerable reaction. Greater success has been reported in the lymphopenic form of hypogammaglobulinaemia (Hitzig & Willi 1961) which is consistent with the view that cellular immunity mechanisms are the most important in graft rejection, and that, whereas many patients with the various forms of immunity deficiency have some deficiency of such mechanisms, it is most profound in these ones. The group of patients with partial cellular immunity deficiency and ataxia telangiectasia are rather slow at graft rejection (Peterson *et al* 1965). Gross defect of cellular immunity with normal humoral response is also associated with survival of homografts (Fulginiti *et al* 1966) consistent with the concept that graft rejection is largely a function of cellular immunity (see Chapter 17).

MORBID ANATOMY OF THE SYNDROMES

The morbid anatomical bases of the syndromes described have been very fully reviewed by Peterson *et al* (1965). In essence, usually the histology is consistent with the functional disturbance. Apart from the effects of chronic infection (bronchiectasis, lung collapse, etc.) the abnormalities are confined to the lymphoid tissue, and are consistent with the view that development of such organs depends on two sources—thymus-derived cells which are concerned with cellular immunity (see Chapter 11) and gasto-intestinal derived cells which are responsible for humoral immunity but which are also to some extent influenced by a humoral function of the thymus (see Chapter 11). The concept of dual origin of such cells, originally demonstrated in the fowl (Warner, Szenberg & Burnet 1962 and many other workers), has apparently some mammalian equivalent (Cooper *et al* 1966). In the lymphopenic form of hypogammaglobulinaemia, there is a conspicuous lack of lymphoid tissue and lymph nodes, generally; what lymph nodes are present show gross deficiency of lymphocytes and plasma cells, very little of the normal architecture of cortex and medullary differentiation, germinal centres, etc.; the cells are mainly reticular in type. The thymus may be so small as to be difficult to find, and it may fail to descend from the neck (Tobler & Cottier 1958). It consists of a small collection of epithelial cells and fibrous tissue, with few lymphocytes, or Hassell's corpuscles (Peterson *et al* 1965).

In other immunity deficiency states the histological defects are more varied. Where immunoglobulins are formed, plasma cells are present, and can be shown by immunofluorescent study to contain the immunoglobulins that are, in fact, being made; for instance, Cruchaud *et al* (1962) showed that the plasma cells of a patient with low level of IgG and high level of IgM reacted for the latter, with very few reacting for the former. Gastro-intestinal lymphoid tissue is particularly deficient in patients with antibody deficiency syndrome and hypogammaglobulinaemia. This is well seen in the appendix (Good *et al* 1962), and the tonsils may be obviously deficient clinically and radiologically as well as on sectioning. This phenomenon parallels closely the appearances of chicks after removal of the bursa of Fabricius. Peripheral lymph nodes may not be grossly deficient in such patients, though the organization of germinal centres is poorly delineated and plasma cells are deficient. In these patients the thymus is usually normal.

In patients with acquired hypogammaglobulinaemia (that is, those with no family history) the appearances may be similar to those described above, but there may be also marked hypertrophy of lymph nodes and spleen, with infiltration with giant cells. Lymphomas may occur, and the thymus, though usually normal, may be the site of a thymoma. The incidence and details of these are reviewed by Peterson *et al* (1965).

Such histological information as there is in the rarer immunity deficiency states are consistent with this outline.

POSSIBLE AETIOLOGICAL MECHANISMS

With such a complicated combination of diseases, clearly one must anticipate multiple aetiologies. In the sex-linked form of hypogammaglobulinaemia, the lymphopenic form and in reticular dysgenesis, there are apparently clear-cut genetic defects, though one must still speculate on their mode of action. The parallel with thymus and bursa ablation experiments suggests that the first may be a gross deficiency of gastro-intestinal derived lymphoid cells, the second a thymus deficiency, and the third a failure of lymphoid cells generally. But there remain many difficulties. For instance, in the sex-linked form, IgM levels can range from low to abnormally high values from family to family, and even in the same family (Soothill 1962) and an individual's defect of production of each immunoglobulin may be very variable (Soothill *et al* 1968). It seems likely that the direct site of gene action is considerably separated from development of cells specialized for immunoglobulin production. If the defect of humoral immunity in the lymphopenic form depends on lack of a humoral factor from the thymus, how is it that isolated cellular immunity with thymus dysplasia can occur also, and it is remarkable that the defects of immunoglobulin production are so similar in this group to those in the sex-linked form. The combination

of quantitative and qualitative defects in the same patient are also odd in the light of these hypotheses.

It is clear, too, that 'acquired' hypogammaglobulinaemia is also sometimes partly a familial phenomenon (see the Inheritance section). The association with

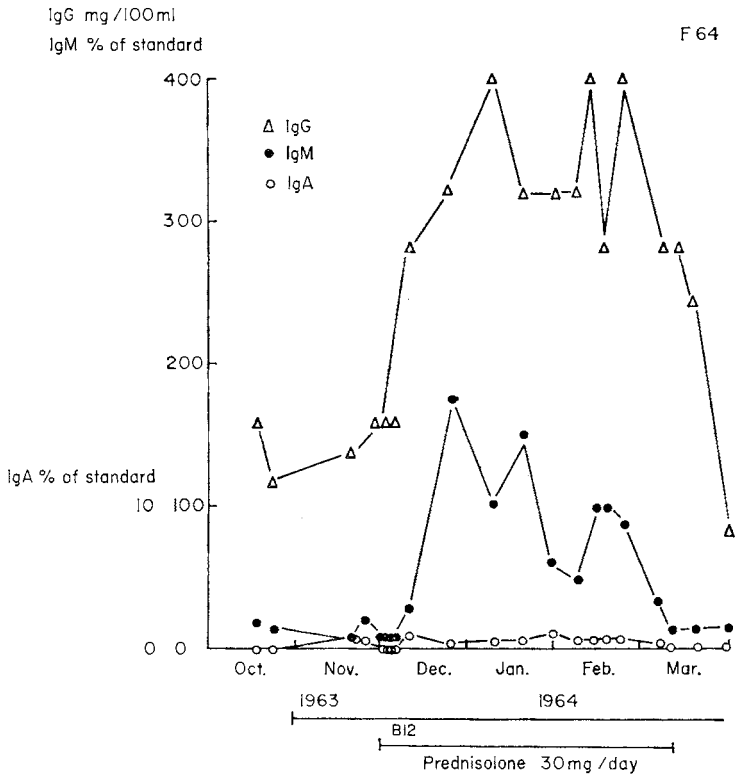


FIG. 19.6. Serial estimations of the immunoglobulins in the serum of a patient with pernicious anaemia and hypogammaglobulinaemia, on treatment with Vitamin B_{12}, and prednisolone. (Soothill 1967—reproduced by kind permission of the Athlone Press).

auto-allergic phenomena in the relatives, and in the patients, raise two possibilities. One, supported perhaps by the production of an auto-allergic haemolytic anaemia in neonatally thymectomized animals (Sutherland *et al* 1965), is that patients with immunity deficiency states are lacking feedback regulatory mechanisms—perhaps thymus dependent—which result in isolated immunologically competent cells breaking through the normal tolerance. Another possibility is that the hypogammaglobulinaemia may be, in itself, an auto-

allergic phenomenon—the immunity mechanism behaving like a snake eating its own tail. In view of this possibility a patient with hypogammaglobulinaemia, pernicious anaemia and anti-gastric parietal cell antibodies was given prednisolone (Soothill 1967); all the immunoglobulins rose (Fig. 19.6), and fell again after stopping the drug. Clearly this possibility needs further study, but if this does represent suppression of an auto-allergic mechanism, one would guess that it would act at some stage earlier than the actual production of immunoglobulins.

The possibility that an excess of Mowbray's factor (Mowbray 1963), an α_2 globulin perhaps of thymus origin, which inhibits antibody production, might lead to some forms of immunity deficiency states, needs further study, perhaps particularly in those with thymoma and those with functionally defective immunoglobulins.

The high incidence of thymoma in acquired hypogammaglobulinaemia suggests that the thymus may play a part here too, but the association of various immunity deficiency states, both 'primary' and 'secondary', with various forms of lymphoid neoplasia raises the possibility that the immunity deficiency is the primary defect, and that the neoplasm is an effect of failure of postulated mechanisms of immunological elimination of abnormal cells (Peterson *et al* 1965). But, in that case, why are they usually tumours of cells of the lymphoid series. The background to this is discussed more fully in Chapter 11.

There are some circumstances in which the aetiology of a form of immunity deficiency seems more firmly based. In congenital rubella, some infants have a characteristic 'acquired congenital' hypogammaglobulinaemia (Soothill *et al* 1966). But even if this can be fairly confidently ascribed to the intra-uterine virus infection, aetiological puzzles remain, not least because, though IgG is deficient, IgM is high. The specific deficiency of thymus and parathyroids, clearly relatable to a basic failure of branchial arch development (Di George *et al* 1968) indicates the role of the thymus in both cellular immunity and humoral immunity, and provides one basic aetiological entity in this field.

It seems likely that such a complex mechanism as the immune response will have defects at different stages (afferent, central and efferent) and at different levels of development of these. Only when techniques are available to study these individually will the complexity become clearer.

INCIDENCE, DIAGNOSIS AND TREATMENT

Incidence

Without better definitions, it is impossible to give a clear idea of the incidence of the various types of defect mentioned. Data on patients with hypogammaglobulinaemia, as defined above, in Britain, collected under the auspices of the

Medical Research Council Working Party of Hypogammaglobulinaemia, provide the most complete information available. The age and sex of eighty-two patients is shown in Table 19.3. Boys predominate over girls by nearly 5:1, but in adults, females predominate. Cohort analysis by year of birth shows that the incidence in Britain of diagnosed male children aged 5 to 9 years exceeds 1 per 100,000 births; this is, of course, a minimal incidence in a frequently fatal, and not obviously diagnosable condition (Squire 1962). The other immunity deficiency states are probably rarer.

Diagnosis

In order to establish a ground for treatment, and to advance knowledge by accurate classification of individual patients, it would be very desirable to have a system for separating those patients with recurrent infection in whom there is a defect in one or more immunity mechanisms from those without. Unfortunately a body of control information is not yet available to attempt this rationally.

Quantitative deficiencies of immunoglobulins can be defined using a gel diffusion precipitin technique (see Chapter 13), and, since Gitlin & Janeway (1956) suggested that infections were particularly troublesome if the IgG concentration were less than 200 mg/100 ml, the Medical Research Council Working Party on Hypogammaglobulinaemia took the view that this provided a *prima facie* case for giving γ-globulin. This still seems rational, but patients with recurrent infection with high levels of IgG than this may have qualitatively defective immunoglobulins. Deficiency of IgM or IgA may point to this, but antibody response testing seems needed, ultimately, and adequate control information is not available. A search for antibodies usually present, such as isoagglutinins and anti-streptolysin O, is useful, but tests of response to administered antigens are also needed.

Similarly, such tests for cellular immunity are needed, but available tests are even less quantitative. Development of contact sensitivity to dinitrofluorobenzene or dinitrochlorobenzene have been used, with some control data (Uhr *et al* 1962) but the normal reaction can be disturbingly severe. Measurement of humoral and cellular response to a number of antigens in different physical forms would seem to give the best chance of testing the immune mechanisms at both afferent and efferent levels. But only dead antigens should be used, in view of the possibility of unexpected susceptibility. To parallel cutaneous delayed hypersensitivity *in vitro*, lymphocyte transformation to phytohaemagglutinin and to antigens, and inhibition of macrophage migration in tissue culture would appear to be promising lines, but many difficulties are still interfering with their quantitative use in human material.

In addition to these specific tests of immune mechanisms, a standard clinical examination, and work-up, is, of course, needed.

Treatment

There are a number of possibles lines of treatment for the immunity deficiency states, with varying relevance to the different forms indicated above, but Table 19.5, shows how relatively ineffective these are.

Chemotherapy of Individual Infections

Gitlin & Janeway (1956) early noted that individual infections responded well to chemotherapy suitably chosen according to the sensitivity of the infecting organisms. This is shown for tuberculosis in Fig. 19.7. Marshall *et al* (1964) reported success in one patient with pneumocystis carinii infection, using pentamidine, and this has since been confirmed. Undoubtedly these various measures are effective and life-saving. But in spite of them, chronic tissue damage results from the frequent occurrence of infections and these patients almost always require more than treatment of individual infections as they occur, to avoid fatal progression.

Table 19.5

Crude death-rates by sex and age
(Note the high death-rate among the female children)

	Children (0–14 years)		Adults		
	M	F	M	F	All
Deaths	6	5	1	5	17
Total at risk	41	9	10	22	82
Death rate %	15	56	10	23	21

(From Squire 1962)

Prophylactic Antibacterial Drugs

Gitlin & Janeway (1956) proposed the use of these and it seems a reasonable line of treatment, though there is no proof of their efficacy.

Replacement of γ-Globulin

Gitlin & Janeway (1956) outlined a rational policy of administering γ-globulin prepared from pooled donors in doses equivalent to 0.025 g/kg body wt/week and reported a clinical impression of improvement in the patients as a result. One would certainly not anticipate spectacular results by any such measures in view of the fact that the patients have all the effects of tissue damage resulting from chronic infection leading to increased susceptibility to infection again, and the replacement treatment cannot be expected to provide a full range of antibodies to all possible infecting organisms, and there would be nothing in the nature of a booster response of antibody which normally occurs in an immunized individual.

Nor is any deficiency of cellular immunity replaced. On the other hand, a conspicuous lack of history of the usual epidemic fevers of childhood in children with hypogammaglobulinaemia receiving γ-globulin treatment strongly suggests that it is providing effective protection against these infections.

Such treatment seems reasonable whenever there is a deficiency of circulating antibodies, whether this is due to a quantitative or qualitative defect of IgG,

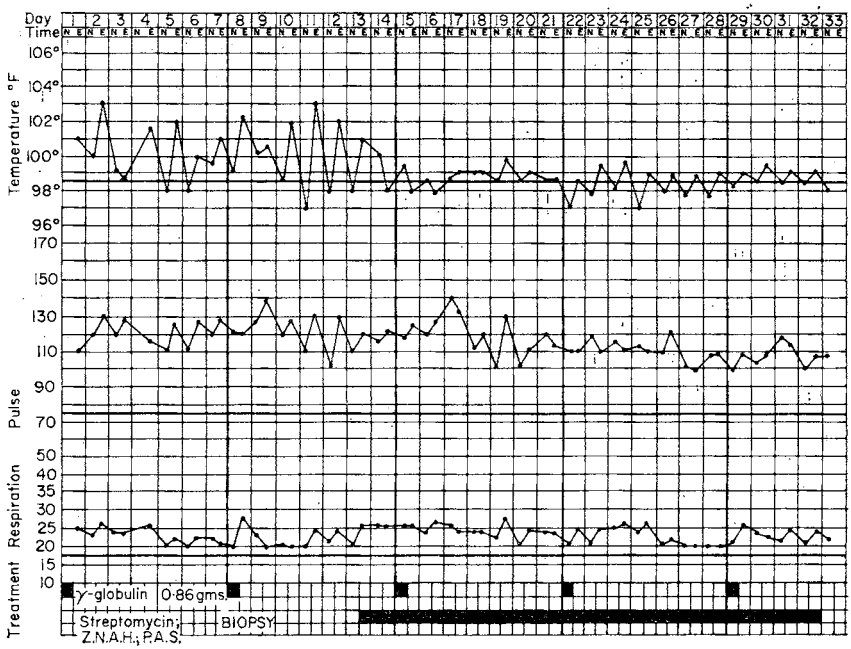


FIG. 19.7. Temperature chart of a boy of 5 years with hypogammaglobulinaemia who had had BCG in infancy, with transient Mantoux conversion, and who had been treated with weekly injections of γ-globulins for 4½ years. He had had fever and gross swelling of the tonsils and cervical lymph nodes for 4 months, which failed to respond to various antibiotics. A cervical lymph node biopsy was done on day 9 for suspected lymphosarcoma. The biopsy histology showed tuberculosis, and *Myco. tuberculosis* were seen and cultured (human strain) from it. He developed radiological evidence of pulmonary miliary tuberculosis on day 12, and chemotherapy was started on day 13 with very rapid response, which led to full recovery.

The chart demonstrates that fulminating tuberculosis may occur in patients with hypogammaglobulinaemia, with normal lymphocyte count, and capacity to react with delayed hypersensitivity, and that, like other acute infections in these patients, it responded normally to chemotherapy.

provided this is not due to loss. There seems little rationale for γ-globulin treatment in those patients with a predominantly cellular defect.

The transient form of hypogammaglobulinaemia is perhaps the most profitable of all to treat, and Davis & Hobbs (1967) have produced suggesting evidence that γ-globulin is effective in protecting premature babies from fatal infections. It is also possible that there may be a case for giving IgM to babies affected with intestinal infections, and IgA in those lacking it in the mucous secretions, but such treatments are only speculative at present.

γ-globulin replacement treatment is not without risk. A characteristic form of reaction, entailing dyspnoea without wheezing, tightness in the chest, distress leading to *angor animi*, fever, and even death occurs within minutes to hours of the injection. Kamme *et al* (1966) have described the clinical features. The mechanism is not clear, but it is not related to any measurable competence of the immune mechanism, and significantly spares boys with affected male relatives (Soothill *et al* 1968). The reactions are similar to ones obtained when γ-globulin is injected intravenously (Barandun *et al* 1962), which may entail fixation of complement by IgG aggregates. A hypothesis consistent with these facts is that reactions occur to intramuscular γ-globulin in some patients with hypogamma globulinaemia as a result of failure of filtering of such aggregates in defective lymph nodes.

Replacement of Deficient Cells

Grafts of immunologically competent cells provide a further possible line of treatment in those who lack mechanisms to reject them. Graft versus host reactions would, of course, be expected, so foetal tissue seems indicated. Grafts of lymph nodes and thymus have taken in such patients and some evidence has been reported of increase in IgG concentration, production of antibodies to antigens against which the donor had been immunized, and increase in circulating lymphocytes (Gitlin *et al* 1964; Hitzig, Kay & Cottier 1965; Harboe *et al* 1966) but all such patients have died, so this approach must be regarded more as a potential for the future in a very small group of these patients rather than as a currently effective form of treatment.

Immunization

Defects of immunity are rarely, if ever, complete in these patients, so it is reasonable to try to protect them against as many infections as possible by active immunization. There is good evidence to believe that the majority of patients with hypogammaglobulinaemia react normally to virus infections, so immunization against them would be likely to be particularly useful. But the phenomenon of progressive necrotic vaccinia is proof that, until it is possible to distinguish with confidence which patients with the various immunity deficiency states will respond favourably to any individual organism, only dead antigens should be used. BCG has also been shown to disseminate in some such patients (Bouton, Mainwaring & Smithells 1963).

Suppression of Causative Mechanisms

The Aetiology section and Fig. 19.6 contain data suggesting that suppression of a postulated auto-allergic mechanism might perhaps have a place in treatment. In the secondary immunity deficiency states, the efficacy of treatment of the underlying cause is clearly established in some instances.

Prevention

Until recently, a eugenic approach seemed about the only one possible in this field, but it now seems likely that immunization of all girls against rubella should prevent post-rubella acquired congenital hypogammaglobulinaemia, as well as the other associated defects.

CONCLUSION

These fascinating defects would appear to provide a means of disentangling some of the relative roles of the various immune mechanisms. Greater detail, and further up-to-date references may be found in the Proceedings of the Third Developmental Immunology Workshop (Good, Miescher & Smith 1968). At present they seem only to add to the complexity, but that is, perhaps, progress.

It has become clear that present means of description of individual combinations of defects which have been described as, for instance, dysgamma globulinaemia types 1 . 2 . 3 . . n, are becoming increasingly cumbersome. A three-tier system of classification is needed, one based on techniques of measurable disturbances of immunity functions and their related symptomatic effects, one on structural concepts (presumably of thymus and lymph nodes), and one on aetiological concepts. Then assertions of correlation, such as many of the ones given in other sections of this chapter, can be exposed to statistical analysis. Currently the structural and aetiological classifications are poorly systematized—essentially lists given in the relevant sections above—but the functional classification, given in Fig. 19.8, can be made rational, when based on the assumption of the basic duality of immune mechanisms, humoral and cellular, and the strong clinical impression that symptomatology in the immunity deficiency states are explicable in terms of defects of one or other or both. The statistical and technical bases for definition of humoral and cellular mechanisms still require development (see diagnosis section above), and this must include quantitative and qualitative defects of mechanisms—both general and antigen-specific. Short cuts to the classification of any individual patient will often be possible—gross deficiency of IgG will mean antibody deficiency syndrome, with or without cellular immunity deficiency. The way in which such defects arise in terms of quantitative or qualitative disturbance of any particular function can be listed, giving the 40 million possibilities, but such detailed diagnosis will rarely be required.

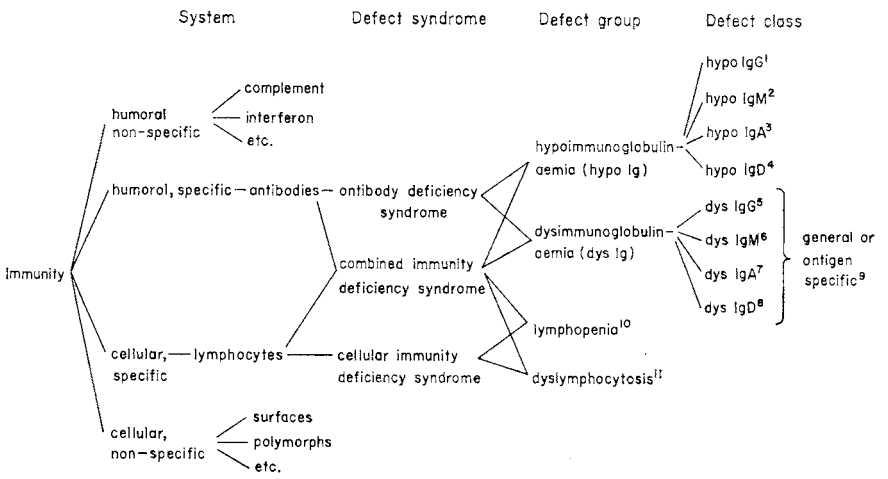


FIG. 19.8. A functional or syndrome classification of the immunity deficiency states, based on the current concept of the duality of specific immunity. Examples of non-specific mechanisms are indicated but defects are not included (see Fig. 1). All patients with defects of specific immune mechanisms, whatever the structural and aetiological type, can be classified into defect syndromes, defect groups (according to quantitative or qualitative defects of each mechanism) and defect class. There is reason to believe that most, if not all, of the eleven parameters (numbered in the figure) on which the defect classes are defined vary independently in these patients, providing! $11 \triangleq 4 \times 10^7$ combinations.

REFERENCES

AISENBERG A. (1962) Studies on delayed hypersensitivity in Hodgkin's Disease. *J. clin. Invest.* **41,** 1964.

ALDRICH R.A., STEINBERG A.G. & CAMPBELL D.C. (1954) Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhoea. *Pediatrics* **13,** 133.

BARANDUN S.H., COTTIER H., HASSIG A. & RIVA G. (1959) *Das Antiköpermangelsyndron.* Basel: Schwabe.

BARANDUN S., KISTLER P., JEUNET F. & ISLIKER H. (1962) Intravenous administration of human gamma globulin. *Vox Sang.* **7,** 157.

BARON S. & ISAACS A. (1962) Absence of interferon in lungs from fatal cases of influenza. *Brit. med. J.* **i,** 18.

BARON S., NASOU J.P., FIELDMAN R.M., OWEN G.M., LEVY H.B. & BARNETT E.V. (1962) Antibody production by hypogammaglobulinaemic patients. *J. Immunol.* **88,** 443.

BLACKBURN R., CHAKERA T.M. & SOOTHILL J.F. (1965) IgM light chain types in suspected macroglobulinaemia. *Clin. Chim. Acta* **14,** 6.

BLETCHER T.E., SOOTHILL J.F., VOYCE M.A. & WALKER W.H.C. (1968) Antibody deficiency syndrome. A case with normal immunoglobulin levels. *Clin. exp. Immunol.* **3,** 47.

BOUTON M.J., MAINWARING D. & SMITHELLS R.W. (1963) BCG dissemination in congenital hypogammaglobulinaemia. *Brit. med. J.* **i,** 1512.

BRUTON O.C. (1952) Agammaglobulinaemia. *Pediatrics* **9,** 722.

BRUTON O.C., APT L. GITLIN D. & JANEWAY C.A. (1952) Absence of serum γ globulin. *Amer. J. Dis. Child.* **84,** 632.

CITRON K.M. (1957) Agammaglobulinaemia with splenomegaly. *Brit. med. J.* **i,** 1148.

COOKE W.T., WEINER W. & SHINTON N.K. (1957) Agammaglobulinaemia—Report of two adult cases. *Brit. med. J.* **i,** 1151.

COOPER M.D., PEREY D.Y., MCKNEALLY M.F., GABRIELSEN A., SUTHERLAND D.E.R. & GOOD R.A. (1966) A mammalian equivalent of the avian bursa of Fabricius. *Lancet* **i,** 1388.

CRABBÉ P.A. and HEREMANS J.F. (1966) Lack of gamma-A-immunoglobulin in serum of patients with steatorrhea. *Gut.* **7,** 119.

CROWDER R.V., THOMPSON W.T. & KUPFER H.G. (1959) Acquired agammaglobulinaemia with multiple allergies and pernicious anaemia. *Arch. intern. Med.* **103,** 445.

CRUCHAUD A., ROSEN F.S., CRAIG J.M., JANEWAY C.A. & GITLIN D. (1962) The site of synthesis of the 19S γ globulin in dysgammaglobulinaemia. *J. exp. Med.* **115,** 1149.

DAVIS J.A. & HOBBS J.R. (1967) Serum γ_G globulin levels and gestational age in premature infants. *Lancet* **i,** 757.

DE VAAL O.M. & SEYNHAEVE V. (1959) Reticular dysgenesis. *Lancet* **ii,** 1123

DI GEORGE A.M., LISCHNER H.W., DACOU C. & AREY J.B. (1968) Congenital absence of the thymus and its immunologic consequences. In GOOD R.A., MIESCHER P.A. & SMITH R.T., ed. (1968). See separate reference.

FIREMAN P., BOESMAN M. & GITLIN D. (1964) Ataxia-telangiectasia: a dysgammaglobulinaemia with deficient gamma$_{1A}$ (beta$_{2A}$)-globulin. *Lancet* **i,** 1193.

FUDENBERG H., GERMAN J.L. & KUNKEL H.G. (1962) The occurrence of rheumatoid factor and other abnormalities in families of patients with agammaglobulinaemia. *Arthritis and Rheumatism* **5,** 565.

FUDENBERG H. & SOLOMON A. (1961) 'Acquired agammaglobulinaemia', with autoimmune haemolytic disease: graft-versus-host reaction? *Vox Sang.* **6,** 68.

FULGINITI V.A., HATHAWAY W.E., PEARLMAN D.S., BLACKBURN W.R., REIQUAM C.W., GITHENS J.H., CLAMAN H.N. & KEMPE C.H. (1966) Dissociation of delayed hypersensitivity and antibody-synthesising capacities in Man. *Lancet* **ii,** 5.

GEWURZ H., PICKERING R.J., MUSCHEL L.H., MERGENHAGEN S.E. & GOOD R.A. (1966) Complement dependent biological functions in complement deficiency in man. *Lancet* **ii,** 356.

GIEDION A. & SCHEIDEGGER J.J. (1957) Kongenitale Immunoparese bei Fehlen spezifischer β_2-Globuline und quantitativ normalen γ-globulinen. *Helv. paediat. Acta* **12,** 241.

GILBERT C. & HONG R. (1964) Qualitative and quantitative immunoglobulin deficiency. *Amer. J. Med.* **37,** 602.

GITLIN D. & CRAIG J.M. (1963) The thymus and other lymphoid tissues in congenital agammaglobulinaemia. (1) Thymic alymphoplasia and lymphoid hypoplasia and their relations to infection. *Pediatrics* **32,** 517.

GITLIN D., HITZIG W.H. & JANEWAY C.A. (1956) Multiple serum protein deficiencies in congenital and acquired agammaglobulinaemia. *J. clin. Invest.* **35,** 1199.

GITLIN D. & JANEWAY C.A. (1956) Agammaglobulinaemia, congenital, acquired and transient forms. *Progr. Hemat.* **1,** 318.

GITLIN D., JANEWAY C.A., APT L. & CRAIG J.M. (1959) Agammaglobulinaemia. In *Cellular and Humoral Aspects of the Hypersensitive States*, p. 375, ed. LAWRENCE H.S. New York: Hoeber-Harper.

GITLIN D., ROSEN F.S. & MICHAEL J.G. (1963) Transient 19S gamma globulin deficiency in the newborn infant and its significance. *Paediat.* **31,** 197.

GITLIN D., VAWTER G. & CRAIG J.M. (1964) Thymic alymphoplasia and congenital aleukocytosis. *Pediatrics* **33,** 184.

GLANZMANN E. & RINIKER P. (1950) Essentielle Lymphocytophthise. Ein neues Krankheitsbild aus der Säuglingspathologie. *Ann. paediat.* **175,** 1.

GOOD R.A., KELLY W.D., RÖTSTEIN J. & VARCO R.L. (1962) Immunological deficiency diseases. Agammaglobulinaemia, hypogammaglobulinaemia, Hodgkin's Disease and Sarcoidosis. *Progr. Allergy* **6,** 187.

GOOD R.A., MIESCHER P.A. & SMITH R.T. (eds.) (1968) Proceedings of the Third Developmental Immunology Workshop. *The Immunologic Deficiency Diseases in Man*, Sanibel Island, February 1967. National Foundation. To be published.

GOOD R.A., ZAK S.J., CONDIE R.M. & BRIDGES R.A. (1960) Clinical investigations of patients with agammaglobulinaemia and hypogammaglobulinaemia. *Pediat. clin. N. Amer.* **7,** 397.

GRANT G.H. & WALLACE W.D. (1954) Agammaglobulinaemia. *Lancet* **ii,** 671.

GUTMANN L. & LEMLI L. (1963) Ataxia-telangiectasia associated with hypogammaglobulinaemia. *Arch. Neurol.* **8,** 318.

HARBOE M., PANDE H., BRANDTZAEG P., TRETER K.J. and HJORT P.F. (1966) Synthesis of donor type of γG-globulin following thymus transplantation in hypo-γ-globulinaemia with severe lymphocytopenia. *Scand. J. Haemat.* **3,** 351.

HEREMANS J. (1960) *Les Globulins Sériques du Système Gamma*, p. 299. Bruxelles: Editions Arscia. S.A.

HITZIG W.H., KAY H.E.M. & COTTIER H. (1965) Familial lymphopenia with agammaglobulinaemia. *Lancet* **ii,** 151.

HITZIG W.H. & WILLI H. (1961) Hereditäre lymphoplasmocytäre Dysgenesie ('Alymphocytose mit agammaglobulinämie'). *Schiez. Med. Wschr.* **91,** 1625.

HOBBS J.R. (1966) Disturbances of the Immunoglobulins. In *The Scientific Basis of Medicine Annual Reviews*. London: Athlone Press.

HOLMES B., QUIE P.G., WINDHORST D.B. & GOOD R.A. (1966) Fatal granulomatous disease of childhood. *Lancet* **i,** 1225.

HUTCHISON J.H. (1955) Congenital agammaglobulinaemia. *Lancet* **ii,** 844 and 1196.

KAMME C., CÖSTER C., HAGELQUIST E., KALÉN N., LINDHOLM H. & GRUBB R. (1966) Side effects of treatment of hypogammaglobulinaemia with gammaglobulin. *Acta med. Scand.* **179,** 679.

KEKWICK R.A., VALLET L., CUTBUSH M., MILLISON P.L., THOMAS A.R., GELL P.G.H. & SOOTHILL J.F. (1961) Estimation of γ-globulin in the serum of patients with hypogammaglobulinaemia. *J. clin. Path.* **14,** 470.

KRIVIT W. & GOOD R.A. (1959) Aldrich's syndrome. *A.M.A. J. Dis. Child.* **97,** 137.

LARSON D.L. & TOMLINSON L.J. (1952) Quantitative antibody studies in man. II. The relation of the level of serum proteins to antibody production. *J. Lab. clin. Med.* **39,** 129.

LARSON D.L. & TOMLINSON L.J. (1953) Quantitative antibody studies in man. III. Antibody response in leukaemia and other malignant lymphomata. *J. clin. Invest.* **32,** 317.

MACCALLUM F.O. (1962) Bacterial and viral antibodies in patients with hypogammaglobulinaemia. *Proc. roy. Soc. Med.* **55,** 397.

MARSHALL W.C., WESTON H.J. & BODIAN M. (1964) Pneumocystis carinii pneumonia and congenital hypogammaglobulinaemia. *Arch. Dis. Childhood* **39,** 18.

MOWBRAY J.F. (1963) Ability of large doses of an alpha$_2$ plasma protein fraction to inhibit antibody production. *Immunology* **6,** 217.

NEZELOF C., JAMMET M.L., LORTHOLARY P., LABRUNE B. & LAMY M. (1964) L'Hypoplasie héréditaire du thymus. *Arch. franç. Pédiat.* **21,** 897.

PAGE A.R., HANSEN A.E. & GOOD R.A. (1963) Occurrence of leukaemia and lymphoma in patients with agammaglobulinaemia. *Blood* **21,** 197.

PARROTT D.M.V. & EAST J. (1964) Studies on a fatal wasting syndrome of mice thymectomized at birth. In *The Thymus in Immunobiology*, p. 523, ed. Good R.A. & GABRIELSEN A.E. New York: Hoeber-Harper.

PEPYS J. (1966) Personal communication.

PETERSON R.D.A., COOPER M.D. & GOOD R.A. (1965) The pathogenesis of immunologic deficiency diseases. *Amer. J. Med.* **38,** 579.

PETERSON R.D.A., KELLY W. & GOOD R.A. (1964) Ataxia-telangiectasia. Its association with a defective thymus, immunologic-deficiency disease and malignancy. *Lancet* **i,** 1189.

RAMOS A.J. (1956) Case Presentation. *J.A.M.A.* **160,** 1317.

Registrar General's Statistical Review for England and Wales (1964).

ROCKEY J.H., HANSON L.A., HEREMANS J.F. & KUNKEL H.G. (1964) Beta-2A aglobulinemia in two healthy men. *J. Lab. clin. Med.* **63,** 205.

ROSEN F.S. & JANEWAY C.A. (1966) The gamma globulins. III. The antibody deficiency syndromes. *New Eng. J. Med.* **275,** 709.

ROWE D.S. & FAHEY J.L. (1965) A new class of immunoglobulins. II. Normal serum IgD. *J. exp. Med.* **121,** 185.

SANGER R. & RACE R.R. (1963) Xg blood groups and familial hypogammaglobulinaemia. *Lancet* **i,** 859.

SCHALLER J., DAVIS S.D., CHING Y.-C., LAGUNOFF D., WILLIAMS C.P.S. & WEDGWOOD R.J. (1966) Hyperglobulinaemia, antibody deficiency, autoimmune haemolytic anaemia, and nephritis in an infant with a familial lymphophenic immune defect. *Lancet* **ii,** 825.

SILVERSTEIN A.M. (1960) Essential hypocomplementemia. *Blood* **16,** 1338.

SONES M. & ISRAEL H.L. (1954) Altered immunologic reactions in sarcoidosis. *Ann. int. Med.* **40,** 260.

SOOTHILL J.F. (1962) The concentration of γ macroglobulin in the serum of patients with hypogammaglobulinaemia. *Clin. Sci.* **23,** 27.

SOOTHILL J.F. (1967) Quantitative disturbances of plasma proteins in disease. In *Scientific Basis of Medicine Annual Reviews*, p. 276. London: Athlone Press.

SOOTHILL J.F., HAYES K. & DUDGEON J.A. (1966) The immunoglobulins in congenita rubella. *Lancet* **i,** 1385.

SOOTHILL J.F., HILL L.E. & ROWE D.S. (1968) A quantitative study of the immunoglobulins. in hypogammaglobulinaemia. In GOOD R.A., MIESCHER P.A. & SMITH R.T. eds. (1968) See separate reference.

SQUIRE J.R. (1960) Hypogammaglobulinaemia in Great Britain. *Acta Haemat.* (*Basel*) **24,** 99.

SQUIRE J.R. (1962) Hypogammaglobulinaemia in the United Kingdom, 1956–61. *Proc. roy. Soc. Med.* **55,** 393.

SQUIRE J.R., HARDWICKE J. & SOOTHILL J.F. (1967) Proteinuria. Chapter in *Renal Disease*, ed. BLACK D.A.K., 2nd ed. Oxford: Blackwell.

SUTHERLAND D.E.R., ARCHER O.K., PETERSON R.D.A., ECKERT E. & GOOD R.A. (1965) Development of 'autoimmune processes' in rabbits subjected to neonatal removal of central lymphoid tissue. *Lancet* **i,** 130.

SUTHERLAND I., MITCHELL D.N. & HART P.D. (1965) Incidence of intrathoracic sarcoidosis. among young adults participating in a trial of tuberculosis vaccines. *Brit. med. J.* **ii,** 497

TEILUM G. (1964) Amyloidosis secondary to agammaglobulinaemia. *J. Path. Bact.* **88,** 317.

TOBLER R. & COTTIER H. (1958) Familiäre lymphopenie mit agammaglobulinämie und schwere moniliasis. *Helv. Paediat. Acta* **13,** 313.

TOMASI T.B., TAN E.M., SOLOMON A. & PRENDERGAST R.A. (1965) Characteristics of immune system common to certain external secretions. *J. exp. Med.* **121,** 101.

UHR J.W., DANCIS J., NEUMANN C.G. (1960) Delayed-type hypersensitivity in premature neonatal humans. *Nature (Lond.)* **187,** 1150.

WALDMANN T.A. & SCHWAB P.J. (1965) IgG (7S gammaglobulin) metabolism in hypogammaglobulinaemia: studies in patients with defective gamma globulin synthesis, gastrointestinal protein loss, or both. *J. clin. Invest.* **44,** 1523.

WARNER N.L., SZENBERG A. & BURNET F.M. (1962) The immunological role of different lymphoid organs in the chicken. I. Dissociation of immunological responsiveness. *Australian J. exp. Biol. and Med. Sci.* **40,** 373.

WEST C.D., HONG R. & HOLLAND N.H. (1962) Immunoglobulin levels from the newborn period to adulthood and in immunoglobulin deficiency states. *J. clin. Invest.* **41,** 2054.

WILLIAMS R.T. (1966) Acquired dysgammaglobulinaemia in a young man. *Clin. exp. Immunol.* **1,** 223.

WOCHNER R.D., DREWS G., STROBER W. and WALDMANN T.A. (1966) Accelerated breakdown of immunoglobulin G (IgG) in myotomic dystrophy: a hereditary error of immunoglobulin catabolism. *J. clin. Invest.* **45,** 321.

WOLF J.K. (1962) Primary acquired hypogammaglobulinaemia, with a family history of collagen disease and hematologic disorders. *New Engl. J. Med.* **266,** 473.

WOLLHEIM F.A., BELFRAGE S., CÖSTER C. & LINDHOLM H. (1964) Primary 'acquired' hypogammaglobulinaemia. Clinical and genetic aspects of 9 cases. *Acta med. Scand.* **176,** 1.

YOUNG R.R., AUSTEN K.F. & MOSER H.W. (1964) Abnormalities of serum gamma$_{1A}$ globulin and ataxia-telangiectasia. *Medicine* **43,** 493.

SECTION IV

THE ALLERGIC STATE AS RESPONSIBLE FOR CLINICAL HYPERSENSITIVITY AND DISEASE

A. ALLERGIC DISEASES WITH GENERAL MANIFESTATIONS

CHAPTER 20

CLASSIFICATION OF ALLERGIC REACTIONS RESPONSIBLE FOR CLINICAL HYPERSENSIVITY AND DISEASE

R.R.A.COOMBS AND P.G.H.GELL

INTRODUCTION

In this chapter, and to a lesser extent throughout this book, we have attempted to use an unequivocal and precise terminology, in which the 'allergic state' is taken to denote a state of *altered reactivity to an antigenic substance*. A main stumbling block to clear thinking has always been the use of the words 'immunity' and 'immune'—implying as they do by their common meaning absolute protection against a noxious agent, or at least the occurrence of some process strictly advantageous to the organism. In many reactions 'with an immune basis' the 'immune' process actually constitutes the disease, while in others, such as tuberculin sensitivity, one cannot say for sure whether in the long run they are disadvantageous or not. In our usage the 'immune state' is specifically the state of *protection against the biological effects of an antigen or organism carrying the antigen*.

On the other hand, in the minds of many practising physicians, the term 'allergy', though a useful and indeed a fashionable one, implies no precise concept; it is used as a rough and ready label for all conditions in which the active reaction of a patient's own tissues, rather than a disordered physiological process like cancer or diabetes or direct damage by an invading organism, determines the manifest lesions of the disease, or at least a large part of them. Side by side

with this rag-bag of generally obscure conditions are those diseases normally dealt with by the clinical allergist—serum sickness, drug sensitivities, hay fever and asthma, various dermatological conditions—and empirically used tests such as the tuberculin and Schick tests, which are immunologically determined.

Our contention is that reason and clarity can only be achieved if we adhere to the suggestion originally put forward by von Pirquet; he coined the word allergy as uncommitted in the clinical sense to either immunity (protection) or to the opposite, paradoxically deleterious, effects of these so-called 'immune' reactions, such as anaphylaxis. This view is expressed with the greatest precision in his paper published in 1906. The whole of this classical paper is translated in Appendix A. The following extracts from it are strictly relevant to the present discussion:

'*The vaccinated person behaves . . . in a different manner from him who has not previously been in contact with such an agent. Yet he is not insensitive to it. We can only say of him that his power to react has undergone a change.*

'For this general concept of a *changed reactivity* I propose the term *allergy*. . . .

'The vaccinated, the tuberculous, the individual injected with serum becomes *allergic* towards the corresponding foreign substance. A foreign substance which by one or more applications stimulates the organism to a change in reaction is an *allergen*. This term—not quite in accordance with philological usage—traces its origin to the word antigen (Detre-Deutsch) which implies a substance capable of giving rise to the production of antibody. The term allergen is more far-reaching. The allergens comprise, besides the antigens proper, the many protein substances which lead to no production of antibodies but to supersensitivity.

'*The term immunity must be restricted to those processes in which the introduction of the foreign substance into the organism causes no clinically evident reaction, where, therefore, complete insensitivity exists*; whether this be due to alexins (natural immunity), or even to some kind of adaption to a poison (Wassermann and Citron).'

Thus the term 'allergy' should be taken to mean the specifically altered state of a host following exposure to an allergen. This altered *biological* state or allergy has in itself no implied connotation as regards the production of either *clinical* hypersensitivity or *clinical* immunity; it may entail either or both simultaneously in the same individual. 'Hypersensitive' and 'immune' are descriptive terms only, useful in indicating a clinical state, and are not mutually exclusive.

Von Pirquet's clear foresight in putting forward such an untethered basic concept allows without any difficulty the inclusion of actively acquired tolerance amongst the allergic phenomena. We would, however, suggest that 'transplantation allergy' would be a more precise designation that 'transplantation immunity' and likewise 'auto-allergic diseases' rather than 'auto-immune diseases'. Moreover, if we can be persuaded to accept the concept of allergy as it was originally stated, it will be seen that the scope of the subject broadens to

contain examples not usually dealt with in textbooks of allergy. Besides the phenomena of true 'immunity' (protection), and the recognized hypersensitivity reactions, there must be included transfusion reactions, haemolytic disease of the newborn, iso-'immune' neonatal purpura, the tentatively grouped 'immunopathological' diseases such as allergic thyroiditis and rheumatoid arthritis, the reactions of homograft rejection and finally the experimentally produced runt disease and actively acquired tolerance.

At the cellular level, the term 'allergized cell' is a useful one, in that it includes changes in the cell of a broadly immunological nature, without implication as to what such changes are: thus the 'committed cell' (committed to respond to a particular antigen) and the 'tolerized cell' (committed not to respond to a particular antigen)—if such a cell exists—both come under the same general heading of 'allergized cells'. Moreover, the 'change' in such a cell can come by the activation of internal mechanisms or wholly from without, i.e. actively or passively: thus we can consider the plasma cell, or the 'sensitized 'cell of Delayed Allergy (Type IV below) actively allergized: the cell which is coated with Reagin (Type I below) or with cytophilic antibody, passively allergized.

Apart from devotion to the rather loose common usage the prime objection to this terminology is often a purely aesthetic one: the sound of the letter 'g', whether hard or soft, is in its combinations phonetically harsh and often comical in English speech. The fault lies primarily with the Greeks, who used the word $\varepsilon\rho\gamma o\nu$ = ergon, to mean work, something which nobody likes.

A Rational Basis for Classification of Tissue-Damaging Allergic Conditions

The conventional basis for classification of diseases, in terms of a clinical syndrome, of an anatomical or a biochemical lesion and so on works well enough in practice, for the purpose of sorting out patients into their appropriate wards and deciding whether to subject them to the tender mercies of a physician or a surgeon. But as von Pirquet realized, a number of quite distinct though all fundamentally 'allergic' processes may be going on simultaneously in one patient, and even in the one local lesion. Therefore, if we are to apply an *aetiological* classification to the conditions discussed in this section of the book we must first distinguish the sorts of allergic process which may be occurring. This has to be done mainly on the basis of experimental work on animals, where the variables can be properly controlled. The next stage is to attempt to sort out which of these processes are going on in any patient with a particular clinically classified disease. In all too few of the diseases considered here is only one kind of allergic process involved; and it is an easy error to assume that, even when one given process is demonstrable, this process, and only this, is responsible for the whole trouble, or indeed for any of it. The mere demonstration of the presence of an antibody characteristic of a clinical syndrome, usually in amounts only

detectable by sensitive tests, is little contribution to our knowledge of the pathogenesis of such a syndrome, however useful in diagnosis: there must be some demonstration, too, that such an antibody in such an amount can in fact contribute to the actual pathological lesions which are found. In many diseases, nevertheless, it is now possible to say not merely that they have, vaguely, 'an allergic basis', but that such and such a well-defined process underlies the lesions.

We feel that any illuminating classification, which is surely necessary for the physician faced today with allergic manifestations seen not only in the allergy clinic but also in general medicine, must indicate and indeed be founded on the mechanism involved. For this reason we set forth a fairly simple, yet we hope comprehensive, scheme which does not divorce the clinical from the academic and laboratory side. As the basis of this classification we have chosen the circumstances of the initial reaction between allergen (or antigen) and antibody or specifically modified or allergized cells (as far as this is known), subgrouping subsequently on other secondary phenomena. Hence this is primarily a classification of initiating mechanisms and not of the subsequent events or the diseases themselves.

CLASSIFICATION OF ALLERGIC REACTIONS PRODUCING TISSUE DAMAGE AND OPERATIVE IN CLINICAL HYPERSENSITIVITY

We consider here only the types of allergic reaction which may produce tissue damage of some kind, whether or not the effect in the long run may be beneficial. There are four main pathways or types of reaction (see Fig. 20.1) by which the animal or individual, 'sensitized' by a previous experience of the allergen, may react and, if the reaction is intense enough, suffer as a result of the allergic state.

Excluded from this classification are certain important types of immunological mechanism. Firstly, immunological tolerance, though an allergic reaction on our definition and though it may be deleterious (if, for example there is specific neonatally induced tolerance to a pathogenic organism), is not responsible as such for any local lesion. Secondly, 'allogeneic reactions', i.e. reactions such as the normal lymphocyte transfer reaction, mixed cell reactions (see Chapter 1), lymphocyte plaquing effects (Möller & Möller 1967), etc., are excluded for the time being since no allergization of the reacting cell apparently takes place, but it reacts according to its own intrinsic nature based on its genome—at least as far as is at present known.

This classification, finally, cuts across the classification according to the nature or source of the allergen, into drug reactions, autoallergic reactions and so on. Whatever the allergen the basic reaction mechanisms are the same.

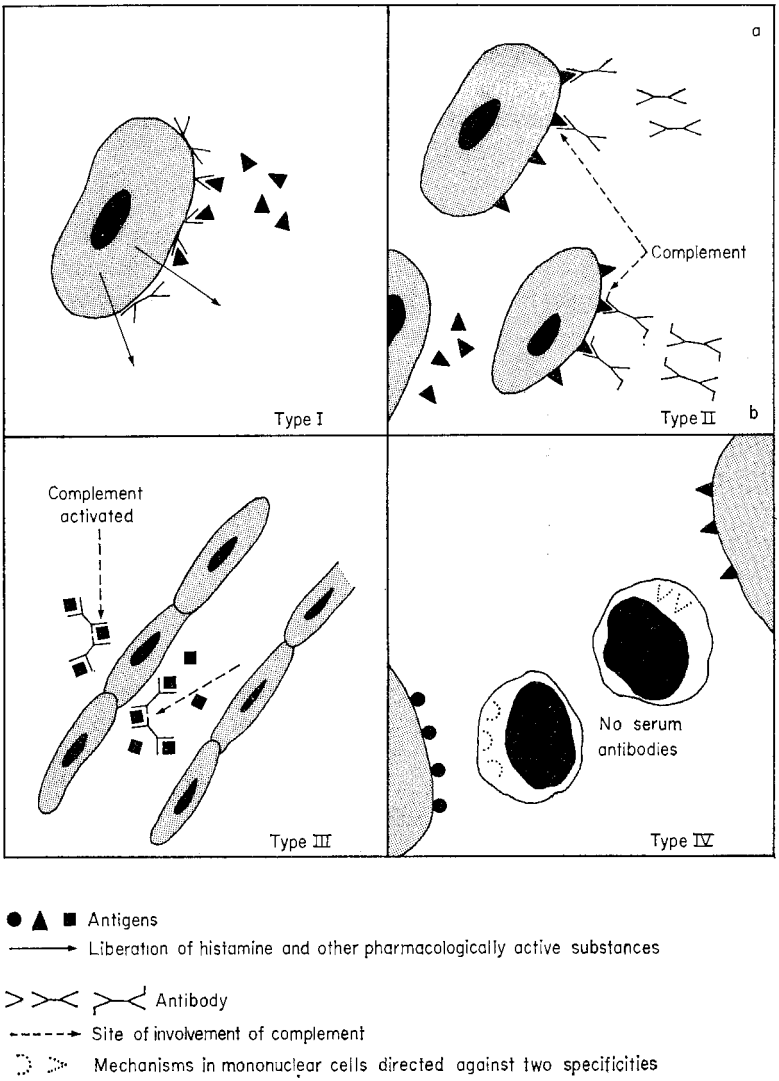


FIG. 20.1. Highly diagrammatic illustration of the four types of allergic reaction which may be deleterious to the tissues and harmful to the host.

Type I. Free antigen reacting with antibody passively sensitizing (allergizing) cell surface.

Type II. Antibody reacting with (a) cell surface or (b) with antigen or hapten which becomes attached to cell surface: complement plays a major destructive role.

Type III. Antigen and antibody reacting in antigen excess forming complexes which, possibly with the aid of complement, are toxic to cells.

Type IV. Specifically modified mononuclear cells (actively allergized cells) reacting with allergen or antigen deposited at a local site.

Definition of the Four Types of Reaction

Type I Reaction (Anaphylactic, Reagin-Dependent)

Initiated by allergen or antigen reacting with tissue cells passively sensitized (allergized) by antibody produced elsewhere, leading to the release of pharmacologically active substances (vasoactive hormones).

Type II Reaction (Cytotoxic)

Initiated by antibody reacting with *either* (a) an antigenic component of a cell or tissue element *or* (b) an antigen or hapten which has become intimately associated with these. Complement is usually, but not always, necessary to effect the cellular damage.

Type III Reaction (Damage by toxic complexes)

Initiated when antigen reacts in the tissue spaces with potentially precipitating antibody, forming microprecipitates in and around the small vessels causing damage to cells secondarily; *or* when antigen in excess reacts in the blood stream with potentially precipitating antibody forming soluble circulating complexes, which are deposited in the blood-vessel walls or in the basement membrane and cause local inflammation.

Type IV Reaction (Delayed, Tuberculin-type, Cell-Mediated)

Initiated essentially by the reaction of specifically modified mononuclear cells (actively allergized cells) containing a substance or mechanism capable of responding specifically to allergen deposited at a local site. The exact mechanism of this type of reaction is still uncertain, but it is manifested by the infiltration of cells, at the site where the antigen is, without the necessary participation of free antibody.

The mechanisms of these four types of reaction are illustrated diagrammatically in Fig. 20.1.

It must be stressed that the circumstances in which any of these four basic types of reaction may be studied in an uncomplicated form may be very special, and may, in fact, be seen only in certain animal species under quite strict experimental conditions. Again it must be emphasized that the pattern seen in any one human disease is often complex, involving not just one but several of the above pathways or responses.

Discussion of the Four Types of Reaction

Type I Reaction (Anaphylactic, Reagin-Dependent)

Care has to be taken in the use of the words 'anaphylaxis' or 'anaphylactic'. Although a Type I response is the essential underlying mechanism in anaphylaxis,

the word has, on occasion, been applied to Type II and even Type III reactions. For instance, the Type II (cytotoxic) reaction produced as a generalized reaction or locally in the skin of a guinea-pig, by the injection of Forssman antiserum has been called passive reversed anaphylaxis (see Redfern 1926, 1928; van den Ende 1940). Again, the Type III (Arthus-type) reaction produced in the skin of a rabbit (and which may also be produced in man) after repeated injections of antigen and the development of circulating antibody, was referred to as an anaphylactic reaction by Arthus himself and was also referred to as 'localized anaphylaxis' by Richet (cf. Arthus 1903).

Our understanding of Type I reactions has been immensely clarified in recent years by studies on the various sorts of antibodies in a number of animal species. We shall first consider, therefore, the nature of the antibodies whose prime characteristic is to fix to cells, and then the subsequent steps which lead to local and general effects in animals and man. It is no longer necessary to deal separately with man and laboratory animals until the final stage where differences between species in reactivity to various pharmacological substances influence the syndromes produced.

Reagin-like antibodies

It is now possible to make a number of precise statements about the nature of 'anaphylactogenic' antibodies.

1. A number of species produce antibodies which are fully comparable to human reaginic antibodies (see Benacerraf 1967) in their tendency to bind strongly and lastingly to the cells of the species which produced them. In some, at least, the antibody is physicochemically similar to human reagin and produced in similar small amounts.

2. In no cases will this sort of antibody bind in the same way to the cells of a different species (with the exception that human reagin will bind to some extent to monkey tissues).

3. The guinea-pig is possible unusual in that non-reaginic antibodies from some species (rabbit, man) will bind to its cells in the same way apparently as its own γ_1-antibodies bind. We therefore have the paradoxical situation that this species binds exclusively one fraction of its own antibodies which may be analogous to *reagins* and also hetero-specific *non-reaginic* antibodies. This property is related to the structure of the Fc. piece of the immunoglobulin molecule in both cases.

4. In practice reaginic-type antibodies are detected in experimental animals by a test essentially identical with the Prausnitz-Küstner test used in man, known as the passive cutaneous anaphylactic reaction (PCA). It should be noted that relatively large amounts of any precipitating antibody, injected intradermally into the skin of any species, will if followed promptly by intravenous antigen produce an inflammatory lesion. This is, however, not a Type I reaction, although

histamine release does occur, but an Arthus (Type III) reaction. The essential point about the PCA reaction is that time must be allowed to elapse sufficient both for fixation of the reagin-type antibody to cells, and for diffusion away of any non-reaginic antibodies present. After this time antigen is injected intravenously, mixed with a dye to demonstrate local oedema formation (Ovary 1958).

Reaginic-type antibodies are relatively thermolabile and there is some evidence that in man some at least belong to a special new class of immunoglobulin, IgE (Ishizaka, Ishizaka & Hornbrook 1967). The extensive experimental data upon which this account is based is lucidly reviewed by Benacerraf (1967) and Ovary (1958).

Damage induced by reagin-type antibodies

Since the cytological and pharmacological aspects of Type I reactions, once the reagin is produced and bound, are discussed in Chapter 22, we shall do no more here than summarize the steps in the process.

1. Apart from the fact that it takes time, little is known about the mechanism and site of binding on the cell.

2. The cells involved: in the blood, about half the hormone release is from basophils and about half from other polymorphonuclear cells (Graham *et al* 1955). It is probable that in the tissues the basophils ('mast cells') play the main part, but it is quite possible that other cells (such as vascular endothelial cells) are also involved. The cell which produces the antibody almost certainly releases it to be secondarily bound by the reacting cells; it does not react itself. (In our terminology, the antibody producing cell is actively allergized, the reacting basophil is passively allergized.)

3. In the guinea-pig injected intradermally with rabbit antibody, competition occurs between antibody and 'normal' gamma globulin (Biozzi *et al* 1959) There are therefore a limited number of binding sites on the cell, which can be occupied either by heterologous IgG or (presumably) by homologous γ_1-antibody, which binds in the same way, as described above.

4. Antigen coming in contact with antibody bound on the cell produces an injury (whose exact nature is still to be elucidated) leading to the release of vasoactive substances, such as histamine. The mode of action of such substances is discussed in Chapter 22; their main effects are local oedema and contraction of smooth muscle. In the guinea-pig, for example, spasm of the unusually well-developed bronchiolar musculature produces intense constriction of the bronchioles with the result that the animal is unable to force air out of its lungs; this is sufficient to cause death by suffocation (acute anaphylaxis). In less acute cases where the animal is not rapidly asphyxiated there is time for the other effects of general hormone release to become manifest. Clinically this is seen as generalized shock.

Various local manifestations of the anaphylactic reaction in the guinea-pig (as in man) can be studied independently by experimental means either *in vitro* or *in vivo*. The contraction of the isolated ileum or uterus of a sensitized animal in the Schultz-Dale test is well known. Less known perhaps is the *in vitro* contraction of a chain of bronchial rings on the addition of antigen (Schild *et al* 1951). In man, many attempts to develop *in vitro* tests have been made in order to avoid the use of the *in vivo* Prausnitz-Küstner test; some of these are described in Chapter 1. It is still not certain whether human antibodies other than classical reagins can sensitize human cells to produce a Type I reaction.

Type I reactions in man

It is not our intention to discuss Type I reactions in man in any but very general terms, since the clinical pictures are presented in later chapters. There are, however, one or two considerations on the antibodies responsible in man which are of importance.

First there are certain individuals, given the name atopic by Coca & Grove (1925), who produce a type of antibody which has *par excellence* the property of passively sensitizing tissue cells, the reagin or atopic antibody. *In vivo* it has very pronounced biological activity, initiating histamine release from passively sensitized cells in the presence of antigen and it may be shown to be present in the serum by means of a passive transfer test, the well-known Prausnitz-Küstner (PK) test. Since this human antibody will not produce a reaction in the skin of the guinea-pig, the reluctance, in this country at least, to do passive transfer tests in normal persons with human serum (for fear of serum hepatitis) makes investigations on this antibody difficult.

General anaphylactic (Type I) reaction in man. The syndrome is one of profound generalized shock. If the patient does not die rapidly, more local signs are manifest such as bronchial asthma, pulmonary oedema and urticaria (see Chapter 23). Such a reaction could result from antigen reaching the circulation of a highly sensitized person in the course of serum or drug therapy, from desensitization procedures with pollen extracts, after bee or wasp stings or from the classic example of hydatid cysts burst during operation. Should 'cot death' in infants be proved to be a modified anaphylactic reaction (Parish *et al* 1960), then absorption of antigen via the lungs must be considered another hazard, although here the shock may result from vasoactive hormones liberated following a Type III reaction (see below).

Local anaphylactic (Type I) reactions in man. As examples of this we have the ordinary prick and scratch diagnostic skin test where a local wheal results around the small site where antigen is introduced into the skin. Hay fever and allergic asthma are again local manifestations of a Type I reaction. These reactions are localized because of the local access and action of the antigen or allergen. It must be remembered that the person nevertheless has a general

sensitization which would be revealed if excessive amounts of antigen were absorbed.

What is not understood is why some people with such a general sensitization experience only hay fever symptoms or none at all when naturally exposed to the antigen as an inhalant, while others experience asthma. This is hard to explain simply as a quantitative effect. Rather it suggests the influence of as yet unknown local predisposing factors (see Chapter 23).

Extensive urticaria may be an expression of cutaneous anaphylaxis. The Prausnitz-Küstner reaction is the comparable reaction in man to that of passive cutaneous anaphylaxis in the guinea-pig, no dye, however, being needed as the histamine wheal is seen clearly in human skin.

Finally, as will be seen from Chapter 22, histamine is by no means the only mediator liberated from cells by the Type I reaction in man, so one must not expect all the symptoms and signs of these reactions to be inhibited by more or less specific anti-histaminics.

An essential element in our definition of Type I reactions is that pharmacologically active substances of a characteristic type are released from the passively allergized cells when acted upon by antigen. Other cytophilic antibodies are known which passively allergize cells (Boyden 1964), but here there is no evidence for release of any vasoactive hormone. The biological role of these cytophilic antibodies is yet to be established, though they may be of importance in the induction of antibody formation.

Type II Reaction (Cytotoxic)

In this type of reaction we have antibody reacting directly through its combining receptors with either an antigenic component of a tissue cell or with an antigen or hapten which has become intimately associated with the tissue cells. The antibody is usually of classical type, i.e. IgG or IgM. The mechanism of destruction depends partly on the nature of the antibody and partly on the kind of cell; complement is frequently involved.

The reaction of antibody with non-cellular membranes and supporting tissues is properly classified here also, although the 'cytotoxic' element of direct damage to tissue cells is absent.

Antibody reacting with an antigenic component of tissue cells

Transfusion reactions. There is a wide familiarity with the lysis of red cells by antibody and complement *in vitro*. In the body, under the circumstances of incompatible transfusion, lysis may occur intravascularly, or the sensitized cells, either with or without the participation of complement, embarrass the reticulo-endothelial system. White cells of the blood may be similarly involved.

In most books on blood transfusion, reactions due to previous sensitization

and red cell incompatibility are not described as 'allergic' although according to von Pirquet's definition they surely qualify. In these books the use of the term 'allergic' is generally confined to those reactions following transfusion which are characterized by urticaria, oedema and bronchial spasm, i.e. to those reactions thought to be caused by traces of soluble foreign antigens in the donor's plasma reacting with atopic reaginic antibody in the plasma of the recipient or *vice versa*. In our way of thinking this latter situation is a Type I reaction, while the reaction due to cell incompatibility and destruction is Type II; both, however, are allergic reactions.

Haemolytic disease of the newborn. This disease of man and of many other animal species (Roberts 1957) in which the mother produces antibodies which, on gaining entrance into the foetus or newborn, cause destruction of the red cells, must, by the same reasoning, be considered an allergic disease. Iso-allergic neonatal thrombocytopenia (see Chapter 28) is a comparable situation.

Lesions produced in tissues by the action of antibody and complement. Antibodies against surface components of the cell may, in the presence of complement, produce specific cell damage (cf. Goldberg & Green 1959). This type of reaction could well play a role in many of the auto-allergic diseases; auto-allergic haemolytic anaemia is an obvious example. However, in auto-allergic thyroiditis Irvine (1960) considers a Type II reaction to play a role. Also in experimental auto-allergic orchitis humoral factors are concerned, in combination with cellular (Type IV) allergy (Brown, Glynn & Holborow 1967). An antigen has also been shown to be shared by human cardiac myofibrils and components of certain streptococci; the possibility of a Type II reaction here may be relevant to the pathogenesis of rheumatic fever (Freimer & McCarty 1966).

Lesions produced by the injection of heterologous antisera against tissues are primarily of this type: e.g. with nephrotoxic sera (see Chapter 34) and anti-'Forssman' sera into guinea-pigs (Redfern 1926, 1928). In the latter case the pathology is one of haemorrhage, necrosis and gross tissue destruction, stemming apparently from the cytotoxic action of antibody and complement on vascular endothelial cells (see Berkinshaw Smith *et al* 1962).

Homograft rejection. The extent to which a cytotoxic reaction is involved in homograft rejection is still an open question: but it is likely that the 'white graft' reaction in a highly allergized animal is of this nature (see review by Stetson 1963 and Chapter 17).

Cytotoxicity and cytostimulation. Logically, the blast transformation of lymphocytes under the influence of antilymphocytic (Ling & Knight 1967) and anti-IgG (Sell & Gell 1965) antisera, are examples of Type II reactions: their immediate relevance is, however, rather to basic processes of antigen recognition and stimulation than to deleterious tissue reactions of the type which we are considering. In these experiments, larger doses than the stimulating dose were often found to be toxic, by a mechanism which is not yet clear.

Conversely, sublytic doses of complement and antibody, instead of being necrotic, may have a stimulatory effect on certain cell activities and even induce cell modulation. In organ culture experiments, antibody to membrane antigens together with complement increased the synthesis and release of lysosomal enzymes, without showing other deleterious effects on the cells; the tissue matrix showed marked pathological changes, due to the activity of the liberated enzymes (Fell *et al* 1966; Dingle *et al* 1967).

Antibody reacting with antigen or hapten adsorbed on or combined with tissue cells

Possibly of great importance in connection with Type II allergic reactions and disease is the rendering of tissue cells temporarily susceptible to the cytotoxic action of antibody and complement by adsorption of an antigen (bacterial product) or hapten (drug) on to such cells. Reaction of the bacterial antibody or antibody to the cell-drug complex might then bring about cellular destruction.

Cytotoxic reactions in drug hypersensitivity. The generalized purpura, which is the form in which the hypersensitivity reaction to the drug Sedormid manifests itself and which has been worked out by Ackroyd (see Chapter 26) is a clear example of a Type II reaction as defined by us. Quinine and quinidine may act similarly.

A comparable syndrome with haemolytic anaemia, instead of generalized purpura, may follow the use of drugs showing an affinity for the red cell (Muirhead, Holden & Graves 1958; Dausset & Contu 1964). Some such mechanism involving the white cells may also underlie certain forms of drug-induced agranulocytosis (Moeschlin 1958; see Chapters 26 and 29).

Acute nephritis. Another possible mechanism, still Type II, which is postulated as an explanation for acute nephritis following streptococcal infection, is that certain cells of the kidney combine with a bacterial product and then become a target for a cytotoxic reaction mediated by complement and antibody against the bacterial product. This and other mechanisms producing renal damage are discussed in Chapter 34. Other situations can also be envisaged where localization of a bacterial product could result in tissue damage by such a process.

It may seem unorthodox to group together under one heading haemolytic transfusion reactions, the reaction produced in guinea-pig skin on injection of anti-'Forssman' serum and purpura due to Sedormid hypersensitivity, but in each case the underlying mechanism is essentially the same, i.e. antibodies of a specifically sensitized or allergic individual reacting with cells and damaging them.

Not only should this realignment clarify the way of thinking about these reactions, but it seems very probable that in the next few years further examples will be found to fall under this heading when mechanisms producing *in vivo* tissue damage receive closer study.

Type III Reaction (Arthus-Type Reactions, Damage by Toxic Complexes)

Basic to the modern conception of this type of reaction is the observation that complexes between antigen and antibody formed *in moderate antigen excess* are locally toxic to the tissues, probably with the participation of complement (for full discussion, see Weigle 1961). The mechanism of such damage is multiple; hormonal, vascular, and cytotoxic complexes are all involved, and the exact histological site where the complexes are produced or lodge entails differences in the overall pattern.

Such complexes between antigen and antibody may be formed in two situations; firstly, in the blood stream, when large amounts of antigen are circulating and antibody is beginning to be produced in the lymphoid system but is immediately combined with antigen as it enters the circulation; and secondly, when antibody is present in the blood and antigen is injected into the tissues in high local concentration—the first being the 'serum-sickness' situation, and second the 'Arthus-type' situation. Serum sickness will be considered in more detail in Chapter 24 and it will be sufficient to say here only that the site of the localization of complexes from the blood stream is naturally in the vessels themselves. In the Arthus-type situation, antigen deposited intra- or subdermally will presumably diffuse from the site, and at some point in the diffusion zone it will meet and combine with blood-borne antibody at the concentration required to produce antigen-excess irritant complexes. After this the mechanism in the two sorts of Type III situation may be identical, although the initial events and exact histological site are different.

Once complexes of the right sort are formed (i) they will tend to produce both local histamine release, and also possibly the release of actively cytotoxic enzymes, (ii) circulating leucocytes will be attracted by them and by complement-induced factors causing accumulation locally, (iii) activation of local vascular endothelium (Biozzi *et al* 1948) will also promote the lodgement of affected leucocytes, platelets and fibrin, with resultant thrombosis and haemorrhage; (iv) owing to the systemic absorption of chemotactic factors the leucocytes become 'sticky' and will tend to aggregate elsewhere, e.g. in the lungs and at other inflamed sites (Stetson 1951). Thus, according to the amount of complexes produced, anything from transient polymorph infiltration and oedema to extensive vascular thrombosis and local necrosis may develop. The late development of plasma cells suggests the occurrence of local antibody formation.

We use advisedly the term 'Arthus-type'. The Arthus reaction as originally described (Arthus 1903) is probably more complicated since there is evidence that both a delayed (Type IV) (Tremayne and Jeter 1955) and possibly also a Type I component are involved. The simple model is the Passive Arthus, in which antibody is injected intravenously and antigen locally. (It should be noted that the routes of injection are different to those of Ovary's 'Passive

Cutaneous Anaphylaxis' reaction, where antibody is injected intradermally.) The Reversed Passive Arthus, in which antibody is injected locally and antigen intravenously has presumably, *mutatis mutandis*, a similar mechanism.

Antigen-antibody complexes may produce damage by a mechanism less direct than the primary irritant effects of the complexes themselves, namely by the activation of complement components to produce anaphylotoxin which among other things releases histamine (see Chapter 14). Indeed, it is possible that the 'cot death syndrome' may be an example of this phenomenon.

Differentiation from Type I Reactions

Since it is a characteristic of a Type I reaction that the antibody is fixed to cells the use of a non-fixing type of antibody, e.g. horse antibody in the guinea-pig, should provide a model in which the anaphylactic type of passive sensitization of cells and histamine release is eliminated. However, histamine release is an element of any inflammatory reaction, particularly where complement is involved; and therefore although Arthus-type reactions are not inhibited by antihistaminics, it is not surprising that some elements in them, in particular the oedema, may be reduced. On the other hand the intensity of a Type III reaction is greatly reduced by eliminating the animal's circulating platelets or polymorphs (Humphrey 1955) or by heparin (Benacerraf & Biozzi 1953). Again the intensity of a Type III reaction is proportional to the level of circulating precipitating antibody, which is not the case in a Type I reaction. Finally, in performing a passive reaction, no interval for fixation to the tissues is required in a Type III reaction, as it is in a Type I reaction.

The important consideration from the point of view of the classification we are presenting is that the initiating antigen-antibody interaction proceeds independently of cells or tissues. These become involved in the reaction and damaged only secondarily, owing either to the primary irritant action of complexes of a particular antigen to antibody ratio or to pharmacologically active agents released under the influence of these complexes.

Significance of Type III reactions in medicine

1. The full Arthus reaction used to be seen quite commonly in man following repeated administration of antitoxic or antibacterial sera derived from animals; for an illustration of a very severe reaction see Kohn, McCabe & Brem (1938). It may be seen today after several injections of horse tetanus antitoxin or as a rarity in persons receiving long-continued courses of materials such as insulin.
2. Similarly serum sickness proper is not so often seen today as in the days of extensive serum-therapy, at least not in severe form. In typical 'delayed serum sickness' occurring in nonatopic individuals a Type III reaction involving circulating complexes probably plays the main role in the pathogenesis. So-called

'immediate serum sickness' is on the other hand an 'atopic' manifestation involving reagins, a typical Type I reaction (see Chapter 24).

3. There is reason to believe that many cases of drug sensitivity, in particular those involving penicillin and sulphonamides, are examples of a Type III serum-sickness-like mechanism (see Chapters 24 and 26).

4. Finally, because the lesions and other findings in experimentally produced 'hyperimmunization serum sickness' in animals are suggestive of a similar mechanism being operative in nephritis, polyarteritis, rheumatoid arthritis, disseminated lupus erythematosus and other diseases, it is possible that when these diseases are more fully understood it will be found that a Type III reaction plays an important role.

Before concluding this section a word should perhaps be said on the significance of the source and supply of antigen in these Type III reactions. If the antigen is administered as a single dose, the lesions should regress and heal as in classical serum sickness. If on the other hand the antigen is given repeatedly, as during treatment with penicillin, or is endogenous, as is presumably the case with the desoxyribonucleoproteinantigen of lupus, the lesions will recur in crops. Corticosteroids will be effective both as 'anti-inflammatory' agents and as damping-down antibody production.

Type IV Reactions (Delayed or Cell-Mediated Allergy)

The Type IV reaction, usually referred to as delayed hypersensitivity, bacterial or tuberculin-type allergy (also underlying contact dermatitis), is an allergic reaction whose importance in human disease is well recognized but not yet well understood. Since it is generally accepted as being due to the activities of cells independent of free antibodies, the term 'cell-mediated allergy' would appear to be an ideal one, were it not that both Type I and Type III reactions, though antibody dependent, also require the mediation of cells. For the present it would appear best to stick to the conventional if unsatisfactory adjective 'delayed', even though this draws attention to only one, operational, element in the complex situation. It is likely, however, that the action of antigen on the cell which is allergized in the delayed way does in fact take longer to be manifest than the action of antigen on, say, a reagin-coated basophil.

It is important at the outset to distinguish carefully between the *induction of the state* of delayed hypersensitivity and its reaction manifestation, the *delayed reaction* or test. Since we are here classifying reaction mechanisms, we shall not consider in detail the induction process; great though the interest of this question, it is still somewhat obscure, and the exact conditions under which we may expect the development of a state of delayed allergy with or without antibody production are really only known for one animal species, the guinea-pig. It is helpful but still a little risky to extrapolate principles established in the

guinea-pig to man; but in practice most workers in human diseases are ready enough to postulate the intervention of delayed allergy as a response to virtually any substance known to be potentially antigenic, and to accept that such reactions are to be expected in the absence of any demonstrable antibodies, the two most important principles derived from animal work over the last twenty years. As for the induction process, however, animal work, although it throws light on how, tells us rather little of why a state of delayed allergy sometimes develops in human diseases and sometimes does not.

The induction of delayed allergy (DA). Animal work on the histology of induction is well reviewed by Turk (1967); in summary we may say that clear-cut differences are detectable in the architecture of lymph nodes reacting either to a delayed (contactant) allergen or to an antibody production stimulus; in particular, germinal follicle and plasma cell formation are much more prominent when antibodies are being produced. As far as the gross processes are concerned, Gell & Benacerraf (1961) discuss the conditions for the experimental production of 'pure' delayed allergy in the guinea-pig; for most of these the use of Freund's adjuvant with tubercle bacilli added is essential, and so they can throw little direct light on natural processes in man, but it is of significance that very small doses and 'poor' antigens do favour DA. Some contactant chemicals, such as dinitro-fluorobenzene, seem however to act in exactly the same way in man as in guinea-pig, and this substance, together with tests using various bacterial and viral antigens of less well-defined modes of activity, are regularly used in testing for the 'sensitizability' of human subjects (cf. Chapter 19).

Although every antigen which has been tested, with the exception of pure polysaccharides, can when appropriately used be shown to induce DA in the guinea-pig (see review by Gell & Benacerraf 1961) either a contact reactant or tuberculin reactivity is generally used in investigative work, especially in man. In addition, the rejection of homografts is usually treated as a Type IV reaction, although it is not certain that this is entirely parallel to delayed responses to soluble or non-living antigens, or that DA is indeed the only mechanism involved.

The tuberculin reaction is the *locus classicus* of the Type IV reaction but as a prototype for analysis of the mechanism it has certain disadvantages, the chief of which are that the complex nature of the tubercle bacillus complicates the study of the conditions initiating sensitization and that the difficulty of a rigid purification and characterization of 'tuberculin' complicates the test. The violent response of the tuberculous animal to parenteral injection of tuberculin was first observed by Koch (1891) and it was not long before it was realized that the judicious intradermal injection of small amounts of tuberculin could be used as a diagnostic test for tuberculosis. The clear separation of this kind of reaction, which we are now calling the Type IV reaction, from the other allergic or 'anaphylactic' reactions has been a long and arduous process.

Manifestation of delayed allergy: the local lesion. Much illuminating work has

been done upon the development of the delayed lesion in recent years and it is now possible to put down a theoretical scheme for the course of events from the injection of minute amounts (1–25 μg) of antigen into a normal area of skin of an allergized animal to the presence 18–48 hours later of an intense inflammatory lesion packed predominantly with mononuclear cells.

1. Antigen injected into the skin escapes in most cases fairly rapidly: By 5 hours only 10–20% of the initial dose persists: this was shown by Norton & Ziff (1966) to be all contained within macrophages. No differences are found between allergized and normal animals.

2. A mild inflammatory reaction occurs at this period which is occasioned by the presence of any high molecular weight potentially antigenic substance in the tissues: as long as no antibodies of any sort are present (e.g. to tubercular carbohydrate) this is similar in normal and in allergized animals; the relative proportions of leucocytes in the lesion are similar to those in the blood (Paz & Spector 1966).

3. In the next phase there is increasing localization of mononuclear cells in the lesion largely though not entirely derived from blood monocytes (cf. Spector 1967) and exudation becomes far greater in the allergized than in the normal animal, in which it soon ceases.

4. This comes about (and here we enter the field of speculation suggested though not proved by the work of Willoughby *et al* (see Schild & Willoughby 1967 and Kolin, Johanovsky & Pekárek 1966)) because a soluble inflammatory hormone is specifically released, from the contact of a few allergized cells present in the non-specific exudate with the antigen held by macrophages. The number of such 'specifically sensitized' (allergized) cells is very small, and the probability is that they are lymphoid cells.

5. Although the cells leave the blood vessels under a non-specific inflammatory stimulus and consist of all types of leucocytes, the mononuclear cells are held at the site while the other cells, in particular the polymorphs, move on and out. There is therefore built up a stage of mononuclear predominance.

6. The specifically allergized cells may be present as a constant proportion of the circulating leucocytes or may possibly be shed into the bloodstream as a result of the primary escape of antigen from the site. In either case they will be present in the 'non-specific' exudate and their subsequent reaction with antigen at the site will lead to more hormone release and more exudation. A 'cascade' reaction is thus built up.

7. When all antigen has reacted with allergized cells (by a mechanism which is still unknown), hormone release stops and the progress of the lesion ceases. The retained mononuclear cells may, however, remain at the site for many days.

This rather complicated analysis of the process is derived from the observations of Spector, Willoughby and co-workers (reviewed by Schild & Willoughby 1967 and Spector 1967) and to explain such essential observations as

those of Waksman & Matolsky (1958), McCluskey *et al* (1963) and Turk & Oort (1963) that the bulk of the cells in such lesions are of host origin when the lesions are produced by the passive allergization of an animal by the injection of actively allergized cells from another compatible donor.

This observation, first made by Landsteiner & Chase (1942), namely that delayed reactivity could be transferred by cells though not by serum, is now considered an essential characteristic of the delayed allergic state, and is our main justification for postulating the 'specifically sensitized (allergized) cell' as the mediator of delayed reactivity. Although it is hard not to conclude that such a cell contains a substance of the nature of an antibody, the term 'cell-bound' antibody often used is misleading partly because there is no definite evidence that any antibody can be extracted and partly because of the confusion with demonstrably cell-bound antibodies such as cytophilic antibodies and reagins.

A substance which is extractable from cells, though exclusively at present from human allergized cells, is the 'transfer factor' of Lawrence and co-workers (1959, 1963). This substance is dialysable and insusceptible to proteolytic enzymes, so cannot be an antibody, yet is able to transfer delayed reactivity. The absence of any animal model still makes intensive work in this interesting field slow and difficult.

Relationship between delayed allergy and antibody production. It is simplest for the time being to regard delayed hypersensitivity and antibody production as independent allergic mechanisms in spite of the close relationship between the two. There is evidence that 'delayed reactivity' and production of serum antibody to the same antigen can coexist, although sometimes 'delayed reactivity' tends to wane and disappear, or at least becomes difficult to demonstrate when appreciable amounts of antibody are being produced (Leskowitz & Waksman 1960). In some cases, at least, this may be due to 'deviation' of antigen by the more rapidly occurring Arthus reaction.

Nevertheless, it would appear that delayed allergy is related, in some way which is still obscure, to the inductive and proliferative phases of the immunological process, while antibody production is of course related to the productive, or rather to the mass-productive, phase. There are analogies which we do not feel to be inept between the early proliferative phases of the secondary response, as studied *in vitro*, and the response of delayed allergized cells to antigen (cf. Oppenheim *et al* 1967). The block would appear to be between the proliferative phase of the secondary response (although some antibody is produced at this stage in suitable systems) and the turning-on of full antibody production by morphologically identifiable plasma cells.

Differentiation from Type III reactions

Theoretically Type IV reactions may be clearly differentiated from reactions of Types I, II and III by the time taken to mount the reaction after injection of

antigen, say, into the skin and by the fact that it occurs in the absence of circulating antibody. From this it may be gathered that it is not possible to transfer the reaction passively by means of serum—although this is possible using cells. Again, the reaction has a fairly characteristic though not diagnostic histology (Gell & Hinde 1951).

In practice, however, it may be exceedingly difficult to differentiate with absolute certainty a Type IV from a Type III reaction. Absence of circulating antibodies may be a pointer and the delay in appearance of the test reaction is certainly a useful guide, i.e. the observation that a reaction is not maximal within the first few hours; although an Arthus reaction in an animal with a rapidly rising antibody titre could show a somewhat similar appearance. The macroscopic appearance may be useful; in a strong Type IV skin reaction, the edges are well defined with a pallid or necrotic centre while a moderate reaction takes the form of a uniform indurated erythema: in contrast the pure Arthus (Type III) reaction usually has a haemorrhagic centre and ill-defined edges.

Histologically the test sites show infiltration with cells, particularly around the small blood vessels, and in a mild reaction without necrosis such cells are predominantly mononuclear, though even in the mildest a few polymorphs occur. Some of the swelling and induration may be put down to the packing of the tissues with such cells, though oedema and hyperaemia also play a part. In general, however, it is not possible or wise to draw firm conclusions as to the 'delayed' nature of a lesion from histological criteria alone.

Role of Type IV reactions in disease

It is possible that a relatively mild delayed reaction may play a part in defence, perhaps by (specifically) stimulating non-specific defence mechanisms (see Chapter 15). A violent, and especially a 'chronic', reaction can certainly cause evident damage to the host, in two ways: firstly, by causing vascular blockage and necrosis, and, secondly, by replacing normal tissue with infiltrating mononuclear cells. There may well be further activities of the specifically modified 'sensitized' cells, which are still unknown to us.

In an intense reaction necrosis may occur, just as in an Arthus reaction, and histologically this is often associated with obvious vascular damage and thrombosis; nevertheless, fairly strong delayed reactions can occur in the absence of microscopic evidence for any events of this sort. Thus, although there may be both a vascular and a 'hormonal' element in the reaction, it seems most likely that the main chronic damage is produced by the infiltration, disorganization and replacement of the tissue by mononuclear cells.

In many 'auto-allergic' diseases, the demonstration of circulating antibodies has been taken as an indication that the antibodies cause the disease. This is unjustified, for two reasons: firstly, because stimulation of the immunological mechanisms may be the mere effect of specific tissue changes which have

themselves a non-allergic pathogenesis; secondly, because the antibodies may be accompanied by a state of delayed sensitivity, and it may be this rather than any antigen-antibody reaction which is at least the initial cause of the lesions.

In concluding this chapter we would apologize for at times appearing to be somewhat dogmatic. This results from trying to keep within our limits of space and from our attempt to emphasize the simplicity and yet comprehensiveness of this classification of the allergic reactions. We would stress again that this is essentially an immunological classification based on the mechanism of the initiating antigen-antibody reaction. Nevertheless, in reflecting the basic immunological mechanism it should prove helpful to the clinician.

REFERENCES

ARTHUS M. (1903) Injections repetées de sérum de cheval chez le lapin. *C. R. Soc. Biol. (Paris)* **55,** 817.

BENACERRAF B. & BIOZZI G. (1953) In *Proc. IV. International Congress of Microbiology.*

BENACERRAF B. (1967) Properties of immunoglobulins which mediate the release of vaso-active amines. Pharmacology Congress, Sao Paulo, Brazil. In Press.

BERKINSHAW-SMITH E.M.I., MORGAN R.S. & PAYLING WRIGHT G. (1962) The action of Forssman antiserum on the blood vessels of the skin of guineapigs. *Brit. J. exp. Path.* **43,** 665.

BIOZZI G., HALPERN B.N. & BINAGHI R. (1959) The competitive effect of normal serum proteins on antibody fixation in passive cutaneous anaphylaxis in the guineapig. *J. Immunol.* **82,** 215.

BIOZZI G., MENE G. & OVARY Z. (1948) *Rev. Immunol. (Paris),* **12,** 320.

BOYDEN S.V. (1964) Cytophilic antibody and delayed hypersensitivity. *Immunology* **7,** 474.

BROWN P.C., GLYNN L.E. & HOLBOROW E.J. (1967) The dual necessity for delayed hypersensitivity and circulating antibody in the pathogenesis of experimental allergic orchitis in guineapigs. *Immunology* **13,** 307.

COCA A.F. & GROVE E. (1925) Studies in hypersensitiveness. *J. Immunol.* **10,** 445.

DAUSSET J. & CONTU L. (1964) A case of haemolytic anaemia following phenacetin allergy. *Vox. Sang.* **9,** 599.

DINGLE J.T., FELL H.B. & COOMBS R.R.A. (1967) The breakdown of embryonic cartilage and bone cultivated in the presence of complement-sufficient antiserum. 2. Biochemical changes and the rôle of the lysosomal system. *Int. Arch. Allergy* **21,** 283.

FELL H.B., COOMBS R.R.A. & DINGLE J.T. (1966) The breakdown of embryonic cartilage and bone cultivated in the presence of complement-sufficient antiserum. 1. Morphological changes, their reversibility and inhibition. *Int. Arch. Allergy* **20,** 146.

FREIMER E.H. & MCCARTY M. (1965) Rheumatic fever. *Scientific American* **213,** 66.

GELL P.G.H. & BENACERRAF B. (1961) Delayed hypersensitivity to simple protein antigens (review). In *Advances in Immunology* **1.** New York and London: Academic Press.

GELL P.G.H. & HINDE J.T. (1951) The histology of the tuberculin reaction and its modification by cortisone. *Brit. J. exp. Path.* **32,** 576.

GOLDBERG B. & GREEN H. (1959) The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumour cells. *J. exp. Med.* **109,** 505.

GRAHAM H.J., LOWRY O.H., WHEELWRIGHT F., LENZ M.A. & PARISH H.H. (1953) Distribution of histamine among leucocytes and platelets. *Blood* **10,** 467.

HUMPHREY J.H. (1955) Reversed passive Arthus reactions in rabbits. *Brit. J. exp. Path.* **36,** 268.

IRVINE W.J. (1960) The cytotoxic factor in thyroid disease. *Scot. med. J.* **5,** 511.

ISHIZAKA K., ISHIZAKA T. & HORNBROOK M.M. (1967) Physicochemical properties of human reaginic antibody. V. *J. Immunol.* **97,** 754, 840.

KOCH R. (1891) Fortsetzung des Mitteilunger über ein Heilmittel gegen Tuberkulose. *Dtsch. med. Wschr.* **17,** 101.

KOHN J.L., MCCABE E.J. & BREM J. (1938) Anaphylactic gangrene following administration of horse serum. *Amer. J. Dis. Child.* **55,** 1018.

KOLIN A., JOHANOVSKY J. & PEKÁREK J. (1966) Histological manifestations of cellular (delayed) hypersensitivity. III. *J. Immunol.* **97,** 621.

LANDSTEINER K. & CHASE M.W. (1942) Experiments on transfer of cutaneous sensitivity to simple compounds. *Proc. Soc. exp. Biol. Med.* **49,** 688.

LAWRENCE H.S. (1959) The transfer of hypersensitivity of the delayed type in man. In *Cellular and Humoral Aspects of the Hypersensitive States,* ed. LAWRENCE H.S. New York Hoeber-Harper.

LAWRENCE H.S., AL-ASKARI S., DAVID J., FRANKLIN E.C. & ZWEIMAN B. (1963) Transfer of immunological information in humans with dialysates of leucocyte extracts. *Trans. Assoc. Amer. Phys.* **76,** 84.

LING N.R. & KNIGHT S. (1967) The effect of antibodies on lymphocytes in vitro. *Ciba Foundation Symposium on Antilymphocytic Serum.*

LISKOWITZ H.S. & WAKSMAN B.H. (1960) Studies in immunization. I. The effect of route of injection of bovine serum albumin in Freund adjuvant on the production of circulating antibody and delayed hypersensitivity. *J. Immunol.* **84,** 58.

MCCLUSKEY R.T., BENACERRAF B. & MCCLUSKEY J.W. (1963) Studies on the specificity of the cellular infiltrate in delayed hypersensitivity reactions. *J. Immunol.* **90,** 466.

MOESCHLIN S. (1958) Agranulocytosis due to sensitization to drugs. In *Sensitivity Reactions to Drugs,* pp. 77–88, ed. ROSENHEIM M.L. & MOULTON L. Oxford: Blackwell Scientific Publications.

MOLLER G. & MOLLER R. (1965) Plaque formation by non-immune allogeneic and semi-isologous lymphoid cells on monolayers of embryo cells. *Nature* **208,** 260.

MUIRHEAD E.E., HALDEN E.R. & GROVES M.T. (1958) Drug-dependent antiglobulin (Coombs) test and anaemia: observations on quinine and acetophenetidid (phenacetin). *Arch. intern. Med.* **20,** 806.

NORTON W. & ZIFF M. (1965) Electron microscopic observations on the localization of antigen in the tuberculin reaction. *Immunology* **8,** 81.

OPPENHEIM J., WOLSTENCROFT R.A. & GELL P.G.H. (1967) Delayed hypersensitivity in the guineapig to a protein-hapten conjugate. *Immunology* **12,** 89.

OVARY Z. (1958) Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. *Progr. Allergy* **5,** 459.

PARISH W.E., BARRETT A.M., COOMBS R.R.A., GUNTHER M. & CAMPS F.E. (1960) Hypersensitivity to milk and sudden death in infancy. *Lancet* **ii,** 1106.

PAZ R.A. & SPECTOR W.G. (1962) The mononuclear cell response to injury. *J. Path. Bact.* **84,** 85.

REDFERN W.W. (1926) A study of the primary toxicity of heterophile immune rabbit serum for guineapigs, and its apparent relation to the phenomenon of anaphylaxis. *Amer. J. Hyg.* **6,** 276.

REDFERN W.W. (1928) Microscopic changes of heterophile skin reaction. *Amer. J. Hyg.* **6,** 585.

ROBERTS G.F. (1957) *Comparative Aspects of Haemolytic Disease of the Newborn.* London: Heinemann.

SCHILD H.O., HAWKINS D.F., MONGAR J.L. & HERXHEIMER H. (1951) Reactions of isolated human asthmatic lung and bronchial tissue to a specific antigen. *Lancet* **ii,** 376.

SCHILD H.O. & WILLOUGHBY D.A. (1967) Possible pharmacological mediators of delayed hypersensitivity. *Brit. med. Bull.* **23,** (1), 46.

SELL S. & GELL P.G.H. (1965) Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with an anti-allotype serum. *J. exp. Med.* **122,** 423.

SPECTOR W.G. (1967) Histology of allergic inflammation. *Brit. med. Bull.* **23**(1), 35.

STETSON C.A. (1951) Similarities in the mechanisms determining the Arthus and Schwartzman phenomena. *J. exp. Med.* **94,** 347.

STETSON C.A. (1963) Role of humoral antibody in the homograft response. In *Advances in Immunology* **3,** 97. New York and London: Academic Press.

TREMAYNE M. & JETER W.S. (1955) Passive cellular transfer of hypersensitivity to serum antigens in rabbits. *J. Immunol.* **74,** 96.

TURK J.L. (1967) Cytology of induction of hypersensitivity. *Brit. med. Bull.* **23**(1), 3.

TURK J.L. & OORT J. (1963) A histological study of the early stages of the development of the tuberculin reaction. *Immunology* **6,** 140.

VAN DEN ENDE M. (1940) Reversed passive anaphylaxis in the guineapig. *J. Hyg.* **40,** 377.

WAKSMAN B.H. & MATOLSKY M. (1958) Quantitative study of local passive transfer of tuberculin sensitivity. *J. Immunol.* **81,** 235.

WEIGLE W.O. (1961) Fate and biological action of antigen-antibody complexes. In *Advances in Immunology* **1,** 283. New York and London: Academic Press.

CHAPTER 21

AUTO-ALLERGY

P. J. LACHMANN

INTRODUCTION

It must have been at just about the time that Ehrlich (Ehrlich & Morgenroth 1901) was advancing the concept of 'horror autotoxicus'—that disease resulting from antibody formation to an organism's own tissue antigens would not occur—that Donath & Landsteiner (1904) described the auto-antibody still known by their name. This antibody binds at low temperature to a defined blood-group antigen ($P+P_1$) on the patient's own red cells and gives rise to complement-dependent haemolysis on warming. Although it is not clear how this antibody is stimulated, its presence is associated with the syndrome of cold haemoglobinuria and is thus related to the pathogenesis in a readily comprehensible way.

In the intervening decades it has become apparent that the occurrence of allergic reactivity towards an animal's own antigens is by no means uncommon and it is widely suspected that in some cases it may play a part in causing disease. Massive speculation and not inconsiderable experimental work has been devoted to the question as to how auto-allergy may arise and how it may be

involved in pathogenetic mechanisms. It is proposed here to give in very general terms some account of these matters. Most of the material mentioned in this chapter can be found more fully discussed elsewhere in the book and, for this reason, references to sources have been kept to a minimum.

THE ORIGIN OF AUTO-ALLERGY

Allergic reactions are the result of only two known phenomena: the formation of antibodies and of specifically allergized cells. In this section, the origin of auto-antibodies is mainly discussed, but it seems unlikely that the factors responsible for the development of cells allergized to auto-antigens are much different.

It is self-evident that the consideration of auto-antibody formation is merely a facet of the general problem of antibody formation and tolerance. More detailed discussion of this topic is to be found in Chapter 11 and, for the present purpose, the following points may suffice.

Firstly, the induction of both antibody formation and tolerance operate at the level not of the antigenic molecule, but of the antigenic determinant—that portion of the antigen, probably generally of a size of roughly 1000 molecular weight, which is able to combine with a single antibody. Protein molecules nearly always have multiple determinants and except where a protein shows quaternary (sub-unit) structure, it may be assumed that these determinants are all different.

Secondly, it appears to be now well established that the outcome of an antigenic stimulus—whether it leads to antibody formation or to tolerance—does not depend solely on the age of the host at the time of stimulation. It is possible both to cause antibody formation *in utero* and to induce tolerance in adult life. A number of other factors are known to be involved. These include:

Dose of antigen

Large doses of antigen can produce tolerance even in adult life. Very large doses of rather weak antigens (like bovine plasma albumin) can even induce tolerance in animals already making antibody. Very low doses of this antigen can also induce tolerance.

Chemical nature of antigen

Certain antigens are much better at promoting tolerance than antibody formation. Certain bacterial polysaccharides and D-amino acid polymers are examples.

Physical state of the antigen

Human or bovine IgG, when given to rabbits, gives rise to antibody formation when aggregated material is present. Injection with monomeric material produces tolerance even in adult animals. This may be a particularly striking

example of a general thesis advanced by Mitchison (1966) that antigens given for the first time give rise to antibody formation when presented to the lymphoid cells by way of the macrophage—i.e. having been phagocytosed; but give rise to tolerance if presented directly to the lymphoid cell.

Route of administration

Mitchison's thesis would also explain why giving antigens into the portal circulation favours the induction of tolerance, as phagocytosable material is then largely cleared in the liver, where there are no lymphoid cells.

Nevertheless, all these other factors notwithstanding, it remains generally true that the age of the host on primary stimulation is an important factor, and that animals generally become tolerant to such antigenic determinants as are accesible to their own reticuloendothelial system in foetal life.

Self-Recognition

It may be gathered from what little has been said above that there is no need to postulate—nor indeed is there any experimental evidence to suggest—some special mechanism by which organisms can 'recognize' their own antigens. The outcome of antigenic stimulation—whether antibody formation or tolerance—would seem to depend on the same factors for auto-antigens as it does for any other antigen.

It is, none the less, perhaps still profitable to consider auto-antigens in two main groups: those to which tolerance is not normally fully established and those to which antibody formation is a result of the breakdown of previously established tolerance. This distinction means only that antigens in the first group will—if extracted and injected in suitable dosage and by a suitable method—give rise to antibody formation in normal animals, whereas antigens in the second group will not. Although this is useful as a basis of discussion, the distinction is quantitative rather than qualitative, and often breaks down in practice. Thus there is undoubtedly an important genetic element in the ease with which animals form antibodies to auto-antigens, even in the first group and adjuvants (*vide infra*) also play an important part in facilitating auto-antibody formation. Furthermore, immunization with related or chemically modified antigens may cause auto-antibody formation even where the autologous antigen does not.

Auto-Antigens which Normally Fail to Establish Complete Tolerance

In the main these are antigens which fail to gain access in early life to the host's reticuloendothelial system. One factor recognized as leading to this failure is the *anatomical sequestration* of the antigen—i.e. it just never meets a reticuloendo-

thelial cell. There are certain 'privileged sites' in the body, of which the anterior chamber of the eye is the prime example, whose lack of the normal lymphatic and vascular connections leads to the situation that even extraneous antigens will not reach the reticuloendothelial system. Antigens in 'privileged sites' therefore do not give rise to antibody formation—or tolerance. If antibody production is stimulated by parenteral administration of the antigen, an allergic reaction in the privileged site can follow.

This state of affairs is to be distinguished from another (pseudo-) type of anatomical sequestration—*basement membrane protection*. Here it is not that the antigen cannot get at the reticuloendothelial system but that the reticuloendothelial system (and antibody and complement) cannot get at the antigen. Thus antibody formation may occur (and therefore this phenomenon is not strictly relevant here), but the antigen is protected against such allergic reactions as would otherwise occur. An example of this is the testis. Animals normally have a cytotoxic antibody directed against their own spermatic tissue, but no cytolysis occurs unless the basement membrane is damaged.

The commonest site of anatomical sequestration is undoubtedly the inside of cells. However, the question of how much tolerance is normally present to intracellular components is complex and may be related to the extent to which the components spill into the circulation on cell death—a point to be considered further below. Experimental evidence suggests that it is organ-specific antigens, in particular those of organs that may show some degree of 'privilege' (eye, brain and testis), but also others that can regularly induce auto-antibody formation if given in Freund's adjuvant, whereas the intracellular materials common to most (and therefore also circulating) cells generally do not.

A second factor leading to the failure to establish tolerance may be termed *biochemical sequestration* and includes two types of phenomenon—one well established, the other rather speculative. The well-established phenomenon is that of the 'hidden determinant'. The idea that as a result of interactions occurring *in vivo* a molecule could acquire new antigenic determinants which are 'hidden' in the native state was proposed by Coombs (1947, 1961) with respect to the fixation of complement and the stimulation of antibodies, the immunoconglutinins, reacting with fixed—but not with free—complement components. Rheumatoid factors, similarly, contain antibodies reacting with fixed, but not with free, IgG and the phenomenon may well be much more general. The more speculative phenomenon is concerned with the survival of the potential antigen in the circulation (or the reticulo-endothelial system). It is reasonable that materials which are extremely rapidly broken down in the circulation should not be antigenic and that no tolerance should be established to them. This may be among the reasons why nucleic acids in normal circumstances are not antigenic, although there is evidence (see Plescia & Braun 1967) that animals are not normally tolerant to the relevant determinants.

Where tolerance to a potential auto-antigen has not been established, stimulation of antibody may be expected to result if the antigen gains access to the reticuloendothelial system. A clear example of this is the formation of antibody to lens protein spilled into the circulation during cataract extraction. Leakage of antigen into the circulation may also account to some extent for antibody formation to intracellular components following tissue damage. Thus, experimentally, antibody production to liver antigens follows treatment with carbon tetrachloride, and clinically antinuclear antibodies have been found for a limited period after pulmonary infarction. It is likely that the amount of leakage is an important factor. Thus, it is now recognized that small amounts of thyroglobulin normally escape into the thyroid lymph without provoking antibody formation. Nevertheless, there is no tolerance to thyroglobulin in as much as injection of this material does give antibody formation.

As an alternative to stimulation by the auto-antigen itself, stimulation may be induced by an exogenous cross-reacting antigen. There are many authenticated examples of this phenomenon. In man antibodies to heart muscle are formed as a result of stimulation by the cross-reacting antigen on the streptococcal plasma membrane; allergic encephalomyelitis can be induced by immunization with heterologous brain-containing vaccines; antibodies to posterior pituitary can result from treatment with heterologous pituitary snuff. In experimental animals it has frequently been found that auto-antibody formation to an 'organ antigen' can be induced by immunization with organ extracts from other species. In fact, the heterologous organs are generally much better than isologous organs in producing auto-antibodies. This is probably associated with the presence of a state of partial tolerance to these auto-antigens, the immunization with cross-reacting antigens being sufficient to break the tolerance. Alteration of auto-antigenic determinants *in vivo* by virus or mycoplasma infection or other agencies such as to make them behave as cross-reacting antigens has been suggested to explain, for example, the auto-antibodies to erythrocytes formed in mycoplasmal pneumonia.

Auto-Antigens to which Tolerance is Normally Established

When auto-antibodies are formed to the surface antigens of circulating cells—as is the case in some forms of haemolytic anaemia—it is clear that a pre-existing state of tolerance has broken down. Similarly, loss of tolerance is most likely to be involved at least to some extent in the formation of such auto-antibodies as, for example, the LE cell factor, which has not been reproduced by experimental immunization.

Since this state of affairs is difficult to reproduce experimentally, the explanations for how it may arise tend to be plausible rather than well established.

It has been generally supposed that changes primarily in the antibody-

forming system itself are involved. Thus, Dameshek & Schwartz (1959) suggested that the auto-allergic haemolytic anaemia associated with malignant reticuloses might be due to somatic mutation in malignant lymphoid cells, resulting in their acquiring the capacity to form the relevant auto-antibodies. Burnet (1959) developed this idea further in terms of the clonal selection theory of antibody formation. This postulates that new lines of lymphoid cell with the potential of forming different antibodies arise continuously by somatic mutation from lymphoid stem cells. Tolerance to an antigen is explained by inhibition or destruction by the antigen of emerging cell lines with the potentiality to produce antibody against it.

If this process of 'immunological homeostasis' is upset—and Burnet suggests that the thymus may be the organ responsible for it and that disturbances here may upset the homeostasis—'forbidden clones' may arise which can produce auto-antibodies. This attractive idea has, however, at least one major difficulty. Mutation being a random process, one would predict from this hypothesis that the formation of auto-antibodies would show a markedly random distribution among potential auto-antigenic determinants. This, however, is not the case. In, for example, systemic LE, antibodies are found not only to a considerable variety of different nuclear components but to multiple determinants in a single component such as the DNA-nucleo-protein. This high degree of multiplicity in auto-antibodies to nuclei is not accompanied by an equivalent frequency of antibodies to other potential auto-antigens (such as, for example, thyroglobulin). It therefore seems unlikely that the origin of these antibodies can be ascribed to a purely random process. It would appear more probable that antigens themselves (or immunologically related material) play some part in the breaking of tolerance.

A further possibility of change in the antibody-forming system is provided by the analogy of graft versus host reactions. If, for example, lymphoid stem cells from an inbred parent strain are transferred to an F1 hybrid the donated cells will be tolerated having no antigens not shared by the F1 host, but they themselves are able to react against the unshared host antigens. This produces a syndrome bearing some similarity both to diseases like systemic LE and to reticuloses like Hodgkin's disease. The possibility that maternal lymphoid stem cells could lie dormant in offspring and become activated on some suitable stimulus (virus infection, irradiation, etc.) is ingenious, but has no experimental support.

The Role of Adjuvants

Adjuvants are materials that—without necessarily being antigenic themselves—enhance the antigenicity of other substances. A considerable number of such materials are known, including metallic salts such as potassium alum, bacterial endotoxin and finely divided silica. However, Freund's adjuvant—an emulsion of antigen in light mineral oil and, if 'complete', with the addition of heat-killed mycobacteria—is probably now the most widely used for experimental purposes.

It produces, besides an increase in circulating antibody, a marked increase in the manifestation of Type IV (delayed) hypersensitivity. In several situations, Freund's adjuvant can lead to the breaking of immunological tolerance, again emphasizing that the distinction between lack of tolerance and breakdown of tolerance is none too clear.

It also gives rise to myelomatosis in mice and to a disseminated arthritis in rats. Many of the organ-specific experimental auto-allergic diseases can be produced only with the aid of this adjuvant. The exact mechanism of its action is unknown, but it is known that it is wax in the mycobacterium that is the active ingredient. Such mycobacterial products, naturally acquired, could be pictured as playing a role in human disease, and may do so with regard to the lesions of tuberculosis itself, but there is no evidence of a particular relation between tuberculosis and other auto-allergic disease.

The Role of Genetic Factors

In man it is well known that the diseases ascribed to auto-allergy are much commoner in women than in men and that certain groups of diseases and the serological abnormalities associated with them tend to run in particular pedigrees. For example, systemic LE, rheumatoid arthritis, hypergammaglobulinaemia (and agammaglobulinaemia) and positive antinuclear antibody and rheumatoid factor tests tend, as a group, to show familial incidence. Another group with raised familial incidence comprises thyrotoxicosis, auto-allergic thyroiditis, pernicious anaemia, idiopathic Addison's disease and the presence of the organ-specific antibodies associated with these diseases.

Similarly in mice, inbred lines (NZB/Bl) and (NZW) have been found that apparently spontaneously develop a Coombs positive haemolytic anaemia and who frequently develop LE cells, positive antinuclear factors and lesions resembling lupus nephritis. It is therefore inescapable that there is a genetic background to the development of auto-allergic phenomena. This would appear to be of the nature of a 'diathesis' or genetic predisposition rather than direct inheritance, and the diathesis for the systemic LE group of diseases appears to be distinct from that of the allergic thyroiditis group. In the predisposed population it seems that a number of environmental factors may provoke the onset of the disorder. This situation would be analogous to the development of haemolytic anaemias in subjects with glucose 6-phosphate dehydrogenase deficiency, where the possession of the genetically determined biochemical abnormality creates a 'population at risk', which is clinically normal but will in response to such diverse environmental factors as eating Fava beans, inhaling the pollen of *Verbena hybrida* or taking various drugs all of which are harmless to the normal population—develop a haemolytic anaemia.

Unfortunately in the case of the diatheses leading to auto-allergic disease the

nature of the genetic predisposition is obscure and even the environmental provoking factors are only incompletely known.

Before leaving the topic of how auto-allergy may arise, it should be emphasized again that it is by no means always an 'abnormal' process even in the sense of being associated with disease. Thus certain auto-antibodies are found almost universally in both man and experimental animals. These include the cytotoxic antibody to spermatic tissue already mentioned; low levels of immunoconglutinin and (in some species) of rheumatoid factors; and an auto-antibody to components of skin, particularly the sweat ducts and the basal layers of the epidermis.

THE PATHOGENETIC MECHANISMS OF AUTO-ALLERGIC DISEASE

So far only the mechanisms by which auto-allergic reactions may arise have been discussed. As has already been mentioned, some auto-allergic phenomena are associated regularly with a particular group of diseases while others are found regularly in the normal population. It is therefore necessary to inquire how auto-allergic phenomena can produce tissue damage. In this respect again there is no essential difference between the allergic response to auto-antigens and to any others. The four types of allergic reaction that cause tissue damage described by Coombs and Gell in Chapter 20 provide the best framework for this discussion.

Type I (Anaphylactic) Reactions

A particular class of antibody—the reagin—is responsible for most Type I reactivity in man. The ease with which reagins are formed is genetically influenced—'the atopic diathesis'. This diathesis carries with it an increased susceptibility to hay fever and asthma but is not associated with an increased incidence of diseases believed to be due to auto-allergy. Furthermore, the pathogenetic mechanism in Type I reactions is basically pharmacological—the result of mediators released from mast cells—and this type of mechanism is also not characteristic of auto-allergic disease. Nor have reaginic antibodies to auto-antibodies so far been described. It seems improbable, therefore, that Type I reactions play much part in auto-allergic disease.

Type II (Allergic Cytotoxicity) Reactions

These are the reactions where antibody reacts with antigens on or attached to a cell membrane—or a basement membrane. If the system is complement binding, cytolysis usually follows: if not, the affected cells may be abnormally sequestered in the reticuloendothelial system. In the case of solid organs other more subtle results may also ensue. Thus organ cultures of chick bones grown in antibody and complement show lysosomal activation with loss of the cartilage matrix.

During this type of allergic reaction, direct damage to the target cell is the main pathogenetic mechanism, inflammatory changes being secondary to such damage.

Where auto-antibodies to cell membrane or basement membrane antigens are found in auto-allergic disease, Type II allergic reactions can in general be expected to play a part in producing the lesions. Thus, auto-antibodies to erythrocytes in auto-allergic haemolytic anaemias and similar antibodies to leucocytes and platelets produce their pathogenetic effects in this way. In auto-allergic thyroiditis cytotoxic antibodies are often found and probably play a part in the lesions of this disease. The long-acting thyroid stimulation (LATS) appears to be an auto-antibody and it may act in a manner analogous to the effect of antibody and complement on the bone organ cultures.

Antibodies to glomerular basement membranes are well known to be able to produce nephrotoxic nephritis in experimental animals. It has recently become apparent that a number of cases of human glomerulo-nephritis—including those patients with 'Goodpasture's syndrome'—are of the same type and show auto-antibodies to glomerular basement membrane. This type of nephritis can be distinguished by the pattern of deposition of immunoglobulin and complement in the glomerulus from the 'immune complex' type of nephritis discussed in the next section.

Antigens which become attached to cell or basement membranes can participate in Type II reactions. Although not strictly auto-allergic in nature sedormid purpura provides a fine example of this, platelets with sedormid loosely bound on them being lysed by an antibody directed against this complex. However, the phenomenon may well be more general than this. Bacterial polysaccharides are often very adept at fixing passively on to cells—a phenomenon of which advantage is taken in passive haemagglutination tests, and antibodies to these may provoke Type II reactions. Some cases of allergic vasculitis are thought to be of this type.

Type III Arthus Reactions

Type III allergic damage is due to the presence of circulating antigen-antibody complexes. This is primarily an acute inflammatory reaction, the damage being mainly vascular and mediated by complement with polymorphs, the essential cellular component.

Serum sickness is the characteristic disease associated with Type III reactions. The lesions of this disease occur at sites of election for the localization of immune complexes. These sites appear to relate to vascular anatomy where this favours retention of complexes—as in the glomeruli, lung and skin—and to regions of increased capillary permeability which also favours the sticking of complexes. Trauma appears to be a significant cause of increased permeability

in vivo and may account for the high susceptibility of synovial membranes and heart valves to serum sickness lesions. In contrast to Type II reactions the localization of lesions thus bears no necessary relationship to the site where antigen is initially released.

In relation to human auto-allergic disease, Type III reactions are probably of importance in relation to certain forms of nephritis and of some of the lesions of diseases like systemic LE and perhaps rheumatoid arthritis.

The characteristics of 'immune complex nephritis' are well known from the work of Dixon and his group (see Chapter 34), and the patterns of deposition of immunoglobulins and complement typical of this condition are seen in a number of types of human nephritis. These are by no means all necessarily auto-allergic, the antigen responsible being often unknown, and occasionally either known or suspected to be exogenous (plasmodial antigens in malaria or drugs like penicillamine), but, for example in lupus nephritis, complexes of nuclear auto-antigens and antinuclear antibodies have been demonstrated. Substantial evidence for the existence and pathogenic role of immune complexes in systemic LE has been accumulated. The part which these complexes play in this disease are likely to be concerned with the perpetuation of the inflammatory reaction—a point to be discussed below.

Type IV (Delayed) Reactions

Type IV reactions are associated with the presence not of antibody but of specifically allergized cells. The pathogenetic mechanism is again largely inflammatory but the inflammation is granulomatous in type and characterized by infiltration by mononuclear cells. The antigens provoking Type IV reactions are more often than not sessile at the reaction site.

In experimental auto-allergic situations Type IV reactions have been implicated in allergic encephalomyelitis and similar 'organ specific' allergic diseases provoked by immunization with extracts of organs in Freund's adjuvant. In man it is plausible to believe that Type IV reactions may similarly be involved in diseases like auto-allergic thyroiditis and perhaps also in the systemic LE group. However, in an outbred species where transferred cells are rejected it is difficult to experiment directly in Type IV situations and clear evidence for this belief is hard to come by.

Transferability of Auto-Allergic Disease

Witebsky (1959) some years ago proposed that conditions analogous to Koch's postulates should be fulfilled before a disease has ascribed to it an auto-allergic cause. Transferability—in this case by serum or allergized cells—from an affected to a normal animal is perhaps the key postulate and is one that has generally been very difficult to achieve. A consideration of the pathogenetic mechanism does, however, make it clear that such difficulty is to be expected.

Only in the case of Type II reactions to readily accessible cells would transfer of serum alone seem likely to reproduce the disease at will. If the cells are normally protected by basement membrane even Type II reactions would not be transferable in this way. In the case of Type III reactions a source of antigen in or accessible to the circulation as well as antibody is required and if the presence of the antigen in the circulation is itself the abnormal event, transfer of antibody would not be expected to produce disease. It is possible that the same is true of Type IV reactions.

The Perpetuation of Inflammation

The natural history of inflammation due to allergic reactions to exogenous antigens is self-limiting. When the supply of antigen is exhausted by

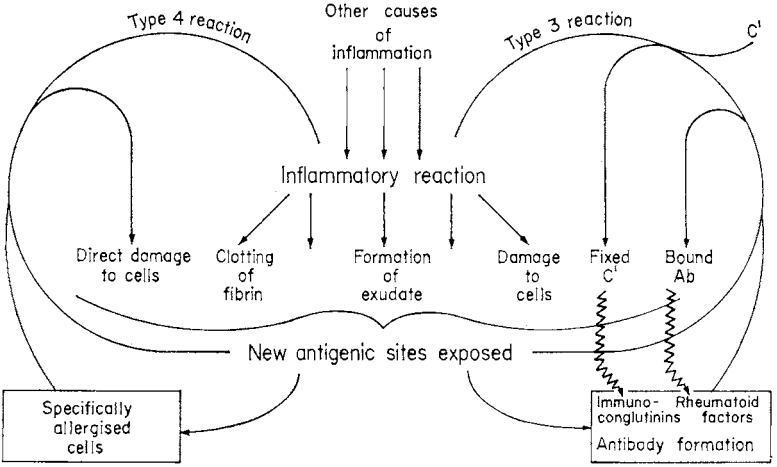


FIG. 21.1. Schema to show how allergic reactions to the products of inflammation can lead to the perpetuation of inflammatory reactions.

(Reproduced from Lachmann P.J. (1967) *The Scientific Basis of Medicine Annual Reviews.* By permission.)

excretion or catabolism, the allergic reaction wanes and the disease disappears. In contrast, the inflammation associated with, for example, the systemic LE group of diseases is characteristically prolonged, and subject to remission and relapse. A possible explanation of this chronicity is that allergic reactions are occurring to the products of the inflammatory reaction itself and therefore allowing the process to cycle: more inflammation giving rise to more antigens, which elicit more allergic reactions, which produce more inflammation, and so on. A schematic version of this is shown in Fig. 21.1.

A number of changes occurring during the inflammatory reaction are listed which are known or thought to produce new antigenic determinants. Damage

to cells may release nuclear and other intracellular components not normally accessible to the reticuloendothelial system as already described. That the clotting of fibrin could be associated with development of allergic reactivity was shown by Dumonde & Glynn (1962) who were able to show Type IV reactivity to autologous fibrin in rabbits immunized with this material in Freund's adjuvant. They also produced an arthritis histologically similar to human rheumatoid arthritis in such animals by implanting fibrin into their joints. Inflammatory exudate used in place of fibrin produced a similar arthritis. Whether it is fibrin itself that is the necessary antigen or whether it is material incorporated into fibrin clot is not certain. The binding of antibody and fixation of complement are known to give rise to auto-antibodies as a result of the exposing of hidden determinants. Type III and Type IV reactions involving these newly exposed antigens can then give rise to more inflammation and more antigens are produced.

It can be seen that this is a mechanism for the perpetuation of inflammation quite independent of how the inflammatory process originally arose. It would therefore help to explain how a sub-population genetically predisposed in some way to produce this type of auto-allergic response unusually readily could develop chronic self-perpetuating allergic inflammation in response to a variety of infective, traumatic or allergic insults, which in the normal population produce only an evanescent response. Weissmann *et al* (1965) have shown in rabbits that repeated intra-articular injection of an apparently non-antigenic lysosome-damaging agent—streptolysin S—produced an arthritis and that this was associated with the appearance of antibodies to intracellular components. These experiments are wholly consistent with the type of scheme outlined above and suggest that, given powerful enough stimulation, even normal animals can be made to react in this way and to reinforce the suggestion that genetic predisposition is likely to be a quantitative rather than a qualitative phenomenon.

IMPLICATIONS FOR HUMAN DISEASE

The Prevention of Auto-Allergy

From what has been said about the origins of auto-allergy, it would follow that there is unlikely to be any specific way of preventing the allergic response to auto-antigens as a class. Nor would this necessarily be a desirable goal. Many auto-allergic phenomena are certainly harmless and some are known to have beneficial functions; for example, immuno-conglutinins increase resistance to certain infections.

Present knowledge of the factors responsible for immunological tolerance makes it feasible by suitable adjustment of dose, physical state and route of administration to induce tolerance to some potential auto-antigens artificially. It

is just conceivable that such a procedure could have a clinical use in subjects who are considered to be at special risk of developing a particular auto-allergy. It would clearly be much more useful clinically—and not only in relation to auto-allergy—if tolerance could be induced in subjects already producing antibody. This has been achieved in mice to bovine plasma albumin by giving extremely high doses of antigen, so that the idea is not impossible. However, there are likely to be great practical difficulties. It is not clear that any antigen can be used in this way; many antigens would be toxic at the very high dosage levels required, and serum sickness could complicate the procedure.

Non-specific immunosuppression can, of course, also be used to suppress an auto-allergic response, but since the allergic responses as a whole have important physiological functions, this approach on a long-term basis is a hazardous one.

In such instances as the systemic LE group of diseases a diathesis is believed to underlie the origin of the particular auto-allergic state. If the nature of this genetic predisposition were clearly known, if it could be recognized in unaffected people and if a method of influencing it could be devised, genuine causal prophylaxis might be possible. At present none of these conditions is met and prevention of known environmental provoking factors is the most that can be done.

Interference with Pathogenetic Mechanisms

There are a number of possible methods of interfering with pathogenetic mechanisms of the different types of allergic reaction. Particularly in the Type III and possibly Type IV reactions where a vicious circle involving allergic reactions to the products of inflammation is believed to act as a perpetuation mechanism, it is possible that temporary interference of this kind may cause the condition to remit.

Thus, *corticosteroids* stabilize lysosomes and reduce the intensity of inflammation produced in response to a Type III reaction.

Interference with *complement action* is another way of attaining this object. This is now possible in at least one way. A factor from cobra venom in association with a protein normally present in human serum forms an enzyme which destroys C'3. This has been shown to prevent Type III reactions *in vivo*. Its clinical use is hampered by the antigenicity of the cobra venom factor. However, it seems likely that other ways of specifically and temporarily inactivating complement will be devised.

Allergic damage due to antigen-antibody complexes may also be reduced in experimental situations by hindering their localization by inhibiting increases of vascular permeability; for example, with antihistamines. This is not effective once localization has occurred and thus makes clinical application difficult. Destruction of antigen in the circulation provides another approach to reducing allergic damage. This can be done only if the antigen is clinically 'unusual' but has been used successfully in penicillin allergy, using penicillinase to destroy the

antigen, and in the auto-allergic situation desoxyribonuclease has been used to destroy desoxyribonucleo-protein antigens.

CONCLUSION

Perhaps the main conclusion about auto-allergy that can be drawn is that it is not an immunological phenomenon apart requiring special hypotheses and postulates to account for it, but that the mechanisms underlying both the origin of allergic responses to auto-antigens and the role they may play in causing tissue damage are basically the same as those involved in the case of exogenous antigens.

The balance of factors determining whether antigenic stimulation gives rise to tolerance or antibody formation are generally such that potential auto-antibodies, for example to spermatic tissue, to some components of skin and to fixed complement, are ubiquitous and a variety of disturbing factors can cause other potential auto-antigens to give rise to antibody formation.

Whether an auto-allergic response is pathogenic or beneficial or neither depends on the circumstances in which antigen-antibody or antigen-allergized cell interaction occurs, and the factors involved are discussed in detail elsewhere in this book. It is suggested that special importance may be attached to auto-allergic reactions directed to the products of inflammation as these may provide a clinically significant mechanism for perpetuating inflammatory reactions.

REFERENCES

BURNET F.M. (1959) *The Clonal Selection Theory of Acquired Immunity*. London: Cambridge University Press.

COOMBS R.R.A. (1947) The antiglobulin and conglutination reactions. Doctoral thesis, University of Cambridge.

COOMBS R.R.A., COOMBS A.M. & INGRAM D.G. (1961) *The Serology of Conglutination and its Relation to Disease*. Oxford: Blackwell.

DAMESHEK W. & SCHWARTZ R.S. (1959) Leukaemia and auto-immunization; some possible relationships. *Blood*, **14**, 1151.

DONATH J. & LANDSTEINER K. (1904) Ueber pariscysmale Hämoglobinorie. *Munch. Med. Wschr.* **51**, 1590.

DUMONDE D.C. & GLYNN L.E. (1962) The production of arthritis in rabbits by an immunological reaction to fibrin. *Brit. J. Exp. Path.* **43**, 373.

EHRLICH P. & MORGENROTH J. (1901) Ueber Hämolysine. V. *Berl. Klin. Wschr.* **38**, 251.

MITCHISON N.A. (1966) Recognition of antigen by cells. *Progress in Biophysics and Molecular Biology* **16**, 1.

PLESCIA O.J. & BRAUN W. (1967) Nucleic acids as antigens. *Adv. Immunol.* **6**, 231.

WEISSMANN G., BECHER B., WIEDERMANN G. & BERNHEIMER A.W. Studies on lysosomes. VII. Acute and chronic arthritis produced by intra-articular injections of streptolysin S in rabbits. *Am. J. Path.* **46**, 129.

WITEBSKY E. (1959) Historical roots of the present concepts of immuno-pathology. In *Immunopathology Symposium 1958*, p. 1, ed. GRABER P. & MIESCHER P. Basel: Benno Schwabe.

CHAPTER 22

PHARMACOLOGICAL MEDIATORS OF HYPERSENSITIVITY REACTIONS

W.E. BROCKLEHURST

RECENT TRENDS OF STUDY

The historic association between histamine and allergy is now recognized as true but misleading. Histamine has many useful functions as a 'local hormone', particularly in the removal of the toxic products of tissue damage, and the processes of growth and repair. Its role in hypersensitivity reactions is now seen in close relation to the inflammatory reaction in any given tissue, with other active substances making substantial contributions to the reactions. The importance of any given substance will obviously be determined by the amount produced and its persistence; it will thus depend upon the nature of the initial antigen-antibody reaction and the presence of stores or precursors of active substances. There will obviously be great differences between different organs, and one would expect to find marked differences between species. Experiments with human tissue are clearly desirable, but have such severe limitations that animal experiments must usually serve as a basis for a much narrower range of clinical experiments in man. In an attempt to follow the reaction in allergic tissue step by step, pharmacologists have used some of the methods of the biochemist to trace the events in the immediate type reaction. These studies have shown that

one of the first events resulting from antigen-antibody combination is the activation of enzymes, or a change in balance between existing active enzymes and their inhibitors. This in turn leads to the release or formation in the tissue of substances having pharmacological activity. The biochemist, for his part, has recently become interested in the possible effects of cathepsins and lysosomal enzymes set free from various types of cell by damage caused by allergic reactions, including the activation of complement, on or near to the cells.

Studies have been directed mainly to reactions of immediate type, but there is also suggestive evidence concerning some of the events of the delayed reaction. The delayed-type reaction must take place *in vivo* and this excludes the new techniques by which we have gained more understanding of the immediate reaction. The most useful way of modifying reactions *in vitro* has been depletion and/or inhibition of the active agent. A recent addition is the use of antisera, but this is obviously not always practicable, and all these approaches may have unexpected complications.

EVENTS PRECEDING THE APPEARANCE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES

The technique which has contributed very greatly to knowledge of these events is the use of chopped tissue in the study of anaphylaxis *in vitro*. Tissue, usually guinea-pig lung, taken from a sensitized animal is chopped into slices or fragments and thoroughly washed in Ringer solution (usually Tyrode's solution). The material can be pooled and then subdivided to give a large number of replicate samples. The undamaged tissue is living and retains its histamine stores well. If this tissue is incubated at 37°C in Ringer solution at pH 7.5–7.9 in tubes gently agitated to ensure diffusion and moderate oxygenation, the addition of the antigen causes a sudden release of some of the stored histamine. The released histamine is easily and accurately assayed biologically on the guinea-pig ileum. The percentage released from the tissue varies in different experiments between 10 and 30%, depending on the level of sensitization of the tissue. In any one experiment the level of histamine release is surprisingly constant in replicate samples, but is readily altered by modifying the treatment of the tissue. Reduction of the histamine yield can therefore be used as an indication of interference with the normal chain of events set off by challenge of the tissue. Very recently human lung obtained at pneumonectomy has been passively sensitized *in vitro*, thus permitting experiments similar to those with guinea-pig tissue. The use of human tissue will explore the relevance of the guinea-pig experiments to human allergy, and it will also make possible direct studies on the nature of sensitizing antibody and the effect of other globulins upon the process of sensitization. The passive sensitization of tissue from the higher apes has also been reported recently. All this work is still at an early stage.

Indications that the Reaction Involves Enzymes

Mongar & Schild (1956, 1957, 1958, 1962) worked extensively with actively sensitized guinea-pig lung. They found that the reaction was dependent on the presence of calcium, that it had a pH optimum at 7.8, and was very dependent upon temperature; at 30°C the release of histamine was very slight, at 38–40°C it was maximal, and after the tissue had been warmed to 44°C for 15 minutes no reaction occurred. They also observed that many antipyretic substances, e.g. phenol, phenylbutazone, cinnamic acid, amidopyrine, phenazone, and salicylate, if present at the time of challenge, prevented histamine release, whilst not preventing desensitization of the tissue. In the course of this work it was shown that iodoacetate, N-ethylmaleimide, azide and dinitrophenol, and also complete deprivation of oxygen could prevent histamine release. Mongar and Schild therefore concluded that the tissue must be capable of oxidative metabolism for the anaphylactic reaction to occur. This has since been interpreted by others as 'anaphylaxis is an energy-requiring reaction', but this statement is not fully justified, as will be discussed later. Several groups of workers using modifications of the technique have confirmed and extended the findings of Mongar and Schild (Högberg & Uvnäs 1960; Chakravarty *et al* 1959, 1960; Yamasaki *et al* 1961; Hayashi *et al* 1960). Moussatche & Prouvost Danon (1956, 1960) also used intermediates and inhibitors of the tricarboxylic acid cycle to show that this source of energy influenced anaphylaxis in chopped tissue.

Diamant (1962) showed that glucose could compensate for the lack of oxygen, indicating that if energy was required it could be supplied by the glycolytic pathway. The substances thought to inhibit histamine release by an effect upon the Krebs cycle may therefore be acting in other ways not necessarily concerned with metabolic energy. Mongar & Perera (1965) found that lack of oxygen and the inactivation of SH enzymes with thiols had an additive effect in inhibiting the anaphylactic release of histamine. This suggests that the tertiary structure of the proteins, which is greatly dependent upon S-S bridges, may be the point at which oxygen lack (considered as changed redox potential) acts. Such an effect might be at the antibody itself or its attachment to the tissue, but would be more likely to affect the enzymic stages triggered by the antigen-antibody union.

Mast Cells

Mota (1957, 1959, 1960) and Boreus & Chakravarty (1960) used the mast cell count as a means of evaluating the severity of anaphylaxis. Nearly all the histamine in practically every tissue is found in the granules held within the mast cells (Riley 1959). These granules contain heparin, and can therefore be stained with basic dyes such as toluidine blue. In anaphylaxis and in many forms of tissue damage these granules are lost from the mast cells which often totally disintegrate. The numbers of identifiable mast cells present in a tissue before and

after challenge will thus give a semi-quantitative evaluation of the severity of the antigen-antibody reaction. Mota studied mast cells in fragments of various tissues, and experimented particularly on sheets of the very thin mesentery supporting the small intestine of the guinea-pig. He has obtained results in agreement with those of Mongar and Schild, and has also shown that the anaphylactic damage to mast cells can be reduced if nicotinamide or other inhibitors of diphosphopyridine nucleotidase are present. It has until recently been assumed that histamine and heparin (or a related acidic polymer) were loosely united in the mast cell granule because they were base and acid, and 'artificial' granules have been produced by mixing the two substances in the presence of zinc to promote binding. The work of Uvnäs (1967) has shown that the granules from rat peritoneal mast cells are quite stable if kept in distilled water or any sodium-free medium, and that over a wide range of concentration sodium and histamine can compete for combination with the heparin in a reversible manner. The granule thus behaves like a bead of ion-exchange resin, with sodium as the sole exchanging cation. It is thus easy to visualize the situation *in vivo*, and to see that histamine will be lost explosively from granules discharged from the mast cells, but gradually and incompletely within cells which have become abnormally permeable to sodium as a result of damage or impaired metabolic activity.

Attempts to Identify Specific Enzymes

Studies on anaphylaxis *in vitro* have contributed much to our knowledge, but the interpretation of results relies upon the specificity of the antagonist or the enzyme inhibitor, and in many instances the degree of specificity is poor or uncertain. This criticism applies particularly to experiments in which high concentrations of inhibitor have to be added, because an increase of 20 mM or more in the ionic strength of Tyrode solution will itself reduce the amount of histamine released in anaphylaxis. High concentrations also invite the danger of inhibition by a subsidiary property of the inhibitor, which will thus lead to incorrect conclusions regarding the mechanism involved. Since the results of Mongar and Schild clearly indicated an enzymic step or steps as intermediate events in the release of histamine, the obvious development was the identification of the enzymes. Austen & Brocklehurst (1961) approached this problem by offering alternative substrates (synthetic esters and peptides), to act in competition with the normal substrate in guinea-pig lung and thus reduce the effectiveness of the events leading to histamine release.

This technique relies upon the readiness of enzyme and synthetic substrate to combine and is not concerned with the ability of the enzyme to split the substrate subsequently. Thus the results in respect of any given enzyme might differ quantitatively from those of the biochemist who is concerned with the splitting of substrate. The method nevertheless provides objective information about the type(s) of enzyme which must be directly involved in the chain of reactions

leading to the release or formation of pharmacologically active products.

By building up a pattern of experimental data showing the relative effectiveness (or failure) of a large number of substrates, Austen and Brocklehurst concluded that an enzyme having the characteristics of chymotrypsin was essential to the reaction, and were able to exclude the enzymes known to be activated from the first, second and fourth components of complement. They obtained suggestive but inadequate evidence that the third component was involved in the reaction. Now that this component of complement has been further subdivided and shown to include a permeability factor when activated, it seems possible that this might contribute directly to histamine release, but it must be remembered that human reaginic antibodies do not activate complement in the usual tests. These results do not mean that other enzymes may not be activated, but indicate that these are not essential factors in the release of histamine and formation of SRS-A during anaphylaxis.

Anoxia and Metabolic Activity

Edman *et al* (1964) investigated the actual levels of oxygen lack needed to prevent the antigen-antibody reaction and found that 50 per cent inhibition is produced by 0.5 per cent oxygen in nitrogen. This inhibition is greatly potentiated if small amounts of cysteine are present during anoxia. From this, and studies of substances able to disrupt or inactivate S-H and S-S groups in tissue, it was concluded that the way in which O_2-lack reversibly influences the tissue is probably by altering these active groups or bonds on the enzymes or their precursors or possibly on the antibody itself. The need for O_2 would then not necessarily be related to metabolic processes, and indeed it is difficult to see how oxidative metabolism could survive the combination of oxygen-lack and CO treatment which failed to prevent the anaphylactic release of histamine in the experiments of Austen and Brocklehurst. The use of very delicate methods for detecting oxygen utilization during the first minutes after challenge with antigen have failed to show any increase in the requirements of the tissue in spite of the rapid release of histamine during this time (Mongar & Perera 1965). These results negate the early work and fall into line with that quoted above. Nevertheless, some of the products of tissue metabolism can influence antigen-antibody reactions since in the guinea-pig small increments of succinic acid enhance anaphylactic histamine release. However, maleic acid is at least as potent as succinic acid in this respect, whereas fumaric acid is inactive, and the effect therefore seems to be at best only indirectly related to the level of available metabolic energy. Furthermore, these substances are without effect in rat tissue and have little effect on passively sensitized human lung. Table 22.1 is an attempt to present in an accessible form the more important observations and their probable meaning. Hypothetical schemes for the actual course of events in the

TABLE 22.1

Procedures which modify the anaphylactic release of histamine

Features	Notes
pH: optimal range about 7.8: barely any reaction at 6.2	pH and temperature dependence confirms the enzymic nature of the reaction
Temperature: optimal range 38–41°C: total inhibition at 15°C and 45°C: 15 minutes at 45°C irreversibly prevents the response of tissue to events subsequent to the Ag-Ab union	
Ionic strength: limited enhancement if moderately hypotonic, inhibition if hypertonic	Raised ionic strength decreases the forces involved in protein interaction
Ca^{++}: total lack prevents reaction; levels below about 1 mEq/litre depress it Mg^{++}: thought to be necessary	Ca^{++} and perhaps Mg^{++} may be involved in activation of enzymes by Ag-Ab complex (cf. complement)
Reaction reduced or abolished by: Reduction in S-H groups (1) by very low O_2 tension (2) by iodoacetate, N-ethyl maleimide, oxidized glutathione, and many other thiol inhibitors including sulphite	Dependence on oxygen and/or glucose varies with the species, and may not be related directly to S-H stability
Inhibitors of chymotrypsin (1) antipyretics and compounds of indole, phenol, nicotinic acid, etc. (2) ester and peptide substrates (3) di-isopropylfluophosphonate	In corresponding tests, inhibitors of trypsin, carboxypeptidase and leucine aminopeptidase do not suppress the reaction
Salicylaldoxime and phlorizin saturated fatty acids pentanoic to dodecanoic (also ε-aminocaproic)	Salicylaldoxime and phlorizin presumed to be acting against the esterase from C'3
Reaction enhanced by: Succinic acid and other substances in the Krebs cycle which are capable of raising the concentration of succinic acid Maleic acid	Effect not understood, and seen chiefly in guinea-pig tissues: much less striking with rat cells and human tissue
No effect with: Cytochrome poisons CO and 2-heptyl 4-hydroxyquinoline N-oxide (dinitrophenol and CN effective only in large doses, and in anomalous fashion)	Dinitrophenol and CN produce their effects by actions not related to cytochromes
Fumaric acid	Component of the citric acid cycle, structurally related to succinic acid, and also to maleic acid which is not part of the cycle

reaction are of little merit because the total number of steps is unknown, and the sequence of those considered to be identified is largely a matter of speculation.

Ions

Calcium ion is essential, since complete removal of Ca^{++} by the chelating agent EDTA* will prevent the release of histamine and the Schultz-Dale reaction, although desensitization occurs. The anaphylactic reaction is reinstated on restoration of the Ca^{++} by adding excess, but this will also restore Mg^{++} by displacing it from the EDTA. Thus we can show that Ca^{++} is essential, but cannot be sure that Mg^{++} is not also necessary, or at least normally involved in hypersensitivity reactions. New chelating agents permit better discrimination and preliminary work suggests that Mg^{++} is necessary in some types of reaction.

The Site of the Reaction

The mast cell provides visible evidence of anaphylactic damage and histamine release both *in vitro* and *in vivo* in many species. When small pieces of mesentery are used in *in vitro* tests, mast cell damage and histamine release occur. Very few other types of cell are present, and it has been concluded that the mast cell contains all the requirements for anaphylactic release of its contained histamine. Speculation that the mast cell is not only the source of histamine, but the specific tissue type where antibody is localized and from which all the processes of the anaphylactic reaction originate, cannot be accepted for several reasons. Firstly, passive anaphylactic reactions can be produced in the skin of rats which have been intensively treated with compound 48/80 to destroy the mast cells. Secondly, visible anaphylactic damage occurs to other cell types. Thirdly, the amount of antibody necessary to sensitize any tissue is so small that localization of uptake at this level cannot be demonstrated. Fourthly, anaphylactic activation of SRS-A has been shown without release of histamine or damage to mast cells.

Workers on isolated mast cells and on destruction of mast cells by relatively specific agents such as compound 48/80, have used the rat because such studies are not satisfactory in the guinea-pig, nor, as far as we know, practicable in other species. The isolated mast cells of the rat contain an active enzyme resembling chymotrypsin, and lose their histamine rather easily. Other species examined have mast cells which probably contain a precursor but do not contain the active enzyme, and are less ready to lose their histamine. Rat tissues may thus prove to be atypical in respect of reactions involving histamine release.

Factors Influencing the Reactions of Different Tissues

We know that antibody is easily and rapidly taken up on tissue, and now think that this is a rather non-specific surface phenomenon which will vary somewhat

* EDTA = Ethylene diamine tetra-acetic acid, disodium salt.

for different cell membranes. If this is true, we must expect that the union of antibody and antigen will occur on the membrane of many if not all types of cell, but that the subsequent events will be dependent on the type of cell and its immediate surroundings. It is evident that cell membranes must differ because of the wide range of reactivity shown by different tissues in contact with the same biologically active agent—e.g. histamine or 5-hydroxytryptamine. This is true even in tissues as closely related as the smooth muscle of the trachea and the bronchioles of the same animal. Histamine does not contract all smooth muscle, neither does 5-hydroxytryptamine. We should therefore expect some variation in the intensity of the initial trigger (antigen-antibody union) and a considerable variation in the effect this has on the cell membrane. This first set of variables might thus be called 'membrane susceptibility'.

A second variable will be the 'nature of the antibody' involved in the reaction. In the last few years the importance of this variable has become more generally recognized especially in animal experiments. The clinician has recognized the existence of reaginic antibody for a long time, but apart from knowing that it could sensitize skin in minute amounts, the chemistry of this antibody has remained quite obscure. The experimental scientist had originally to be content with whole serum or the total globulin fraction of serum for passive sensitization and we now know that this must have produced mixed reactions which were often misleading. The use of purer fractions in recent years has greatly clarified the situation and has shown that complement-requiring γ_2-globulins act quite differently from human reaginic antibody, whereas γ_1-globulins have much in common. Further fractionation and characterization of γ_1-globulins is still necessary, and a method for separating reaginic antibody without the use of Zn^{++} would be welcomed. We are now in an intermediate stage; enough progress has been made to show what has to be done, but we still have to find out how to do it, and we may not be sure that it has been done properly even then. Fortunately, methods in immunochemistry and protein separation are advancing quickly and by the time these remarks are read, it is to be hoped that they will already be out of date. There is now evidence that in the guinea-pig different antibody globulins are responsible for the activation of each of the major mediator substances of anaphylaxis (Stechschulte *et al* 1967). Differentiation in other species may be anticipated.

The next variable might be headed 'intermediate reactions', and this would include the activation of enzymes. If complement is activated, as in reactions of Arthus type, the range of active enzymes and products will be wide, but practically independent of the site in the body. Reactions involving principally reaginic or γ_1-antibodies must be largely dependent upon the cells to which these antibodies are fixed. Enzyme activation could result from changes in ionic environment or the effects of co-enzymes or unmasking of active groups. Whilst there must be a general scheme of enzymes in all living cells, the special

functions of each type of cell make it safe to assume that the pattern of enzyme activation will vary from one tissue to another.

The next phase concerns pharmacologically active mediators and the number and quantity of these will obviously depend both on the stores or precursors of such mediator substances, and on the effectiveness of the enzymes able to free or form them. The range and persistence of the active enzymes will thus determine in very large measure the severity and the characteristics of the whole reaction.

Finally, the mediators (possibly with some contribution by the intermediate reaction and also some from the primary membrane reaction) produce the clinical picture. Here the difference between tissues will largely be one of physiology, that is, the range of possible effects which a mediator *can* produce in one particular tissue, for example skin or gut or bronchiole. In addition, there will be modifying factors such as the proximity between susceptible cells and the active mediator, and the rate of destruction or dilution of the mediator in the tissue spaces. In the case where mediation is by polypeptides, the active level may rise or fall very sharply, since it depends on the minute-by-minute rates of formation and destruction. In most other instances, release or formation is apparently fairly rapid, and destruction thereafter reduces the level progressively. A less obvious factor which may limit the range of the effect of a mediator, but incidentally prolong its local action, is the adsorption of the substance close to its site of formation. In sum, there would be great differences between the observed reactions in different tissues even if the same amounts of the same mediators were freed in each. With the increasing number of known mediator substances, the range of effects becomes correspondingly multiplied and the overall picture infinitely more complex.

FINAL MEDIATORS OF TISSUE REACTIONS IN ANAPHYLAXIS

We may reasonably assume that a substance is mediator of an effect observed in a sensitized tissue challenged with antigen if the following points can be demonstrated:

1. That addition of the substance can mimic the effect.
2. That the substance is present at an effective level in the tissue at the time of the reaction.

Additional evidence, which might be acceptable instead of 2, would be:

(*a*) Abolition of the effect by a moderate concentration of an antagonist possessing a high degree of specificity.

(*b*) Reduction of the effect by depletion of the substance or its precursor in tissue (or if blood-borne, in the whole animal).

The exclusion of any particular substance from a reaction is much more

difficult, and may well be deduced from clinical experience rather than experiment.

Histamine

There are several useful reviews of the reasons for connecting histamine with anaphylaxis (e.g. Dragstedt 1941; Dale 1950, 1952; Feldberg 1961). The first intimation of such a connection was by Dale & Laidlaw (1910), yet the final clear connection with clinical practice did not come until the work of Schild *et al* in 1951. This work was an application of the classic methods of pharmacology to prove that histamine was set free when living bronchioles and lung tissue from human asthmatic subjects were challenged *in vitro* by the various specific allergens. This work showed clearly that histamine was released from human tissue *in vitro* in the same experimental procedure as had been previously used for guinea-pig tissue, but the failure of these workers to find any additional substance led them to attribute an important role to 'intrinsic histamine', which we would not now accept (see later).

The well-known pharmacological actions of histamine are as follows:

1. Contracts many smooth muscle organs including human bronchioles.
2. Given locally (e.g. by iontophoresis or intradermal injection) causes an increase of vascular permeability and constriction of small venules leading to oedema. Larger amounts cause spasm of both arterioles and venules of the capillary bed.
3. Intravenous infusion in man, causes headache (with a sensation of pressure) and flushing of the face, with some fall of carotid blood pressure and some tightness of the chest. There is a considerable increase of acid gastric secretion. The release of endogenous histamine by chemical releaser substances causes similar effects (Lacomte 1957). The rise in haematocrit reading indicates loss of fluid from the circulation into the extravascular compartment.
4. Increases nasal and lacrymal secretions and is thought to increase secretion of bronchial glands.

Thus the local release of histamine could easily be the cause of urticarial conditions, hay fever and angio-neurotic oedema, and also of the bronchospasm seen in acute anaphylactic shock.

Antihistamine drugs

The clinical conditions listed above are effectively controlled by potent modern antihistamine drugs in very many cases, and this fact directly implicates histamine and, moreover, suggests that it is the sole major causative agent. Unfortunately, this conclusion is not well founded, because the antihistamine drugs are not entirely specific, and are usually used near the highest tolerable level, so that their other actions may be responsible for some part of the clinical improvement. This likelihood is strengthened by the facts that experimentally the

anti-allergic potencies of drugs do not follow the order of their anti-histaminic potency, and that many drugs successful clinically are not the most potent anti-histaminics, but have numerous other actions. These unspecific actions include local anaesthesia, atropine-like activity, antagonism of 5-hydroxytryptamine and reduction of tonus in certain smooth muscle preparations. The most general of these is the local anaesthetic potency, which is often as great as that of xylocaine. This may be the cause of sedation experienced as a side-effect, and could also help to account for the reduction of pruritis and the axon reflex flare in skin reactions. The other properties, such as antagonism of acetylcholine, vary enormously between different drugs. It is clearly probable that such a special property may be particularly useful to supplement the antagonism of histamine in treatment of the symptoms of a particular type of allergic reaction, depending on the site or the tissue involved.

The physiological role of histamine

It used to be thought that in spite of its ubiquity histamine had no physiological role except in gastric secretion. It is now recognized that the release of histamine locally in trauma or other forms of tissue damage assists in the mobilization of phagocytes (Goszy & Kato 1957) and stimulates or facilitates tissue repair. The latter effect may be indirect, the important factor being the release of ground-substance material from the mast cells; however this may be, the depletion of histamine stores, or the local application of antihistaminics, retards healing and gives a weaker repair after incisions (Boyd & Smith 1959; Kahlson 1960, 1962). These observations may indeed offer a teleological explanation of how histamine comes to be involved in anaphylaxis. The mechanism begins as a line of defence, with products giving physiological advantages, but when grossly magnified the same process can cause grave distress to the body by an excess of those same products. One may therefore question the desirability of using antihistamine drugs continuously over long periods, but in acute allergic manifestations *excess* histamine is released, and it is logical to counteract its effects by specific drugs. Antihistamine drugs alone may not be enough to suppress all symptoms because histamine may be only one of several active agents, as will be shown later.

If there was not already wide experience showing the safety of antihistamine drugs, we would have misgivings about using them, since the work of Kahlson and of Schayer (see later) has shown that histamine is formed locally in response to physiological demand, as in the foetus and in granulation tissue.

Histamine Metabolism

The importance of histamine must be considered in the light of evidence regarding the dynamics of histamine release, destruction and resynthesis. These findings are mainly to the credit of Schayer (1959, 1961), who has developed an isotope-labelling technique to follow these physiological events. For instance,

intravenous ^{14}C histidine is decarboxylated *in vivo* to give ^{14}C histamine, which can then be estimated quantitatively after repeated recrystallization with carrier histamine. The advantages of the technique are that physiological amounts are used, and the animal is not subjected to stress, restraint or trauma, quite apart from the usual advantages that labelled material can be followed with certainty and considerable quantitative precision. Furthermore, the final products of metabolism can be separated by chromatography and identified by parallel comparison with known materials, they can then be quantitatively estimated by the ^{14}C label, and this can also show the presence of unknown end-products. Schayer's important results include the following:

Excretion

Histamine ingested in food or formed in the gut by bacteria, is all excreted, much of it being deaminated in the wall of the intestine before it even reaches the blood stream. The excretion products vary greatly with species. In man the urine contains imidazol-acetic acid (IAA), methyl IAA, and IAA riboside, showing that deamination by mono-amine oxidase, before or after conjugation, is the main metabolic pathway. Very little is excreted unchanged, and all of this has been formed in the body.

Storage

Histamine which is stored in mast cells, and which constitutes nearly all of the content of the tissue, is formed from histidine, and is presumed to be formed in the cell. The turnover of the stored histamine is slow because this seems to be an emergency supply, but once freed this histamine is treated as extrinsic and is not returned to storage, but detoxicated and excreted, in the same way that injected histamine is handled in the body.

Utilization

Many tissues (and possibly all), have a fairly high turnover of histamine formed in or near the vascular endothelium by histidine decarboxylase. This is not stored, and the rate of destruction is high, therefore it contributes little to the amount of histamine extracted from tissue. The activity of histidine decarboxylase is increased as a result of stress (including cold) and any traumatic or chemical or other histamine-releasing process to which the animal is subjected. Kahlson has shown that it is present in high concentration where growth is rapid, e.g. regeneration of tissue, wound healing, foetal development and malignant tissue. Schayer considers that it is probably a local regulator of circulation at the level of the capillaries, thus reminding us of the views held by Dale and Lewis at least 40 years ago. Kahlson's work shows that an enormous increase of histidine decarboxylase occurs in tissue undergoing rapid growth before adequate vascularization has occurred, suggesting that nutrition by increased lymph flow may be controlled by histamine acting as a 'local hormone'.

Histidine decarboxylase

When tissue is repeatedly subjected to histamine release, as in repeated trauma, or frequent treatment with chemical releasers, the histidine decarboxylase rises during the resynthesis and restoration of histamine stores, and continues higher than normal afterwards (Schayer 1960). Since repeated histamine release is inevitable in all allergic states it seems likely that allergic tissue will have abnormally high histamine synthesis and a correspondingly raised level of active histamine continually present. Unless tachyphlaxis occurs, such tissue would be expected to have a lowered threshold of response to histamine from mast cells or other extrinsic sources, and might also be expected to inactivate the extra histamine at a comparatively slow rate. This would offer an explanation of the many reports that asthmatics respond to lower doses of histamine and other substances than do non-asthmatics, or, correspondingly, give a response where normal subjects do not.

Possible action of glucocorticoids

The striking anti-allergic action of the glucocorticoids is probably due to many separate effects, which include the increased stability of many membranes such as those of endothelial and smooth-muscle cells and of lysosomes, as well as a general reduction of enzyme activity. It is also known that the levels of tissue histamine fall, that tissue repair is slowed and that antibody synthesis is reduced under the influence of the anti-inflammatory corticoids. Depression of enzyme synthesis by glucocorticoids would produce particularly striking changes where the symptoms were enhanced because an enzyme was abnormally active, as postulated regarding histidine decarboxylase in chronic allergic states. This is, of course, only one of several possible ways in which the corticoids might act. Two obvious ones are, firstly the suppression of the enzymic events triggered by the union of antigen with antibody and leading to the release of active substances, and secondly the decreased reactivity of all tissues to the active products produced.

Slow-Reacting Substances

Occurrence and role

There is remarkably little of clinical interest to add to the author's 1962 review of SRS-A (slow-reacting substance of anaphylaxis). The chemical structure of this substance is not yet known, and its clinical significance remains uncertain. Nevertheless, there is a strong case for believing that in asthma this substance may be of great importance in the production of prolonged bronchospasm.

There are many 'slow-reacting substances'—the term simply implies a material of unknown composition which can cause the relatively slow contraction of an isolated smooth muscle preparation. The one now called SRS-A was first noted by Kellaway & Trethewie (1940). They found that when the lungs of

a sensitized guinea-pig were perfused through the vascular tree with Tyrode solution at 37°C, anaphylactic bronchospasm was caused by adding antigen to the solution and at this time biologically active substances were detectable in the effluent perfusate. When tested on the guinea-pig ileum, the perfusate caused a contraction which resembled that produced by histamine, but the subsequent relaxation was much slower than that after pure histamine. The main contraction was attributed to histamine, and the persistent late effect to a subsidiary contraction caused by a 'slow-reacting substance'. Brocklehurst (1956) unmasked the SRS by the use of antihistamine drugs, and by parallel quantitative assay against bradykinin, substance P, and 5-hydroxytryptamine showed that it differed from them. As the pattern of pharmacological activity compiled from tests on many different tissues did not correspond to that of any substance previously described, the suffix -A was added to indicate that this was a particular slow-reacting substance associated with anaphylaxis. Even in relatively large doses, it fails to cause the contraction of many types of smooth muscle, but quite small doses cause bronchoconstriction in isolated human bronchioles. This contraction is not inhibited by mepyramine or clinically tolerable levels of atropine. Thus the occurrence of SRS-A could explain the failure of antihistaminic drugs in allergic asthma. It can also account for the mepyramine-resistant component of the anaphylactic reaction of the isolated bronchioles from an asthmatic patient, which was observed by Schild *et al* (1951), but attributed to 'intrinsic histamine'. SRS-A is formed in many tissues, notably in lung and blood vessels, as a result of an antigen-antibody reaction in the tissue itself. *In vitro* the greatest amount is formed in the first 5 minutes after sudden brief exposure of sensitized tissue to antigen, but it it still detectable 1 hour later. When excess antigen is left in contact with the tissue the release of SRS-A is more prolonged. Little or none can be extracted from unchallenged sensitized tissue.

SRS-A obtained by perfusion of human asthmatic lung taken fresh after pneumonectomy for carcinoma of the bronchus (and in other cases from tissue fragments of asthmatic lung) has been compared with the SRS-A from guinea-pig, which is the usual source. In purification and pharmacological tests the two are indistinguishable. A table indicating the relative importance of histamine and SRS-A in asthmatic bronchospasm can be calculated from the actual amounts of each coming from the tissue, and the ratio of the equi-active doses on isolated human bronchioles. For instance, if during one particular minute 4 μg histamine and 400 units of SRS-A are found in the effluent perfusate, and it takes 0.2 μg and 4 units respectively to produce equal contractions of the chain of bronchioles, it follows that we have twenty doses of histamine and 100 doses of SRS-A set free during the same minute. The results of such calculations show that only during the first 3 or 4 minutes after antigen is histamine present at more effective levels than is SRS-A, and that 5 minutes later, the response to SRS-A is twice that to histamine, and by 30 minutes is six times as great. There are other considerations,

such as the very persistent effect of SRS-A, and suggestive evidence that it is adsorbed in tissue near the site of its formation, which make it likely that the relative effectiveness of SRS-A is even greater than these values suggest. There is no good evidence yet for or against the participation of SRS-A in any reaction other than asthma, but it would seem to be a likely factor in food allergy at least, because the gut (of guinea-pig) will both form and respond to SRS-A. Marquis (1967) has recently found that three or four rather large doses intravenously produce lethal cardiovascular effects in the rat and the guinea-pig. The rat shows great congestion and haemorrhage in the duodenum, similar to that seen when rats die from anaphylactic shock. There is only little congestion in the intestine of the guinea-pig, but the rise in thoracic venous pressure seen prior to death shows that there is either a toxic effect on the heart itself, or an increase in pulmonary vascular resistance leading to right heart failure, which is the classic picture of anaphylactic death in the rabbit.

Chemistry

SRS-A is an acidic substance, very soluble in water. It readily associates with lipids, and the complex then acquires some degree of solubility in organic solvents. After purification it shows a marked tendency to adsorb on glass or agar or any solid medium, and for this reason can best be separated by electrophoresis using a density gradient. It has resisted destruction by a wide range of enzymes and is only slowly destroyed at 20°C and pH 2 or pH 13, or by boiling at pH 7.5. It is quite quickly destroyed by organic peroxides and by bacterial action. The methods for purification of SRS-A have utilized extraction in ethanol containing some water, which excludes the protein and some lipids and salt, solubility in ether from weakly acid solution, or adsorption on to charcoal, silicic acid or alumina. Usually a combination of these methods is employed (Änggard *et al* 1963; Brocklehurst 1962; Marquis 1967; Collier & James 1967). Thin-layer chromatography of partly purified SRS-A on silicic acid has shown that the activity is often associated with lecithin, and that after treatment with α-phospholipase the activity remains with the lysolecithin (Änggard *et al* 1963). Other methods of purification and chromatography give a product in which phosphorous cannot be detected but some lipid constituents are present (Marquis 1967). It is possible to assay less than 0.1 μg per ml of purified material on the isolated guinea-pig ileum, which is the most sensitive test preparation. SRS obtained by perfusion of the cat's skin with the histamine-releaser compound 48/80, and the 'SRS-A' found in the peritoneal washings of the rat or guinea-pig following a local antigen-antibody reaction, have both been compared with authentic SRS-A from lung, by the most discriminating chemical purification and pharmacological tests available, and no difference has been observed. It must be concluded that SRS-A can be formed by mild tissue damage other than allergic shock.

Inhibition

As yet no studies have been reported with reasonably potent and *specific* inhibitors of SRS-A, but all the bronchodilators in common use are effective. It is notable that as soon as short-lived bronchodilators (e.g. adrenaline) are metabolized or washed away, the SRS-A contraction returns. Calcium exerts a definite but limited antagonism *in vitro*. Homochlorcyclizine is only a weak antagonist, and has many other properties (Fisherman *et al* 1960): flufenamic acid is a moderately potent antagonist, but has general anti-inflammatory properties (Collier & James 1967). Drug-screening schemes now often include tests for SRS-A antagonism, so the development of effective drugs may be expected. It is said that some promising compounds exist already, so full investigation of the pharmacology of SRS-A should soon be possible.

5-Hydroxytryptamine (5-HT: Serotonin; Enteramine)

There is no direct evidence connecting 5-HT with human anaphylactic conditions, although it is important in some species. For some time it was thought that 5-HT might be the cause of asthmatic bronchospasm in man because 5-HT given as an aerosol was bronchoconstrictor in guinea-pig, dog and cat, and sometimes in man. The results of recent trials of new drugs having strong anti-5-HT action show that this property does not suppress asthma.

Bradykinin

Bradykinin is a basic nonapeptide. It has been known for many years that when the pseudoglobulin fraction of plasma is digested with pure trypsin, bradykinin (or kinins of similar chemistry and pharmacological properties) is formed, and that chymotrypsin will degrade the peptide further and destroy its biological activity (Rocha e Silva 1951). The substrate (bradykininogen) has been located in the α_2-globulin fraction. Plasma, which contains both the substrate and the precursors of suitable enzymes, can be activated by glass so that bradykinin is formed and later destroyed (Margolis 1960). Bradykinin is the best known and most fully characterized of a group of similar substances referred to as plasma kinins (Lewis 1960; Rocha e Silva 1960, 1963). This substance is formed *in vitro* from plasma following contact with glass or processes associated with clotting or by treatment with trypsin or certain proteolytic venoms. It causes contraction of many, but not all, types of smooth muscle, is vasodepressor, causes increased vascular permeability, and is associated with increased secretion of sweat and salivary glands (Schachter 1959).

Participation in allergic reactions

Considerable fluctuations in the protease-antiprotease balance of blood during anaphylaxis have been recognized for many years, and it seemed highly probable

that bradykinin would reach effective concentrations at least in some organs. Detection presents some difficulty because of the ease with which bradykinin may be formed inadvertently by the experimenter.

In recent years there has been clear evidence that kinins are formed during anaphylactic shock in animals and allergic asthma in man (Beraldo 1950; Brocklehurst & Lahiri 1961; Sicuteri & Periti 1963; Jonasson & Becker 1966; Spragg *et al* 1966) and further evidence that it contributes significantly to the symptoms of shock in rats and guinea-pigs at least.

The enzyme which forms bradykinin is derived from tissue such as lung and skin but apparently not from the blood, and whilst the antigen-antibody reaction frees this activity, histamine does not. There may be several such enzymes including one found in leucocytes. Since the precursor of bradykinin is present in the plasma as a small globulin, some at least must be present in lymph and this amount will increase with raised vascular permeability. Release of suitable enzymes from cells damaged by an allergic reaction or resulting from activation of complement is therefore virtually certain to form bradykinin, but the production of symptoms will depend very much on the rate of destruction. We know that destroying enzymes must normally be present, because intravenous injection of bradykinin produces only short-lived reductions in blood pressure or increased vascular permeability. Destruction by kininases in the lung is very striking, but the distribution of this activity in most tissues, and the effect of allergic reactions on relative rates of formation and destruction, are unknown. In human skin, bradykinin causes redness, oedema and itching, it also stimulates pain receptors (Armstrong *et al* 1957). When given by aerosol it produces bronchoconstriction in the guinea-pig and in man (Collier *et al* 1960; Herxheimer & Stresemann 1961). During asthma the increase of vascular permeability and the possible stimulation of the bronchial mucus glands may be particularly important.

Acetylcholine (ACh)

The inclusion of this substance among the transmitters is prompted only by the need to explain the usefulness of atropine, because ACh has never been implicated directly in any allergic reaction in tissue. For instance, it is not found in the effluent from isolated physostigmine-treated lung during anaphylaxis. Parasympathetic nerves supply the secretory tissue of the respiratory tract and stimulation of the vagus causes bronchoconstriction. It must be assumed that secretion is controlled and smooth muscle tone is maintained by normal parasympathetic activity associated with sensory endings in the lung. There is no doubt that stress, whether physical or psychological, can unbalance the autonomic nervous system, and it has been reasonably assumed that vagal activity increases during an asthma attack. The use of atropine will suppress both bronchial tone and secretion to subnormal levels and will also abolish any

effects which parasympathetic over-activity would otherwise cause. This is rational therapy, but does not mean that ACh is a product of the hypersensitivity reaction. It thus seems to be unwise to study the effects of ACh as a model of asthma, because even if the pattern of response is nearer to the natural picture than that produced by any other substance, all the available evidence shows that the underlying pharmacology is quite different, and the effectiveness of various treatments will differ correspondingly.

The Prostaglandins

This group of unsaturated hydroxy aliphatic acids has been extensively studied in the last 5 years and found to be widely distributed in most tissues. Their pharmacological action is strongest on the smooth muscle of the intestine and uterus, and weak on bronchial muscle* and blood vessels. Recently a small fraction of the biological activity diffusing out of tissue subjected to an anaphylactic reaction *in vitro* has been tentatively identified with prostaglandin. At present it seems unlikely that substances of this group are important in the bronchospasm of asthma, but they could contribute to allergic reactions in the viscera.

The Permeability Factors

G_2 alpha-globulin

Since the properties of this substance were reviewed by Wilhelm in 1956, 'permeability globulins' have received little attention. $G_2\alpha$ is a blood-protein which produces a great increase in vascular permeability by an enzymic mechanism, and is only active when separated from the other plasma proteins. There is no direct evidence that it plays a part in allergic or inflammatory reactions, but there is a strong possibility that it does, especially where the permeability of blood vessels has already been altered by histamine, bradykinin, or lymph node permeability factor.

Lymph node permeability factor (LNPF)

One of the characteristics of allergic reactions is the accumulation of leucocytes. There is an influx of eosinophils to the site in chronic reactions of immediate type, and a very great influx of monocytes which roughly parallels the development of the tuberculin response. These effects can only be due to chemotaxic material produced as part of the reaction. The nature of these materials is unknown, and studies on chemotaxis are notoriously difficult (Harris 1961), but since polypeptides have been shown to possess this property, we have some justification for supposing that the enzymes active during antigen-antibody reactions form leucotaxic products from the proteins of the tissue and lymph

*Recently $F_2\alpha$ prostaglandin has been shown to contract human bronchiolar smooth muscle *in vitro* (Collier *et al.* Demonstration to the British Pharmacological Society, January, 1968).

which will vary with the site and the enzymes involved and thus give the various observed patterns of leucocyte infiltration. The monocytes themselves contain a very potent permeability factor, and there is evidence that this is important in the production of delayed reactions (discussed by Schild & Willoughby 1967). LNPF has no important biological property other than leucotaxic activity and its effect on vascular permeability. It is present in association with RNA, but is not destroyed by ribonuclease. It is antigenic, indicating some degree of species specificity. An important finding is that antibody to LNPF does not produce leucopenia, but greatly suppresses the tuberculin reaction and contact sensitivity to dinitrochlorbenzene. Local injections of salicylate or intravenous indomethacin suppress the extravascular leakage of dye caused by intradermal injection of LNPF, as they do the tuberculin reaction. The release of LNPF from monocytes may be more complicated than merely damage of antibody-bearing cells on contact with antigen, since release from tuberculin-sensitive cells by tuberculin does not take place *in vitro*. The monocytes from lymph nodes or spleen seem to be the best source of LNPF, but similar permeability factors have been extracted from liver, thyroid and other tissues. The discovery of LNPF is the first important step towards unravelling the pharmacology of the delayed type of skin reaction, and similar substances may well be involved in less clearly defined examples of antigen-antibody damage to cells.

Therapeutic Measures

Much new knowledge has been acquired in recent years concerning the details of hypersensitivity reactions, but so far none of this has provided any significant advance in therapy. In fact, there has been a tendency to use drugs having a wider range of action (e.g. antagonism to histamine, 5-hydroxytryptamine, acetylcholine, etc., and often being bronchodilators also) rather than having few and more specific actions as would seem to be the ideal. Drugs providing such an effective blanket must inevitably bring a corresponding range of side-effects. The trend probably reflects the swing away from unitarian ideas centred on histamine towards the acceptance that several agents contribute to allergic reactions, and suspicion that many remain unrecognized. Proof that this is an unduly pessimistic view must await effective antagonists against the agents at present known. The present aim should be to provide both the research worker and the clinician with such tailor-made antagonists, if possible without many other actions. The three major agents encountered in immediate-type reactions are the base histamine, the acidic substance SRS-A, and the nonapeptide bradykinin. These are all so different that a single antagonist to all three is most unlikely and thus three separate treatments will probably be needed. This is important, for it may quite well be that each of these agents separately contributes so much to the total reaction that antagonism of only one of them will provide unconvincing relief, and even antagonism of two simultaneously might still leave an

embarrassingly severe reaction. For this reason the pharmacologist will continue to have doubts until the effects of each known substance can be annulled, and the clinician must be aware that several active agents may each require individual treatment by drugs, and not be too ready to discard the drug for which specific activity is claimed, because of its inability to control the whole range of symptoms.

REFERENCES

ÄNGGARD E., BERGQUIST U., HÖGBERG B., JOHANSSON K., THON I-L. & UVNÄS B. (1963) Biologically active principles occurring on histamine release from cat paw, guinea-pig lung and rat mast cells. *Acta physiol. Scand.* **59,** 97–110.

ARMSTRONG D., JEPSON J.B., KEELE C.A. & STEWART J.W. (1957) Pain-producing substance in human inflammatory exudates and plasma. *J. Physiol.* **135,** 350.

AUSTEN K.F. & BROCKLEHURST W.E. (1961) Anaphylaxis in chopped guinea-pig lung. III. Effect of carbon monoxide, cyanide, salicylaldoxime and ionic strength. *J. exp. Med.* **114,** 29.

AUSTEN K.F. & BROCKLEHURST W.E. (1961) Anaphylaxis in chopped guinea-pig lung. II. Enhancement of the anaphylactic release of histamine and slow reacting substance by certain dibasic aliphatic acids, and inhibition by monobasic fatty acids. *J. exp. Med.* **113,** 541.

AUSTEN K.F. & BROCKLEHURST W.E. (1961) Anaphylaxis in chopped guinea-pig lung. I. Effect of peptidase substrates and inhibitors. *J. exp. Med.* **113,** 521.

BERALDO W.T. (1950) Formation of bradykinin in anaphylactic and peptone shock. *Amer. J. Physiol.* **163,** 283.

BOREUS L.O. & CHAKRAVARTY N. (1960) Tissue mast cells, histamine and 'slow-reacting substance' in anaphylactic reaction in guinea-pig. *Acta physiol. Scand.* **48,** 315.

BOYD J.F. & SMITH A.N. (1959) The effect of histamine and a histamine-releasing agent (compound 48/80) on wound healing. *J. Path. Bact.* **78,** 379.

BROCKLEHURST W.E. (1956) A slow reacting substance in anaphylaxis—'SRS-A'. *Ciba Symposium on Histamine,* p. 175. London: J. & A. Churchill.

BROCKLEHURST W.E. & LAHIRI S.C. (1961) The production of bradykinin in anaphylaxis. *J. Physiol.* **160,** 15P.

BROCKLEHURST W.E. (1962) SRS-A and related substances. *Progr. Allergy* **6,** 540.

CHAKRAVARTY N., HÖGBERG B. & UVNÄS B. (1959) Mechanism of the release by compound 48/80 of histamine and of a lipid-soluble smooth-muscle stimulating principle ('SRS'). *Acta physiol. Scand.* **45,** 255.

CHAKRAVARTY N. & UVNÄS B. (1960) Histamine and a lipid-soluble smooth-muscle stimulating principle ('SRS') in anaphylactic reaction. *Acta physiol. Scand.* **48,** 302.

COLLIER H.O.J., HOLGATE J.A., SCHACHTER M. & SHORLEY P.G. (1960) The bronchoconstrictor action of bradykinin in the guinea-pig. *Brit. J. Pharmacol.* **15,** 290.

COLLIER H.O.J. & SHORLEY P.G. (1960) Analgesic antipyretic drugs as antagonists of bradykinin. *Brit. J. Pharmacol.* **15,** 601.

COLLIER H.O.J. & JAMES G.W.L. (1967) Humoral factors affecting pulmonary inflation, during acute anaphylaxis in the guinea-pig in vivo. *Brit. J. Pharmacol. Chemother.* **30,** 283–301.

DALE H.H. & LAIDLAW P.P. (1910) The physiological action of beta-iminazolylethylamine. *J. Physiol.* **41,** 318.

DALE H.H. & LAIDLAW P.P. (1911) Further observations on the action of β-iminazolylethylamine. *J. Physiol.* **43,** 182.

DALE H.H. (1950) The pharmacology of histamine. *Ann. N.Y. Acad. Sci.* **40,** 1017.

DALE H.H. (1952) The mechanism of anaphylaxis. *Acta allerg., Kbh.* **5,** 191.

DIAMANT B. (1962). Observations on anaphylactic and compound 48/80 induced histamine release from guinea-pig and rat lung tissue *in vitro*. *Acta physiol. Scand.* **55,** 11.

DRAGSTEDT C.A. (1941) Anaphylaxis. *Physiol. Rev.* **21,** 563.

EDMAN K.A.P., MONGAR J.L. & SCHILD H.O. (1964) The role of SH groups and oxygen in the anaphylactic reaction of chopped guinea-pig lung. *J. Physiol.* **170,** 124.

FELDBERG W. (1961) Allergische Reaktionsmechanismen. *Arch. klin. exp. Derm.* **213,** 243.

FISHERMAN E.W., FEINBERG S.M., FEINBERG A.R. & PRUZANSKY J.J. (1960) Homochlorcyclizine: an antiallergic drug with multiple clinical properties. *J. Allergy* **31,** 232–6.

GOZSY B. & KATO L. (1957) *Studies on Phagocytic Stimulation*. Montreal: Therien.

HARRIS H. (1961) Chemotaxis, ed. HARRIS R.J.C. *Exp. Cell Res. Suppl.* **8.**

HAYASHI H., TOKUDA A. & UDAKA K. (1960) Biochemical study of cellular antigen-antibody reaction in tissue culture. I. Activation and release of a protease. *J. exp. Med.* **112,** 237.

HERXHEIMER H. & STRESEMANN E. (1961) The effect of bradykinin aerosol in guinea-pigs and in man. *J. Physiol.* **158,** 38P.

HÖGBERG B. & UVNÄS B. (1960) Further observations on the disruption of rat mesentery mast cells caused by compound 48/80, antigen-antibody reaction, lecithinase A and decylamine. *Acta physiol. Scand.* **48,** 133.

JONASSON O. & BECKER E.L. (1966) Release of kallikrein from guinea-pig lung during anaphylaxis. *J. exp. Med.* **123,** 509–22.

KAHLSON G. (1960) A place for histamine in normal physiology. *Lancet* **i,** 67.

KAHLSON G. (1962) New approaches to the physiology of histamine. *Perspect. Biol. Med.* **5,** 179.

KELLAWAY C.H. & TRETHEWIE E.R. (1940) The liberation of a slow-reacting smooth-muscle stimulating substance in anaphylaxis. *Quart. J. exp. Physiol.* **30,** 121.

LECOMTE J. (1957) Liberation of endogenous histamine in man. *J. Allergy* **28,** 112.

LEWIS G.P. (1960) Active polypeptides derived from plasma proteins. *Physiol. Rev.* **40,** 647.

MARGOLIS J. (1960) The mode of action of Hageman factor in the release of plasma kinin. *J. Physiol.* **151,** 238.

MARQUIS V.O. (1966) The role of SRS-A and other active substances in allergic and inflammatory conditions. Ph.D. thesis, Edinburgh Univ.

MONGAR J.L. & PERERA B.A.V. (1965) Oxygen consumption during histamine relief by antigen and compound 48/80. *Immunology* **8,** 511.

MONGAR J.L. & SCHILD H.O. (1956) Effect of antigen and organic bases on intracellular histamine in guinea-pig lung. *J. Physiol.* **131,** 207.

MONGAR J.L. & SCHILD H.O. (1957) Inhibition of the anaphylactic reaction. *J. Physiol.* **135,** 301.

MONGAR J.L. & SCHILD H.O. (1957) Effect of temperature on the anaphylactic reaction. *J. Physiol.* **135,** 320.

MONGAR J.L. & SCHILD H.O. (1958) The effect of calcium and pH on the anaphylactic reaction. *J. Physiol.* **140,** 272.

MONGAR J.L. & SCHILD H.O. (1962) Cellular mechanisms in anaphylaxis. *Physiol. Rev.* **42,** 226.

MOUSSATCHE H. & PROUVOST-DANON A. (1956) Inhibition by sulphydryl blocking agents of the guinea-pig gut reaction to anaphylaxis and to anaphylatoxin. *Naturwissenschaften* **10,** 227.

MOUSSATCHE H. & PROUVOST-DANON A. (1960). Influence de l'anaerobiose et de differentes tensions d'oxygene sur la liberation d'histamine dans la reaction anaphylactique *in vitro*. *Experientia* **16,** 118.

MOTA I. (1957) Action of anaphylactic shock and anaphylatoxin on mast cells and histamine in rats. *Brit. J. Pharmacol.* **12,** 453.

MOTA I. (1959) Effect of antigen and octylamine on mast cells and histamine content of sensitized guinea-pig tissue. *J. Physiol.* **147,** 425.

MOTA I., DIAS DA SILVA W. & FERREIRA-FERNANDES J. (1960) The inhibition of mast cell damage and histamine release in anaphylaxis by pyridine and diphosphopyridine-nucleotidase inhibitors. Comparison with compound 48/80. *Brit. J. Pharmacol.* **15,** 405.

RILEY J.F. (1959) *The Mast Cells.* Edinburgh: Livingstone.

ROCHA E SILVA M. (1951) Bradykinin—mechanism of its release by trypsin and kallikrein. *Arch. int. Pharmacodyn.* **88,** 271.

ROCHA E SILVA M. (1960) Biochemistry and pharmacology of bradykinin in polypeptides which affect muscles and blood vessels. Ed. SCHACHTER. Oxford: Pergamon Press.

ROCHA E SILVA M. (1963) Bradykinin. *Ann. N.Y. Acad. Sci.* **104,** 190–210.

SCHACHTER M. (1959) *Polypeptides which Affect Smooth Muscles and Blood Vessels.* Symposium Publications. London: Pergamon.

SCHAYER R.W. (1959) Catabolism of physiological quantities of histamine *in vivo*. *Physiol. Rev.* **39,** 11.6

SCHAYER R.W. (1960) Relationship of induced histidine decarboxylase activity and histamine synthesis to shock from stress and from endotoxin, *Amer. J. Physiol.* **198,** 1187.

SCHAYER R.W. (1961) Significance of induced synthesis of histamine in physiology and pathology. *Chemotherapia* **3,** 128.

SCHILD H.O., HAWKINS D.F., MONGAR J.L. & HERXHEIMER H. (1951) Reactions of isolated human asthmatic lung and bronchial tissue to a specific antigen. *Lancet* **ii,** 376.

SCHILD H.O. & WILLOUGHBY D.A. (1967) Possible pharmacological mediators of delayed hypersensitivity. *Brit. med. Bull.* **23,** 46.

SICUTERI F. & PERITI P. (1963) Liberazione di plasmachinine e di istamina e allergia da farmaci. *Folia Allergol* **10,** 523.

SPRAGG J., AUSTEN K.F. & HABER E. (1966) Production of antibody against bradykinin; demonstration of specificity by complement fixation and radio-immunoassay. *J. Immunol.* **96,** 865–71.

UVNÄS B. (1967) Metabolic and non-metabolic processes in the mechanism of histamine release from mast cells. In AUSTIN K.F. & BECKER E.L. *Biochemistry of the Acute Allergic reactions.* Blackwell, Oxford; see also 'Release processes in mast cells and their actuation by injury'. *Ann. N.Y. Acad. Sci.* **116,** 880 (1964)

STECHSCHULTE D.J., AUSTEN K.F. & BLOCH K.J. (1967) Antibodies involved in antigen-induced release of slow reacting substance of anaphylaxis (SRS-A) in the guinea-pig and rat. *J. exp. Med.* **125,** 127–47.

WILHELM D.L. (1956) Serum globulins as mediators of pathological changes of capillary permeability. *Proc. R. Soc. Med.* **49,** 575.

YAMASAKI H., MURAOKA S., SUGIYAMA M. & ENDO K. (1961) Mechanism of histamine-release inhibition by nicotinamide in *in vitro* antigen-antibody reaction. *Jap. J. Pharmacol.* **11,** 54.

CHAPTER 23

THE PATHOGENESIS OF ASTHMA, HAY FEVER AND ATOPIC DISEASES

A. W. FRANKLAND

ALLERGY AND ATOPY

The term 'allergy' indicates that a patient has a changed reactivity as a result of an antigenic stimulus. It became obvious that in man, allergic diseases might well include so many clinical conditions that in 1923 Coca introduced the word atopy meaning 'strange disease'. Coca later defined atopy as a 'type of hypersensitiveness peculiar to man, subject to hereditary influence, presenting the characteristic immediate whealing type reaction, having circulating antibody reagin, and manifesting peculiar clinical syndromes such as asthma and hay fever' (Coca 1923).

In Chapter 20, when dealing with classification of allergic diseases, it was pointed out that an aetiological classification based on the initial reaction of the allergens in the tissues meant a classification of mechanisms and not of diseases. A clinician is faced with a disease whose mechanism is often not understood and it is for this reason when referring to one group of allergic diseases the word

atopy seems a useful one. There are, however, many difficulties confronting us when considering Coca's definition. It may be useful to classify separately the spontaneously occurring diseases in man such as hay fever and asthma from induced states of sensitivity in the experimental animal which can be referred to as anaphylactic. We know that the sensitized anaphylactic dog, rabbit, guinea-pig and rat behave differently from each other and it used to be thought that it would be unwise to argue that atopic man behaved in a similar manner to the anaphylactic animal. It now seems that induced sensitization in man and induced sensitization in the animal are very similar, with the dog behaving clinically much more like man than the anaphylactic guinea-pig. It is easy to sensitize most animals anaphylactically. Man, too, can be sensitized by an injection. The clinical disease of 'serum sickness' caused by injection of horse serum was accurately described by v. Pirquet and Schick (Pirquet & Schick 1905). The joint pains, fever, urticaria and blood picture were so characteristic that the disease could have a name while the spontaneously occurring disease of hay fever and asthma have another general name, atopy, to describe them. It thus came about that anaphylaxis was the disease of the experimental animal, serum sickness was induced sensitivity in man and the atopic diseases occurred spontaneously in man. This classification of diseases rather than mechanisms has served the purpose of stressing that man when atopic does have peculiar antibodies present. These reaginic antibodies are present in all the tissues and in blood serum and give the characteristic wheal and flare reaction of the 'immediate' type (Type I) and can be transferred to normal non-sensitized man.

It must be remembered that skin-sensitizing antibodies can be produced in a variety of ways in normal and atopic people. We cannot be certain, however, that the reaginic antibody of the atopic is the same as the skin-sensitizing antibody of the non-atopic which follows injections from insects or of penicillin or from drugs and nematodes such as ascaris (Terr & Bentz 1965). Atopic man if he is horse sensitive will show Type I reactivity to horse scurf and often to the components of horse serum. Induced serum sickness due to horse serum can occur in the atopic and the non-atopic. It would seem that the reaginic-like antibodies formed in serum sickness occur in association with some of the symptoms of serum sickness such as urticaria, while a Type III mechanism in which antigen-antibody complexes are formed gives rise to the other symptoms of serum sickness of leucopenia, joint pains and lymphadenopathy (see Chapter 24). Now that it is possible more accurately to characterize the reaginic antibodies, it should be possible to study what happens in an atopic patient who is not sensitive to horse serum but is made so by injection of horse serum albumin. Stanworth (1963) points out that the 'horse asthmatic' can often react violently to the administration of horse serum fractions, whereas patients who have serum sickness do not become sensitive to horse dander. It is on such clinical observations that the immunologists are able to build tentative theories about reagin

formation. But as minor antigenic components are probably responsible for the formation of reagin-like antibodies, there seems hardly any justification to come to definite conclusions when highly pure antigens are not available. Ortiz (1966) has described how he became sensitized to rabbit serum when carrying out passive transfer tests on himself.

Hay fever arising spontaneously in animals is rare, but it does occur (Wittich 1941). Dogs who have hay fever have positive skin tests to pollen and react with urticaria, sneezing and wheezing towards pollen in a similar manner as does man. Moreover, Patterson & Sparks (1962) have shown that it is possible to transfer to the skin of a normal dog a wheal and erythema sensitivity by means of a heat-labile factor in the serum of a dog with spontaneously occurring pollen sensitivity. Experimentally, Feinberg & Chopra (1966) have been able to induce a condition not unlike serum sickness but which they call the quasi-atopic state in the guinea-pig. We may conclude that atopic man may not after all be so very different from animals in whom similar complaints also may arise spontaneously or can be induced.

Local as well as general anaphylaxis has been known to occur in sensitized animals since the classical experiments of Arthus in 1903. Whether the Arthus-type phenomenon ever forms the basis for clinical syndromes in man has not as yet been fully investigated. Perhaps such an antigen(s) as produced by *Aspergillus fumigatus* can produce in different clinical conditions reaginic antibodies and/or circulating precipitating antibodies with local vascular damage. It would seem that any organic dust can give rise to a Type III response in the lungs of susceptible patients (Pepys *et al* 1964) (Chapter 36).

There might be ways of distinguishing the 'allergic' from the 'non-allergic' patient other than looking for specific antibodies. Laborde *et al* (1953) suggested that 'allergic' patients were unable to bind histamine in their sera like 'non-allergic' individuals. This power of serum to bind histamine they called 'histaminopexy', but Kirtchev & Frankland (1965) were unable to confirm this finding when comparing 'allergic' and 'non-allergic' individuals.

Hereditary Factors

In 1916 Cooke and Van der Veer and subsequently many other authors have stressed the hereditary nature of the allergic diseases. Yet there seems to be no good evidence that the atopic diseases are inherited. Sometimes there does seem to be very suggestive clinical evidence of inheritance of the atopic diseases and perhaps Schwartz's (1952) evidence is so far the best available to suggest that asthma is an inherited disease and that there is a genetic relationship between asthma and vasomotor rhinitis. Also he found that Besnier's prurigo and hay fever (seasonal) were genetically related to asthma. Urticaria and angioneurotic oedema were genetically linked in females. At present it is impossible to fit in any genetic hypothesis to the existing data. The association between atopic

eczema and respiratory allergy (asthma and hay fever) seems well substantiated from many workers' observations, including those of Ratner & Silberman (1953), who found that 59% of children with atopic eczema later have respiratory allergies. Rajka (1960), in an extensive study of 1200 patients with atopic dermatitis, once more points out that it is not the allergic manifestation that is inherited but the allergic disposition. Some of the observed differences in inheritance may stem from our basic lack of knowledge of behaviour of genes. Intermittent dominance may occur because the limit between dominant and recessive genes is not very sharp since the difference between homo- and heterozygotes in certain 'hereditary' diseases in only quantitative. Rajka's data on identical twins showed considerable discordance in all respects and atopic heredity could only be demonstrated in the ancestors of about half the cases. The influence of hereditary atopic factors on the combination of atopic dermatitis with respiratory manifestation or the age of onset of the dermatitis did not seem to be greatly influenced by an hereditary atopic factor.

In a study by Schnyder (1960) of 361 families, the findings strongly support the hypothesis of a single, autosomal, dominant gene with reduced penetrance. It was concluded that sporadic cases of atopy were genetically determined. He, too, found that bronchial asthma, atopic rhinitis and different types of atopic dermatitis were genetically closely connected but that drug idiosyncrasy, contact eczema and the major part of cases of urticaria, bore no genetic relationship to the atopic group because these diseases were no more frequent in the atopic group than in normals.

In general when considering possible inherited diseases, the genetic basis may be single genes or it may be multifactorial. Environmental influences can be expected to contribute appreciably or substantially to the variation of traits whose genetics is multifactorial (Roberts 1961). The importance of quantitative as well as specific factors was well illustrated when studying the development of locust sensitivity (Frankland 1953). Insects are strong sensitizers and it was found that if an already atopic individual was put into an environment of locusts such as obtained in the insect-breeding rooms, then a specific sensitivity towards locusts developed in a few months in most patients who were 'allergy prone'. Spoujitch (1960) has shown that asthma and other allergic manifestations in individual cases and in various regions are the result of several factors, some known and some not understood. He points out that in Yugoslavia the percentage of skin-test-positive individuals is high—20 to 40% while the percentage of persons showing allergic manifestations was low—3 to 10 %. Latent allergy made into manifest allergy depends too upon an increased sensitivity of the shock organ to histamine and other mediators. Tiffeneau (1958) uses histamine and acetylcholine in inhalation tests to distinguish between asthma and other diseases causing airway resistance of the lung.

It seems, however, that as the clinicians cannot even agree on a definition of

asthma, and as all that wheezes is not asthma and all that wheezes is not allergy, it would appear to be impossible to state whether a disease which cannot easily be defined can be proved to be inherited. Unfortunately, dermatologists do not agree on a definition of eczema particularly as it affects the infant. Moreover, the typical so-called allergic nose may or may not be 'allergic'. Coca's atopic diseases hay fever and asthma thus tend to be defined in terms of the 'characteristic whealing reaction'. A definition of diseases based on the presence or absence of reaginic antibodies, in theory is excellent, but as these antibodies have not yet been characterized and as the allergens are not standardized, quite obviously difficulties will arise in any definition of a clinical disease if it is based on the presence or absence of a 'positive skin test'.

Kallio *et al* (1966) studied whether there was a familial tendency to develop skin-sensitizing antibodies to grass and ragweed pollens. It was shown that the ability to develop skin-sensitizing antibodies is familial and probably genetically determined. In such a study the ability to form reaginic antibodies was followed and not the symptoms of hay fever.

Van Arsdel & Motulsky (1949) pointed out that with a 16.7 percentage frequency of allergy in the population, if the medical background of even five members of a family is known, the probability of allergy being reported might be 60% on the basis of population incidence.

We are left with the thought that as nearly all the population can become allergic to tubercle bacilli, why does not all the population become allergic to pollen? A person is not sensitized on first coming in contact with pollen, it generally takes 3 to 30 years before clinical sensitivity develops. The requisite conditions for antibody formation seem to arise by chance but once established they persist for longer or shorter periods of time. This suggests that what occurs is either a somatic mutation which may or may not be inheritable, or that some kind of adaptation analogous to the production of drug resistance in bacteria may occur (Davies 1958).

Reaginic Antibody

While Coca originally stated that reaginic antibodies were peculiar to man, we now know that the Northwestern colony of atopic dogs have antibodies that are physicochemically similar to those in atopic man (Patterson *et al* 1963). Monkey skin and intestine can be sensitized by human reagins so that monkey but not other animals can be used in passive transfer of reaginic antibodies. The half-life of persistence of reagins in monkey skin was found to be only 30 hr, while for human skin about 14 days (Augustin *et al* 1966). It has also been found that there is a good correlation between allergen-induced histamine release from reagin-sensitized human lung or from monkey gut. Stanworth (1963) in a comprehensive review of the biological and physicochemical properties of reagins stresses the need to develop an *in vitro* method of assaying antibody activity. He

suggests that ultimately the key will be in the identification of the antigenic determinants against which the reagin molecules' receptor groups are directed. It seems likely that the carbohydrate groupings of the glycoprotein allergens will likely play a critical role. Because no *in vitro* tests have been developed, Stanworth & Kuhns (1965) have carried out a critical study of the factors influencing the P-K test. This test, it was shown, was as accurate as many other tests used to estimate other types of antibodies, but in man there always remains the possibility of transmitting infective hepatitis so it seems likely that monkeys, although expensive, will be used in future experiments. Reaginic antibody is heat labile and therefore differs from the blocking antibody that is formed during hyposensitization. Unfortunately neither the original size of the skin test nor the amount of blocking antibody that is formed following a series of desensitizing injections bears any direct relationship to the degree of clinical sensitivity of the patient.

There has been much controversy about whether the reaginic activity followed the IgG or IgA globulins. It seems likely from work by Ishizaka & Ishizaka (1966) that reaginic activity is largely associated with a unique immunoglobulin IgE. As little as 0.001 μgN was enough to demonstrate the P-K reaction. Their work does not exclude the possibility that reaginic antibody in some patients may be associated with IgA globulins. Measurement of immunoglobulins quantitatively has been used in the diagnosis of atopic eczema by Varelzidis and his colleagues (1966). It must be remembered that the skin-sensitizing antibodies occurring spontaneously may not only differ in different individuals, but they may be different from those that are induced in serum sickness or from insects, penicillin, drugs and ascaris. Now that it is possible to characterize reaginic antibodies more accurately, any such difference in skin-sensitizing antibodies, if they exist, should be possible to measure. It will be particularly interesting to see whether the already atopic individual who develops skin-sensitizing antibodies during serum sickness can form two different kinds of skin-sensitizing antibody.

Feinberg, Dewdney & Temple (1965) have shown that by injecting very small (0.01 ml) amounts of foreign serum into the skin of the guinea-pig, 7–10 days later produced a generalized erythema of the skin with oedema of the ears, eyelids, muzzle, feet and genitalia and bristling of the facial hairs. The onset of the symptoms is accompanied by a 24–48 hr rise in blood eosinophils, followed by a basophilia of similar duration. Subsequently Feinberg & Chopra (1966) have shown that it is possible to provoke positive reactions on challenging the nose or conjunctiva with the appropriate allergen. Even if the guinea-pig can have an induced serum-sickness-like disease as well as being made anaphylactic, it seems doubtful whether it is correct to use the word atopic when describing such symptoms in the guinea-pig. Attempts to characterize human reaginic antibodies have been amazingly unsuccessful in that we still have to rely on man or monkey to show their presence. Some deny that it is even an antibody and

that what we read about in Appendix B of this book only demonstrates that something in the blood of the atopic patient can be transferred.

Transference of Antibody

It is worth while considering briefly whether or not reaginic antibodies can be transferred directly from mother to young. Primates behave very differently from other animals in transmission of immunity before and after birth. We know that in rats and mice most of the transmission of immunity takes place after birth and continues until near the end of lactation. In ruminants there is no transmission before birth but antibodies are transmitted in the colostrum for the first 36 hr. Man resembles animals in showing the phenomenon of selective transmission (Brambell 1961). Many maternal antibodies, including antitoxins, antistreptolysins, etc., are found in the cord serum at concentrations equal or exceeding the maternal concentrations while other antibodies such as typhoid 'O' agglutinins are either absent from the cord blood or present in very low concentrations. Transplacental transference of γ-globulins occurs from the mother to the foetus. Antibodies associated with IgG are freely transferred while the IgM which constitutes 5–10% of the total γ-globulin and which is itself heterogeneous, does not pass the placenta. Reaginic antibodies responsible for human allergic disease also fail to pass the placenta. Certainly Brambell's studies in man show that the failure of reaginic antibodies to pass the placenta does not indicate that such antibodies are macroglobulins. All that we can say in our present state of knowledge is that potentially harmful antibodies are held up by the placental barrier by a mechanism not yet fully understood. Sherman *et al* (1940) not only showed that reaginic antibodies failed to pass the placental barrier, but noted that in two out of six colostra of allergic mothers there was a small amount of skin-sensitizing activity (1/100 to 1/1000 of P-K activity of the serum). As saliva also contains reaginic antibodies, it would seem that these antibodies can cross some cell membranes. It would seem that it must be the chemical configuration of the serum protein which ultimately decides whether transmission across foetal membranes will occur.

Fixation of Reaginic Antibody

Reaginic antibodies have the property of fixing to tissues, but we are not yet certain to which cells they become attached. They react with their specific antigen (allergen) and give rise to clinical symptoms of the atopic diseases. Unfortunately, the biological reaction that results in such a union has many qualitative and quantitative characteristics which are not understandable when applied to clinical practice. There are so many factors that determine the availability of antigen and antibody that skin testing may give many perplexing results (see Chapter 7).

Chan (1963) showed that in two subjects whose sera were apparently equally

active, as judged by PK testing, there were concentrations of 0.8 and 29 μg of reaginic antibody per millilitre of serum. The levels of blocking antibody were stated to be 1.5 and 66 μg/ml respectively, so that the ratio of blocking antibody to reaginic antibody was about 2:1 in each case. This finding may give the clue to those patients who very easily, even with low dosage of injected allergen, develop systemic generalized reactions. Although it has been noticed that at the beginning of a course of injections, the size of skin test may increase, any change in the ratio of blocking antibody to reaginic antibody has not yet been studied in detail.

Occasionally children and adults are seen who have classical symptoms of sneezing, eye irritation and asthma due to pollen and in whom, during the first few months of the complaint, have negative skin tests towards the presumed allergens. (A skin test is 'negative' when no immediate wheal and flare response is obtained towards a pollen extract, using the highest concentration of skin-testing material that normally gives a positive skin response in a patient with hay fever.) Within a few months the skin test becomes positive only to the highest concentration. Whether the patient does or does not have injection treatment the skin test will react positively over a period of 1 or 2 years to a ten- or thousand-fold increase in dilution. It does not necessarily follow that the patient becomes clinically more sensitive.

Illustrative Cases

A female operatic singer aged 24 years was seen in the summer of 1960 at the height of the grass-pollen cloud with what appeared to be her first summer of severe hay fever. The skin tests, performed when she had not recently taken any symptomatic drugs, gave a doubtful positive response to the highest concentration of mixed grass pollens. The symptoms disappeared as the pollen count diminished. Seen 6 months later, the skin test to the highest concentration of grass pollens was markedly positive. She was then advised to have a course of pre-seasonal injections. Still she had some hay fever in the summer of 1961 but was able to sing throughout the summer. Six months later in the winter, her skin test gave a positive response to a ten-fold dilution of pollen extract.

Immunologically this patient seems unable easily to fix antibodies in her skin. In other words at the beginning of sensitization, skin tests in no way could be used to judge quantitatively the clinical symptoms. More commonly the converse is seen when clinical symptoms have disappeared but positive skin tests remain.

A male patient aged 44 was first seen in 1947 with marked symptoms of seasonal hay fever. The symptoms corresponded to the pollination of the grasses. He had positive skin tests to all the pollens of grass, trees and weeds against which he was tested. Grass-pollen extract was used in the first year of injection treatment. For the second and third year of injection treatment he was given a mixed extract of the appropriate grass and weed pollens. The patient's symptoms during the summer improved so that after 3 years of injection treatment it was decided to use symptomatic treatment only. He has been followed up each year since and slowly his symptoms have become less severe. Yet his skin tests are as marked as they were in 1948.

THE SHOCK ORGAN

The placenta shows selective transmission of antibodies and clinically these antibodies show a selective organ fixation. Normally the 'shock organ' is at the site of entrance of the allergens, but sometimes the shock organ occurs at an unusual site. Grass pollen in the air typically causes, in a sensitive patient, eye irritation, rhinorrhoea and sneezing. Antibodies are fixed in these tissues so that antigenic pollen causes an immediate reaction at the site of entry. There may be local urticaria from pollen on the exposed skin if the skin is abraded. Some patients during the height of the season are going to develop asthma due to their sensitivity. These patients do not normally grow out of their sensitivity without injection treatment. We have no means immunologically of distinguishing these patients from those who are not going to get asthma and who after a few years normally grow out of their complaint. Statistically if a patient has had hay fever for 15 years or more, the symptoms are going to persist or develop into asthma if this has not already occurred. Clinically the pollen asthmatic is 'more sensitive' but immunologically we cannot distinguish the hay fever patient with or without asthma.

We will be discussing in some detail later the clinical conditions that occur in the atopic diseases. It is to be expected that a food would cause symptoms in the gastro-intestinal tract but sometimes according to Rowe & Rowe (1963) the gastro-intestinal tract may have become the main shock organ for an inhalant allergen. It is a not uncommon clinical experience that patients who are sensitive to the inhalant yeasts, e.g. sporobolomycete, are quite unable to eat gravy made with a yeast extract (Marmite, etc.) The patient may have an immediate uncomfortable feeling in the mouth but normally the symptoms that occur following ingestion are those of asthma. The acutely fish-sensitive atopic patient develops swelling of the mouth when a mouthful of fish is taken. If swallowed, the throat swells and there is gastric mucosal oedema and vomiting. If the food is able to pass the pylorus, abdominal cramps and a watery diarrhoea occur. Inhalant symptoms may occasionally arise when an acutely sensitive patient passes a shop where fish is being fried. There is no necessity to postulate that egg protein in the milk of the breast-fed baby sensitizes it. The baby must have met many millions of molecules of egg protein when its mother has used them in cooking, long before coming across the first egg yolk (or white) as a food. When the skin is the shock organ of an allergic reaction, 'generalized' urticaria occurs, yet it seems strange that the urticaria remains amazingly localized in different parts of the body. Also if swelling of lips or eyelids occurs, sometimes only one lid or half of one lip is involved. There is no easy explanation for this very localized oedema.

McKee (1966) when investigating the familial occurrence of allergy found that patients with seasonal allergic complaints were more susceptible to the common

cold than the non-atopic individual. He postulated that the allergic shock organ showed some difference in susceptibility of the mucous membrane to infection. It is notoriously difficult for an allergic patient to distinguish between an allergic rhinitis and an infective cold and for this reason McKee's postulation may have arisen from faulty symptomatic recordings from patients. Indeed, the patient with seasonal pollinosis in spite of gross hypertrophy of the nasal mucosa, rarely has an infective sinusitis. It would seem that the atopic may even develop less, rather than more, chances of local infection. This is in complete contrast to the group of patients who have nasal polyposis with or without asthma who are non-atopic, and who often have infective sinusitis.

SENSITIZING ANTIGENS

There is no method to distinguish between a pollen that is very allergenic or one that is hardly ever allergenic. In London more pollen is shed from plane (*Platanus*) trees than is shed by the grasses (Hamilton 1959). Yet of 2930 cases of pollinosis investigated in a London clinic (Frankland 1955) only 0.2% were clinically tree-pollen sensitive. In contrast I have one patient whose only sensitivity by skin testing and clinically is to plane pollen, another who only reacts to plantain (*Plantago*) and another who only reacts to nettle (*Urtica*) pollen with clinical symptoms. The conifers produce vast quantities of buoyant pollen, yet pine pollen normally does not cause clinical allergy. The cypress (*Cupressus*) and juniper (*Juniperus*) may cause allergic symptoms. Sometimes patients are seen who clinically and by skin tests give positive responses to the pollens of all of one family, e.g. the catkin-bearing trees (*Amentaceae*). Such patients may get symptoms from February onwards when the hazel (*Corylus*) begins to flower until the summer time. Some of these patients clinically may not be sensitive to the common sensitizers like grass pollen. Sometimes a patient has positive skin tests to many pollens of one family but clinically is sensitive to only one. I have one such patient who has symptoms in April up to the beginning of May who clinically is only sensitive to silver birch (*Betula*). With the help from the daily pollen counts which are carried out at the Wright-Fleming Institute by the Allergy Department, it is often quite easy to pick out the clinical sensitivity of patients who keep a daily record of their symptoms, with very little reference to their skin tests. The skin in an atopic individual is a very sensitive test organ in that dilutions of 10^{-12} can give a specific response, but quantitatively the skin test is grossly inaccurate. It cannot be over emphasized that until we have standardized allergens and have standard methods of performing skin tests with such allergens it will be impossible to characterize reaginic antibodies. Ultimately we have to use skin tests as the means of identification of allergens and antibodies because as yet we have no *in vitro* method whereby reaginic antibodies can be identified.

CLINICAL SYNDROMES

Atopic Eczema

This condition generally starts about the age of 6 months, often beginning on the face and later on has a predilection for the flexor surfaces of the limbs. Sometimes the disease disappears at the age of about 2 years only to reappear especially at puberty. Sometimes there is no tendency towards spontaneous recovery.

This itching and often oozing complaint is ill understood by the dermatologists who refer to it by names other than atopic dermatitis. Constitutional eczema, flexural eczema, neurodermatitis, diathetic eczema, Besnier's prurigo and atopic dermatitis are all names for a skin condition which is easily influenced by many factors some of which are causal and many only aggravating. Positive skin tests are frequently obtained to foods and inhalants but the allergic management of atopic eczema is disappointing (Frankland 1958). It will be found that over half the infants with the complaint will give positive skin tests to egg. Most of these children will not eat eggs. When forced to eat a boiled egg, vomiting occurs followed by diarrhoea. The lips and tongue and throat become swollen, asthma occurs and there may be a generalized nettle rash. A mother who first gives her baby an egg will often try a little egg yolk on a spoon. It is so often this egg yolk that produces alarming symptoms of a generalized allergic reaction. Generally egg-white sensitivity remains for a longer time than egg-yolk sensitivity. This may be the reason for the often repeated statement that it is the egg white that causes the egg sensitivity. Mothers who have allergic babies will often testify that they know that egg yolk causes trouble. In the same way that patients can have general patterns of sensitivity, individuals so often have their own specific pattern. Examples of this have already been given with the tree pollens. So far as foods are concerned an example can be quoted of identical twins; both are allergic—this in itself is unusual (Frankland 1958), but what is more unusual is that one can eat egg yolk and one can eat egg white.

In a food sensitivity it is sometimes quite easy to denature the antigen by cooking. The egg-sensitive child can, if very sensitive, not eat anything containing egg. Later on the usual tendency is towards lessening of sensitivity so that eggs when well cooked (denatured) in cakes, puddings, etc., can be enjoyed without any symptoms. Later still egg can be eaten if fried or scrambled but not if lightly boiled. Egg sensitivity persisting or developing in adult life is rare, but recently two asthmatic women aged 49 and 50 years were seen who had both developed sensitivity to egg yolk. Clinically and on skin testing this was their only sensitivity.

Some food sensitivities remain throughout life. Perhaps the commonest foods causing acute allergic trouble in adults are nuts and fish. Some patients find themselves sensitive to one fish only, more usually the sensitivity is spread to include all fish. However, if the fish is autoclaved it is often no longer

antigenic, so tinned fish, e.g. salmon, but not cooked fresh salmon, can be eaten. The original experiments of Prausnitz & Küstner concerned the transfer of fish sensitivity (see Appendix B). Although 70% of patients with an atopic eczema have reaginic antibodies present, patients know that exacerbations of eczema are more likely to follow emotional disturbances, climatic changes or sweating rather than from some allergic insult. The stigmata of atopy rather than allergy are found in these patients. There is a tendency to vasoconstriction of the skin capillaries so that the skin always appears white. Children and adults have such white faces that they are often wrongly thought to be anaemic. When the skin is firmly stroked a white and not a red line appears in many of the atopic eczematous patients. This white dermographism is very easy to demonstrate and a similar paradoxical vasoconstriction occurs after an intracutaneous injection of acetylcholine (Lobitz & Campbell 1953).

The prelude to the wheeze is the sneeze, the prelude to the sneeze is often atopic eczema. Reaginic antibodies are found in all these complaints as suggested in Coca's definition of atopy. Unfortunately, atopic eczema is only rarely successfully treated using allergic management. There seems no doubt that, when urticaria is produced in a patient with an eczematous tendency, the result of urticaria irritation is an exacerbation of the eczema.*

Urticaria and Angioneurotic Oedema

These dermatoses have the same aetiology and the same pathogenesis often alternating in a patient. The intensely irritating urticaria can occur anywhere on the body, appearing and disappearing in minutes or hours. Sometimes the attacks occur daily for months, but often they have an unexplained periodicity recurring at short intervals. In angioneurotic oedema the lesion is non-pruriginous, but like urticaria its appearance is often sudden and transient. There may be involvement of eyelids, lips, tongue and larynx. Even if the explanation were that the swelling occurs in tissues with most mast cells and therefore in a situation of high tissue histamine, this does not explain the unilateral localization of the swelling to one eyelid or to one lip.

Non-Reaginic Urticaria

There are many causative factors which may be involved in urticaria, but unfortunately it is often very difficult to prove by any procedures the importance of suspected factors. Often the cause has been blamed on an alimentary origin

* It seems significant that in boys with the congenital sex-linked agammaglobulinaemia with γ-globulin levels below 10 mg%, atopic dermatitis occurs without any wheal and erythema-type sensitivity. Moreover, it is impossible to induce the immediate-type sensitivity in such patients using injections of Ascaris extract. Yet a delayed sensitivity to Ascaris is developed. It may be that 'atopic eczema' involves delayed-type allergy rather than the immediate-type allergy which is so often present (Peterson *et al* 1962).

and certain foods such as milk, chocolate, strawberries and shellfish are thought to be causative. Inhalant allergens have occasionally been implicated. During the first 30 or 40 years of this century, the role of bacteria and the importance of the toxic focus were held to be of predominating importance. The role of so-called microbial allergy has never been proved although this rather loose concept is still held to be important by some workers, especially those who notice the benefit derived from removal of foci of infection, and giving bacterial vaccines. Yet, endocrine and psychological factors would seem to play a dominant role in most cases of chronic urticaria, especially when the complaint occurs in women. Sometimes physical agents such as heat and cold or mild trauma may provoke the reaction. It would seem as if cellular trauma in any form can spill out histamine and related substances in such sufferers. None of these suggested causes is based on an antigen-antibody reaction and therefore should not in any way be considered 'allergic'. Skin testing would not be of any value in attempting to find a cause. It seems that in a few cases of exercise urticaria as well as in heat, cold and light sensitivity, there is a very definite familial history of a similar complaint based on an inherited enzymatic defect and therefore fundamentally not allergic.

It is a common clinical observation that strawberries will produce an acute attack of urticaria. Yet positive skin tests to strawberries do not usually occur. There could be two reasons for this, either the antigenic testing fluid is too weak or has some unstable components present that do not allow a sufficiently active material to react with the corresponding antibody, or the oedema is not based on an antigen-antibody reaction. This seems the more likely explanation. Many adults know that they are liable to very severe anaphylactic-like attacks with gross urticaria, vomiting and diarrhoea after eating shellfish. The reaction does not always follow eating the suspected food and this observation suggests that it is not an allergic reaction. This reaction to shellfish does not seem to occur more often in those who are atopic and who cannot eat fish. Again it would seem that the reaction is more a metabolic upset than an immunological one. Fish is one of the commonest foods to cause allergic reactions in atopics and differs from other foods in that this sensitivity quite often remains to adult life from infancy. Reaginic antibodies are always easy to demonstrate in such patients. The clinical state of collapse in an adult after eating shellfish must not be confused with a rather similar picture seen in infants who are food sensitive. Infants who allergically can be very sensitive to cow's milk can be brought to the point of death if made to have a drink of cow's milk.

Another difficulty that arises in allergic man, and particularly in cases of urticaria due to foods, is that although a food may be responsible for the allergic symptoms, no antibodies can be found towards the offending food—the so-called non-reaginic food allergy. Elimination diets are the only reasonable method of discovering the offending foods. Whether non-reaginic allergy is a

good term to use can only be decided when we have a better knowledge of the mechanism of some of these reactions. It is perhaps in drug allergies that most advances in the mechanism have been made (see Chapter 26).

Allergic Urticaria

There thus remains to be considered that group of patients in whom it could be said that the symptoms of urticaria are basically allergic in that the reaction is due to a specific immunological mechanism.

Any patient who has a humoral allergy may develop urticaria. These patients often have eczema, hay fever and asthma; at the same time they will show immediate positive skin tests and circulating reaginic antibodies can be shown to be present. When a child has become cat sensitive, the cat may carry out its own skin test as its claws are dug into the skin and in a few minutes an irritating urticarial wheal develops. The rough tongue of a dog as it licks the dog-sensitive patient will often produce a local irritating patch of urticaria. A child with seasonal hay fever may play out of doors in the summer when pollen is in the air and on the vegetation. The pollen settles on the skin and minute abrasions of the skin sometimes produce a very severe generalized urticaria on the exposed parts. A local irritating urticaria of the vulva due to pollen occasionally occurs in young girls before puberty (Mitchell, Sivon & Mitchell 1948).

Foods can produce urticaria particularly of the giant type. That one man's food is another man's poison has been known for thousands of years. The association between food sensitivities and atopic eczema has already been considered. A food that has reaginic antibodies towards it, in a very high dilution, will not be eaten by the sensitive individual. Thus the acutely egg-, fish- or nut-sensitive patient feels an immediate tingling in the mouth when the offending food reaches the mouth. The food will be spat out immediately and subsequently will not be taken as a food except by mistake. An egg sensitive child, years after it has grown out of its egg sensitivity will remain faddy about eggs and probably refuse eggs in all form. Sometimes, however, eggs will be eaten and even enjoyed in small quantities when reaginic antibodies are still present towards egg. Presumably at this stage of sensitivity, eggs are doing no harm though it is doubtful whether they are doing much good. The response is often quantitative because too much egg will produce even at this stage, when the sensitivity is disappearing, some allergic symptons.

Drug-Induced Urticaria

We can generalize by saying that the immediate whealing response and passive transfer tests for demonstrating Prausnitz-Küstner antibodies are very rarely seen in allergic drug reactions (Frankland 1962), In some cases of penicillin sensitivity, reaginic antibodies towards penicillin are present. These are the patients who are

very sensitive to penicillin and in whom skin tests themselves can cause severe constitutional effects. Occasionally sulphonamides, aminophylline and other drugs will give an immediate positive (Type I) skin test. Although aspirin is the commonest drug to cause urticaria, yet we have no immunological test to prove such a sensitivity. It is unwise for any patient who has urticaria to take aspirin because Warin (1960) has shown that some patients with urticaria are quantitatively made worse by aspirin. Aspirin under these conditions is acting not specifically as an antigen, but in some non-specific way. Penicillin often seems to act in a rather similar manner in that an induced penicillin urticaria can continue for over 18 months, waxing and waning in severity until it finally does not recur. There is no doubt that in rare cases, small amounts of penicillin in the daily milk supply can act as an unsuspected cause for the continuation of the symptoms.

It seems unlikely that drugs of low molecular weight could in themselves give rise to reaginic antibodies and it seems likely that before they can become allergenic these drugs combine with body proteins to become haptens. Many attempts have been made to make complex allergens by incubating the patient's serum with the drug. Such methods have been successful in producing immediate positive skin tests to various drugs, while Rajka (1957) combined penicillin with γ-globulin and was able to obtain positive immediate skin responses in thirty-four out of forty cases of suspected penicillin sensitivity.

In summary we can state that urticaria and angioneurotic oedema are in some cases specifically produced or made worse by drugs, but only rarely are reaginic antibodies demonstrable in this type of drug sensitivity.

In general studies on cells of sensitised patients would seem to be the method of choice when investigating drug induced urticaria (see Chapter 26).

Papular Urticaria

This complaint is also known as prurigo strophulus, lichen urticatus, Brocq's prurigo simplex or urticaria papulosa. This is a separate morbid entity from angioneurotic oedema and urticaria. It is common in young children in the spring and summer. The rash usually occurs on the arms, legs and buttocks. The salient features are small urticarial papules. The urticarial reaction diminishes as the papules become more distinct. Often a vesicle forms on top of the papules. This becomes excoriated and infected from scratching. The irritation comes and goes. It is especially liable to recur when the skin becomes heated with exercise, during a hot bath or when in bed at night. Although there have been many theories about the possible aetiology of this complaint, there seems to the writer to be no doubt that it represents a phase of sensitization to biting insects and as such is of considerable interest to the immunologist. There is no direct evidence to show that the atopic individual differs in his response to biting insects from the so-called normal non-allergic. First we will consider what happens to patients who become sensitive to stings. Some patients when stung by bees or wasps

develop a severe constitutional reaction. This sort of reaction develops on the second sting or after many stings. The patients note that with each succeeding sting the reaction tends to become more severe. In Great Britain between six and twelve people die each year following such stings. The reaction in its severe form is very like that shown by a guinea-pig having an acute anaphylactic reaction. The skin becomes hot and irritating, urticarial wheals break out over the body, swollen eyelids and lips and laryngeal oedema are followed by a fall in blood pressure, loss of pulse and unconsciousness. Involuntary defaecation and urination (as in the anaphylactic guinea-pig) precedes death. It would seem as if immunity has gone wrong. Whether such a reaction occurs more often in a person with an atopic constitution is impossible to state with certainty. Over 70% of over a hundred cases of bee- and wasp-sting sensitivity seen by the author have no other stigmata of atopy. There would seem to be good presumptive evidence that histamine is primarily responsible for most of the symptoms of

TABLE 23.1

Man's Reaction to Insect Bites

	Immediate reaction	Delayed reaction
Stage 1	−	−
2	−	+
3	+	+
4	+	−
5	−	−

acute shock because in three of the author's patients, the disease urticaria pigmentosa is also present. Patients with urticaria pigmentosa have an increase of mast cells in the skin and therefore have an increase of histamine available to be spilled out. Normally slight stroking of the skin will cause the pigmented spots to wheal and flush. These patients who have become sensitive to wasp (2) and bee (1) stings, and who become unconscious after a sting, one presumes have available more histamine than a normal subject. The aspirin-sensitive patient with urticaria pigmentosa will also have a very severe reaction if aspirin is ingested, but this effect is not specific for aspirin in patients with urticaria pigmentosa as has been suggested (Frankland 1957).

As a wasp sting contains many painful and whealing substances, it is easier to study the effect of mosquitoes or bugs taking their blood meal from man to investigate the local effect on the skin. The results of such experiments have been previously reported when using the bug *Rhodnius prolixus* (Frankland 1955). The pattern of sensitization that develops would seem an ideal way to investigate some of the differences between the immediate (Type I) and delayed (Type IV)

type of sensitivity. Using a bug such as *Rhodnius* which feeds directly from the blood capillaries (like the mosquito) the saliva only will be acting as the antigen. At first no local response is produced. After a varying number of bites a delayed papule is produced. It is this papule which represents the stage of papular urticaria. Histologically the skin is shown to be oedematous with an inflammatory infiltration of neutrophils and eosinophils. Following subsequent bites the time of maximal local induration changes from 2 days to 5 or 6 hr. When this stage is reached there appears with the next bite an immediate wheal and flare reaction but no delayed reaction. On subsequent bites the immediate reaction at first becomes larger but finally the reaction disappears (Table 23.1).

A few people instead of losing their sensitivity become more sensitive with the development of generalized urticaria and laryngeal oedema. Presumably death could occur with a minute amount of saliva of the insect. (*Rhodnius* may be peculiar in its sensitizing properties compared with mosquitoes, because in two laboratories using *Rhodnius* a dangerous degree of sensitization has arisen.)

It would seem to the writer that because a very marked delayed reaction was followed by the first 'immediate' reaction with the next bite, the difference between delayed- and immediate-type sensitivity may basically be quantitative rather than qualitative.

Asthma, Nasal Polyposis and High Blood Eosinophilia

Before considering allergic asthma due to extrinsic causes, this would seem to be a suitable opportunity to discuss those patients who have the triad of high blood eosinophilia, nasal polyposis and asthma. Drug sensitivity, particularly aspirin sensitivity, is common in this group. In Rackemann's (1947) original classification of asthma, he referred to one group as 'intrinsic'. These asthmatics typically began their complaint over the age of 40 years, no obvious cause could be found for their complaint and nasal polyposis and drug sensitivity were common. The word 'intrinsic' has come to mean among other things that the asthma had no definite allergic cause and antibodies usually could not be demonstrated in patients with this type of asthma. Rackemann has since realized (Rackemann & Edwards 1958) that many cases of 'extrinsic' allergic asthma if followed up for a sufficient number of years become 'intrinsic' in type. Yet Jimenez-Diaz (1955) has stressed that in only 25 per cent of cases of asthma are the clinical symptoms based on an antigen-antibody reaction. Does this mean that the remaining 75 per cent are based on a mechanism other than sensitization? Or does it mean we are unable in the present state of our knowledge to recognize the antigen-antibody mechanism? Clinicians have tended to define asthma as an allergic disease (Unger 1945). It is much better to use a definition of asthma in which neither the mechanism nor the patho-physiology has to be defined because asthma is not

one disease and the patho-physiology differs with different types of asthma. It might be said at this point that the author does not object to workers having different definitions for asthma, but it is most important for each writer to give his definition.

Asthma can be defined thus: 'Asthma refers to the condition of subjects with widespread narrowing of the bronchial airways, which changes its severity over short periods of time either spontaneously or under treatment, and is not due to cardiovascular disease.'

Before proceeding with any other discussion about asthma we must for a short time consider the eosinophil cell. It seems to be the hallmark of the allergic reaction yet we do not know its function. In patients with seasonal hay fever as their only allergic manifestation, there is a blood eosinophilia during the summer when symptoms are present, but not during the winter when there is no antigenic stimulation. Eosinophils are found in nasal smears, in asthmatic sputum and in the tissues where allergic reactions are occurring. It is therefore of considerable interest that in those patients with nasal polyposis and asthma, there is not only a blood eosinophilia but also the nasal polypi themselves have many eosinophilic cells in them. Yet we are unable to find any offending antigen in these patients. Prognostically it is very important for the clinician to distinguish between various types of asthma on the one hand and air obstruction and air trapping due to recurring bronchitis and emphysema. In the latter conditions the patient has a cough, is breathless with exertion and has attacks of wheezy respiration. Undoubtedly there are many different histopathological reasons why parts of the lung parenchyma are permanently destroyed with resulting emphysema and airway resistance. If we think in clinical terms that 'asthma' means a reversible airway obstruction, then in uncomplicated asthma the airway obstruction is completely reversible—one of my asthmatic patients is an Olympic runner. Asthmatic patients may, however, have infective episodes with bronchitis and pneumonia. It seems likely that in later life, especially with the likelihood of infective episodes in the chest increasing, the asthmatic will eventually develop some degree of emphysema. Some asthmatics seem to develop emphysema at an early age. The question then arises how many of these patients previously had 'asthma'. The difficulties of diagnosis between various combinations of asthma, bronchitis and emphysema is not only of academic interest because of the very different therapeutic methods used and prognostic outlook.

The anti-inflammatory steroid drugs help patients with asthma. So marked is this effect that Bukantz (1961) considers that in any definition of asthma there should be included the words 'benefited by the anti-inflammatory steroids'. The reason that steroids benefit asthma is not known, but at least there would seem to be grounds for believing that the very marked effect of the corticosteroids on tissue and blood eosinophils may give some clue as to the function of the eosinophil cell in asthma.

It was found that when patients were admitted to hospital in acute breathless attacks not due to cardiac disease, that bronchial biopsy gave a fairly specific histological picture to allow bronchitis to be distinguished from asthma (Glynn & Michaels 1960). The essential histological finding in patients with asthma was a subepithelial collection of eosinophil cells. This finding was absent from the bronchitics who showed a marked hypertrophy of the serous-secreting cells. Samter (1959) has probably followed up more cases of nasal polyposis and asthma than anyone else. Although he has usually constructive ideas he is worth quoting on the cause of eosinophilia. 'Eosinophils are found in nasal and bronchial tissues after certain antigen-antibody reactions have taken place; but neither antigen nor antibody alone, neither histamine releasers nor histamine itself, nor antihistamine drugs, nor any other chemical mediators which are released during antigen-antibody reactions have induced eosinophilia in experimental animals. Function and fate of eosinophils are still unknown.' While local and peripheral blood eosinophilia has been studied for many years, it is only recently that the mast cell has been studied, but unfortunately more in the rat than in man. In man the mast cells are one, but probably only one, of the sources of histamine. There seems to be no doubt that we will in the future look not only for eosinophil cells but also for mast cells in sputum and nasal smears in suspected allergic patients (Shioda & Mishima 1966). Yet it is claimed that most of the blood histamine in man is carried by the blood basophils (Graham *et al* 1955). Shelley (1962) devised a test which he claimed showed that basophil degranulation occurs when allergen is added to either human or rabbit leucocytes that have been incubated with allergic sera. Unfortunately many subsequent workers have been unable to repeat Shelley's work because of the spontaneous basophil degranulation that occurs in the controls. Ridges & Augustin (1964) have devised an *in vitro* test to measure reagins, but like Shelley's test, other workers have had difficulties in reproducing this test (see Chapter 1).

Talmage (1957) too has pointed out when discussing the complexity of allergic injury that this depends primarily on the complexity of the physiochemical properties of the three major components of the reaction: the allergen, the antibody and the cell. The substances that are released during an allergic reaction are discussed elsewhere (Chapter 15) as is the mechanism of their release. It seems apparent that the release of histamine in the anaphylactic animal is different in different species. In man the release of histamine involves chemical and enzymatic reactions which are not yet fully understood, while the importance of heparin, serotonin and slow-reacting substances have perhaps in the past been minimized while stressing the importance of histamine release. The disappointing clinical results in the atopic diseases of the so-called antihistamine drugs would seem to indicate that the antigen-antibody reaction releases a complex chain of reactions with the production of pharmacologically active agents only one of which is histamine.

Allergic Asthma

Asthma not due to discoverable allergic causes has been described in some detail. We must now consider the pathogenesis of allergic asthma in the atopic individual. Clinically the dominant causes of asthma can be considered under the three main headings of (i) infective, (ii) psychological and (iii) allergic. Usually, the asthmatic attacks are not produced by one cause, but by many causes and the dominant cause alters with different age groups (Williams 1959). In a child under the age of 7 years we generally find that infective and emotional causes are dominant. These children by skin tests may be shown to have reaginic antibodies towards specific inhalant allergens, but their importance is subclinical. It seems strange that at a time a child is growing out of food sensitivities (e.g. egg), the inhalants such as pollens which are being absorbed by everyone only sensitize the atopic. It is tempting to guess that the allergens are in fact haptens so that if this theory is correct, then the allergic individual differs from the non-allergic in possessing some tissue moiety which can alter the atopic antigens. On this theory the non-atopic might be made atopic by tissue injury, especially nasopharyngeal infection. Removal of nasal polypi by operation is quite often followed by asthma. Severe upper or lower respiratory infection in patients with rhinitis seems often to be the start of asthma. Yet these patients have no discoverable reaginic antibodies. It may be that we are not using the correct antigens to test such patients. The old idea of being allergic to one infecting organism has been brought up to date by the work of Hajos (1960). She has prepared specific extracts of various organisms obtained from nasopharyngeal and sputum specimens. Skin tests are performed with these organisms and notice is taken of both the 'immediate' and 'delayed' responses. Provocation inhalation tests of atomized solutions of the organisms are given and any degree of airway obstruction produced is measured. The work is in its preliminary stages, but at least it does seem that a more rational approach to bacterial allergy is now being made, and more will be heard on the general importance of bacteria acting not only as antigens but as allergens (Frankland 1961).

Bronchial provocation tests have been much used to seek out the possible diagnosis of specific allergic asthma in confirmation of positive skin tests. Unfortunately the provocation used may be given in an unphysiological manner. A concentration of pollen allergen can be inhaled in 30 sec which normally is inhaled in 24 hr. Much can be learnt from such procedures. Not only is information obtained from known inhalant allergens but also it is possible to gauge whether the patient after a series of injections is less sensitive (Citron *et al* 1958). We learn that an allergenic inhalant insult in the morning can cause typical allergic asthma 14 or 16 hr later. The mechanism of this delayed response when one would expect the reaction to be maximal in 15 min, is not understood.

Antibiotics are causing an added interest to the possible importance of

Candida albicans as an atopic allergen. Itkin & Dennis (1966) showed that *C. albicans* was capable of producing asthma on bronchial testing if positive skin responses were present. But even normal subjects may, on bronchial challenge to an *E. coli* endotoxin, develop airway obstruction. Some strains of *C. albicans* contain endotoxin-like fractions and so do some dust extracts. The positive skin test and the positive bronchial provocation test may therefore have nothing to do with an atopic or an allergic reaction. However, it now seems likely that there are many organic dusts that give rise not to a Type I but to a Type III reaction (Pepys *et al* 1964). The patient complains of a wheezy cough in certain dusty environments. A specific sensitivity is present, although reagins are absent, but precipitins towards specific antigens if looked for are present.

It has been known for over 20 years that it is possible to shock a guinea-pig by a nebulized antigen. Halpern (1950) showed that the antigen given by inhalation after a suitable interval would kill the guinea-pig with acute respiratory obstruction apparently in the same way as an inhalation of histamine. Histologically, however, the appearances were quite different. Asthma provoked by histamine produced a pure bronchospasm without oedema or eosinophilia. Asthma provoked anaphylactically produced not only bronchospasm but also oedema in the fibro-connective zone between the muscle and cartilage. Eosinophils also appear in the oedema fluid. Although studies using isotope-labelled antibodies suggest that the distribution of antibodies is widespread, we still do not know why some pollen-sensitive patients do or do not develop pollen asthma when they are sensitive to pollen. It is possible that the capillary endothelium may protect an organ. If an overdose of a pollen extract is given, a constitutional reaction occurs. Quite often there is very little local reaction at the site of injection and neither is there generalized urticaria, but only asthma. It would seem that the pollen allergens can circulate through the skin without establishing contact with the antibodies. Thus a constitutional reaction is a focal reaction in tissues which in any individual represents the site of the allergic disease. The 'shock organ' in any patient may thus depend upon the lack of this hypothetical substance in the organ, and conceivably this trait could also be inheritable.

In allergic asthma the antibodies are fixed in the bronchial mucous membrane and normally the exposure to the specific allergen is by inhalation. We presume that a non-stable enzyme system is set into motion with the production of histamine and other active substances. Histamine then exerts its pharmacological action of contraction of smooth muscle, increased capillary permeability and stimulation of mucous secretion. It is not known exactly where the antigen-antibody reaction takes place, but from the work of Warren & Dixon (1948) using ^{131}I-labelled globulin, it seems that it is in connective tissue proper where antigen-antibody union takes place with release of histamine. This ground substance is a highly complicated matrix which can take up molecules and ions rather like an exchange resin. It would seem that ground substance could prevent

histamine from diffusing while an antigen-antibody reaction might encourage its diffusion.

It is certain that although we do not know the exact mechanism of histamine release in bronchial asthma, histamine has been studied more than any of the other chemical mediators. Heparin may be important in the anaphylactic reaction; it certainly is in the dog. Heparin is also in large amounts in the mast cells which hold on to the histamine. Yet antihistamine drugs do not help allergic asthma (Frankland & Gorrill 1953). This means that other substances such as 5-hydroxytryptamine—serotonin—may be important. Yet antiserotonin drugs, like the anti-histamine drugs, are of no use in asthma in man. The 'slow reacting substance'—SRS-A—of Brocklehurst (1956) which forms in atopic man might explain why antihistamines are not effective therapeutically in man. There well may be other chemical mediators which cause the clinical entity of asthma.

We must also remember that parasympathetic impulses induce bronchoconstriction and secretion of mucus. Under strong parasympathetic control, a patient might react to a relatively small dose of histamine, while if the patient was predominantly under adrenergic control, larger doses of histamine might be required before overcoming sympathetic resistance. These ideas gave rise to the term 'nervous diathesis' or 'vagotonic' patient. Atropine sulphate inhibits smooth muscle constriction and secretion of mucus induced by acetylcholine. Why then do atropine sulphate or antihistamine drugs, which often have a marked anti-acetylcholine effect, not help asthmatic patients? The explanation may be that the essential phase of cholinergic asthma (if it exists) is due to vascular reactions and oedema of bronchial tissue. This tissue oedema would be little affected by atropine sulphate. Experiments to determine the role of vagal afferents from the lung in man both in the control of breathing and the sensation of breathlessness, have shown that man is unique among mammals (Guz 1966). Bilateral block of the vagus nerves had no effect whatever on rate, tidal volume or pattern of air flow. In view of these ideas, I would make a strong plea that the word 'bronchospasm' is not used when referring to asthma. This word seems to indicate a knowledge of the pathogenesis of asthma, which at least seems unlikely in many if not most forms of asthma.

THE CHEMICAL NATURE OF ALLERGENS

It seems strange that the immuno-chemists have so far been unable to find any distinguishing feature common to allergens in general that distinguishes them from other antigens. Immunologically pure allergens in the past have not been used to study the various properties of allergens and antibodies in allergic patients, but with the methods now available, chemically pure substances can be prepared. These must be tested against patients whose only sensitivity is towards

such a pure allergen, so that the antibody towards such an allergen can be characterized. In this country most work on the purification and standardization of allergens has been done on horse scurf (Stanworth 1957) and on grass-pollen allergens (Augustin 1959). During the last decade many immunological studies have used various gel-diffusion methods to measure antigenic components. Rabbits can be immunized with pollen to produce precipitating antibodies towards pollen. Attempts have been made to standardize pollen extracts using such a precipitating system. Moreover, immuno-electrophoresis has been used to compare human serum against rabbit serum and pollen solutions against anti-pollen rabbit serum. It has been found that pollen antigens exhibited a wider range of electrophoretic mobilities than serum proteins, but in general they were of a comparable complexity. Eight, ten or more antigens can be distinguished in this way. But are these antigens allergens? We now have a lot of evidence that these precipitating antigens do not represent the allergens responsible for sensitivity. Pollen extracts can be sterilized for 10 minutes at 100° without loss of activity—this method was originally used by Noon in 1911. This procedure destroys the well-characterized so-called A line which has been used to standardize pollen solutions. It is found that this antigen which is not a pollen allergen is particularly unstable and breakdown products and/or aggregates are produced which no longer precipitate with antisera to the original extracts, but act as inhibitors. Piper (1955) claimed successful hyposensitization in hay fever with a few injections of large doses of pepsin-treated grass-pollen extracts. In theory, destruction of the reactive allergens without interfering with the desensitization of the individual, would be a great step forward. Piper's experiment was uncontrolled so it seemed very important to do a properly controlled experiment along similar lines. This showed (Frankland & Augustin 1962) that inactivated pollen extracts give very poor clinical results compared with standard skin active pollen extracts.

The ideal method of hyposensitizing an asthmatic has yet to be found. Multiple injections with aqueous extracts has been the method of choice for over 50 years. The hope that water in oil emulsions would be a safe and effective method of hyposensitization giving one or a few injections has not been realized (Pearson 1965). Only a few doctors use emulsions as the method of choice for hyposensitization. An alum-precipitated pyridine complex has been formulated which seems to be an advance in injection therapy, in that relatively few injections are necessary (Frankland & Noelpp 1966). However, the ideal method free from any unpleasant untoward effects has not yet been devised. If we use allergens that have been denatured and do not give a positive skin test, they are not effective in treatment. When we use any conventional method we may cause most unpleasant generalized constitutional reactions, and when an overdose is given by mistake the patient can die in a few minutes like an anaphylactic guinea-pig.

MECHANISM OF ACTION OF HYPOSENSITIZATION

Since we do not know the sites of formation, nor indeed has there been general agreement as to the nature of the skin-sensitizing reaginic antibody, there are considerable difficulties in our understanding of the possible mechanism whereby a series of injections gives clinical benefit to a patient. *In vitro* methods for measuring the beneficial responses in allergy have been the goal of many investigators. Subjective clinical impressions of the patient and his doctor may be very faulty in gauging results of injection treatment of an allergic complaint such as hay fever, unless a very careful double blind control trial is carried out. However, many reports of objective *in vitro* methods to study various injection treatments have been reported. In spite of work suggesting the opposite view there is now no doubt that there is no correlation between pollen reagin and pollen haemagglutinating antibody titres. The limitations of very sensitive haemagglutination procedures in evaluating the immune responses to repository injections of ragweed emulsions have been well documented (Friedman, Spiegelman, Blumstein, Gershenfeld & Fishman 1961). Anti-ragweed antibodies were measured in pre- and post-treatment sera by haemagglutination procedures. Both the bis-diazotized benzidine method and the tannic acid method with rabbit erythrocytes coated with ragweed extract were used. There was no correlation of serum antibody titres obtained with any of the haemagglutination procedures used or between the clinical effects or injection dosage used.

CONCLUSION

Advances in the understanding of the pathogenesis of the atopic diseases will only come when standardized methods using 'pure' antigens and antibodies are available. It is to be hoped that the immunologists, physiologists and pharmacologists with the new techniques available will be able to characterize for the clinician these impure substances.

REFERENCES

ARTHUS M. (1903) Injections repetées de serum de cheval chez le lapin. *Compt. rend. Soc. Biol.* **55,** 817.

AUGUSTIN R. (1959) Grass pollen allergens. III. Their differentiation from the other pollen antigens by immunoelectrophoretic studies in relation to skin reactivity, enzymic digestions, heat and pH stabilities. *Immunology* **2,** 230.

AUGUSTIN R., O'SULLIVAN S.A. & CONOLLY R.C. (1966) Reagins as the anaphylactic antibodies of man. *Acta allerg.* **21,** 430.

BRAMBELL F.W.R. (1961) The transmission of antibodies from mother to foetus. Problems concerning the transmission of immunity from mother to young. *Proc. Roy. Soc. med.* **54,** 992

BROCKLEHURST W.E. (1956) A slow reacting substance in anaphylaxis, SRS-A. In *Ciba Foundation Symposium on Histamine*, p. 175, ed. WOLSTENHOLME G.E.W. & O'CONNOR C.M. London: J. & A. Churchill.

BUKANTZ S. (1961) Personal communication.

CHAN P.C.Y. (1963) Ph.D. thesis, Univ. London, London.

CITRON K.M., FRANKLAND A.W. & SINCLAIR J.D. (1958) Inhalation tests of bronchial hypersensitivity in pollen asthma. *Thorax* **13**, 229.

COCA A.F. and COOKE R.A. (1923) On the classification of the phenomena of hypersensitiveness. *J. Immunol.* **8**: 163

COOKE R.A. & VAN DER VEER A. (1916) Human sensitization. *J. Immunol.* **1**, 201.

DAVIES G.E. (1958) Allergic reactions as hazards in the use of new drugs. In *The Evaluation of Drug Toxicity*, p. 58, ed. WALPOLE A.L. & SPINKS A. London: J. & A. Churchill.

FEINBERG J.G., DEWDNEY J.M. & TEMPLE A. (1965) Serum sickness in guinea pigs. *Int. Arch. Allergy* **27**, 175.

FEINBERG J.G. & CHOPRA S. (1966) A quasi-atopic experimental model in the guinea pig. Information Exchange Group No. 5, Memo. 163.

FRANKLAND A.W. (1953) Locust sensitivity. *Ann. allerg.* **11**, 445.

FRANKLAND A.W. & GORRILL R.H. (1953) Summer hay fever and asthma treated with antihistaminic drugs. *Brit. med. J.* **i**, 761.

FRANKLAND A.W. (1955) The treatment of seasonal hay fever. Thesis for D.M., Oxford.

FRANKLAND A.W. (1955) Über die Entwicklung einer experimentell induzierten Uberempfindlichkeit gegen Insekten (Rhodnius). *Allergie und Asthma* **i**, 229.

FRANKLAND A.W. (1957) Release of histamine in urticaria pigmentosa. *Lancet* **i**, 1040.

FRANKLAND A.W. (1958) Allergy. In *Modern Trends in Paediatrics*, p. 179, ed. HOLZEL A. & TIZARD J.P.M. London: Butterworth.

FRANKLAND A.W. (1961) Microbic flora and therapy with bacterial vaccines in chronic bronchitis with secondary asthma. *Folia allergol.* **8**, 200.

FRANKLAND A.W. (1962) Skin testing and desensitization to drugs. *Proc. Roy. Soc. med.* **55**, 46.

FRANKLAND A.W. & AUGUSTIN R. (1962) Grass pollen antigens effective in treatment. *Clin. Sci.* **23**, 95.

FRANKLAND A.W. & NOELPP B. (1966) Summer hay fever treated with aqueous and alum-precipitated pyridine extracts. *Practitioner* **196**, 766.

FRIEDMAN H., SPIEGELMAN J., BLUMSTEIN G., GERSHENFELD M. & FISHMAN A. (1961) Serological evaluation of immune responses to repository injection of ragweed emulsion. *Ann. allerg.* **19**, 991.

GLYNN A.A. & MICHAELS L. (1960) Bronchial biopsy in chronic bronchitis and asthma. *Thorax* **15**, 142.

GRAHAM H.T., LOWRY O.H., WHEELWRIGHT F., LENZ M.A. & PARISH H.H. (1955) Distribution of histamine among leucocytes and platelets. *Blood*, **10**, 467.

GUZ A. (1966) In *Breathlessness*, p. 65, ed. HOWELL J.B.L. & CAMPBELL E.J.M. Oxford: Blackwell.

HAJOS M.-K. (1960) A comparative study of skin test and bronchial tests with bacterial solutions in infective bronchial asthma. *Acta allerg., Kbh.* **15**, 517.

HALPERN B.N. (1950) L'asthma. Rapports du 2 Congrés Internationale de l'asthma, 23. L'expansion.

HAMILTON E.D. (1959) Studies in air spora. *Acta allerg., Kbh.* **13**, 143.

ISHIZAKA K., & ISHIZAKA T. and HORNBROOK N.M. (1966) Physiochemical properties of reaginic antibody. V. correlation of reaginic activity with γE-globulin antibody. *J. Immunol.* **97**: 840

ITKIN I.H. & DENNIS M. (1966) Bronchial hypersensitivity to Candida albicans. *J. Allergy* **37,** 187.

JIMENEZ-DIAZ C. (1955) Aetiological analysis of primary bronchial asthma. 2nd Int. Congr. Allerg., Rio, Brazil.

KALLIO E.I.S., BACAL H.L., EISEN A. & FRASER F.C. (1966) A familial tendency toward skin sensitivity to ragweed pollen. *J. Allergy* **38,** 241.

KIRTCHEV P. & FRANKLAND A.W. (1965) The histamine binding power of human serum from allergic and non-allergic subjects. *Acta allerg.* **20,** 153.

LABORDE C., PARROT J.L. & URQUIA D.A. (1953) Le pouvoir histaminopexique du serum sanguin. Technique de mesure. *Presse Med.* **61,** 1151.

LOBITZ W.C. & CAMPBELL C.J. (1953) Physiologic studies in atopic dermatitis (disseminated neurodermatitis). *Arch. Derm. Syph.* **67,** 575.

MCKEE W.D. (1966) The incidence and familial occurrence of allergy. *J. Allergy* **38,** 226.

MITCHELL W.F., SIVON I. & MITCHELL J.H. (1948) Vulvo-vaginal pruritus associated with hay fever. *Ann. allerg.* **6,** 144.

NOON L. (1911) Prophylactic inoculation against hay fever. *Lancet* **i,** 1572.

ORTIZ F. (1966) Serum sickness in man. A case due to rabbit serum. *J. Allergy* **37,** 274.

PATTERSON R. & SPARKS D.B. (1962) The passive transfer to normal dogs of skin reactivity, asthma and anaphylaxis from a dog with spontaneous ragweed pollen hypersensitivity. *J. Immunol.* **88,** 262.

PATTERSON R., CHANG W.W.Y. & PRUZANSKY J.J. (1963) The Northwestern University colony of atopic dogs. *J. Allergy* **34,** 455.

PEARSON R.S.B. (1965) Depot injections in treatment of hay fever and pollen asthma. *Brit. med. J.* **ii,** 1148.

PEPYS J., LONGBOTTOM J.L. & JENKINS P.A. (1964) Vegetable dust pneumoconiosis. Immunologic responses to vegetable dusts and their flora. *Amer. Rev. Resp. Dis.* **89,** 842.

PETERSON R.D.A., PAGE A.E. & GOOD R.A. (1962) Wheal and erythema allergy in patients with agammaglobulinaemia. *J. Allergy* **33,** 406.

PIPER C.T. (1955) Hay fever and general practititioner. *Med. J. Aust.* **i,** 303.

PIRQUET C.F. & SCHICK B. (1905) *Die Serumkrankheiten.* Leipzig: Deuticke.

RACKEMANN F.M. (1947) A working classification of asthma. *Amer. J. med.* **3,** 601.

RACKEMANN F.M. & EDWARDS M.C. (1958) Is intrinsic asthma a reversible disease? *J. Allergy* **29,** 528.

RAJKA G. JR. (1957) Studies on urticaria of serum sickness type due to penicillin, using penicillin combined with gamma globulin as allergen. *Proc. 11th int. Congr. Derm. 1960* **3,** 554. Stockholm, *Acta dermato-venereologica.*

RAJKA G. (1960) Prurigo Besnier (atopic dermatitis) with special reference to the role of allergic factors. *Acta dermat. venerol.* **40,** 285.

RATNER B. & SILBERMAN D.E. (1953) Critical analysis of the hereditary concept of allergy. *J. Allergy* **24,** 371.

RIDGES M.P. & AUGUSTIN R. (1964) An *in vitro* test for atopic reagins by double-layer leucocyte agglutination. *Nature (Lond.),* **202,** 667.

ROBERTS J.A.F. (1961) Human genetics and the public health. *Proc. Roy. Soc. med.* **54,** 841.

ROWE A.H. & ROWE A. (1963) Bronchial asthma. Its diagnosis and treatment. Springfield, Illinois: Charles C Thomas.

SAMTER M. (1959) Pathophysiology of bronchial asthma. In *International Textbook of Allergy,* p. 224, ed. JAMAR J.M. Copenhagen: Munksgaard.

SCHNYDER U.W. (1960) Neurodermatitis-asthma-rhinitis. A genetic allergological study. *Int. Arch. Allergy (Suppl.)* **17.**

SCHWARTZ M. (1952) Heredity in bronchial asthma. *Acta allerg. Kbh.* **5,** Suppl. II.

SPOUJITCH V. (1960) The complexity of allergic reactions. *Acta allerg., Kbh.* **15,** Suppl. VII, 112.

SHELLEY W.B. (1962) New serological test for allergy in man. *Nature (Lond.),* **195,** 1181.

SHERMAN W.B., HAMPTON S.F. & COOK, R. A. (1940) The placental transmission of antibodies in the skin sensitive type of human allergy. *J. exp. med.* **72,** 611.

SHIODA H. and MISHIMA T. (1966) The significance of mast cells in nasal smears. *J. Allergy* **37,** 321.

STANWORTH D.R. (1957) The isolation and identification of horse-dandruff allergen. *Biochem. J.* **65,** 582.

STANWORTH D.R. (1963) Reaginic antibodies. In *Advances in Immunology,* p. 181, ed. DIXON F.J. & HUMPHREY J.H. London: Academic Press.

STANWORTH D.R. & KUHNS W.J. (1965) Quantitative studies on the assay of human skin sensitizing antibodies (reagins). An examination of factors affecting the accuracy of the Prausnitz-Kustner (P-K) test. *Immunology* **8,** 323.

TALMAGE D.W. (1957) Allergy and immunology. *Ann. Rev. of Med.* **8,** 239.

TERR A.I. & BENTZ J.D. (1965) Skin sensitizing antibodies in serum sickness. *J. Allergy* **36,** 433.

TIFFENEAU R. (1958) Hypersensibilité cholinergo-histaminique pulmonaire de l'asthmatique; relation avec l'hypersensibilité allergénique pulmonaire. *Acta allerg., Kbh.* **12,** Suppl. V, 187.

UNGER L. (1945) *Bronchial Asthma.* Springfield, Illinois: Charles C Thomas.

VAN ARSDEL P.P. & MOTULSKY A.G. (1949) Frequency and hereditability of asthma and allergic rhinitis in college students. *Acta gent et statist med.* **9,** 101.

VARELZIDIS A., WILSON A.B., MEARA R.H. & TURK J.L. (1966) Immunoglobulin levels in atopic eczema. *Brit. med. J.* **ii,** 925.

WARIN R.P. (1960) The effect of aspirin in chronic urticaria. *Brit. J. Derm.* **72,** 350.

WARREN S. & DIXON F.J. (1948) Antigen tracer studies and histologic observations in anaphylactic shock in the guinea-pig. *Amer. J. med. Sci.* **216,** 136.

WILLIAMS D.A. (1959) Allergy in asthma. *Brit. med. J.* **ii,** 880.

WITTICH F.W. (1941) Spontaneous allergy (atopy) in the lower animal. *J. Allergy* **12,** 3.

CHAPTER 24

SERUM SICKNESS AND ACUTE ANAPHYLAXIS IN MAN

J. MORRISON SMITH AND P.G.H. GELL

INTRODUCTION

Serum sickness now causes little morbidity in countries where active protection against diphtheria and tetanus is widely employed in childhood. In the five years 1961–65 in Birmingham only three cases of diphtheria occurred. There is, however, no alternative at present to serum treatment of diphtheria, although doubt has been expressed recently about the value of serum in the treatment of tetanus (Vaishnava *et al* 1966). When diphtheria was common and when scarlet fever was also treated with horse serum in doses up to 200 ml, serum sickness was very common and occurred in up to 50% of patients given such treatment.

The occurrence of serum sickness was related to the size of the dose of serum given and decreased as more concentrated serum preparations became available. It was also related to the route of administration, being more common in patients given part or all of the serum intravenously. In the 1930s, when large fever hospitals gave serum to several thousands of patients each year—and the doses used were commonly 10–20 cc.—intramuscular injection of 10 cc. resulted in about 10% of patients developing serum sickness, and with 20 cc. about 30% developed serum sickness. When intravenous serum was given, 45–50% of patients usually developed serum sickness. When a second dose of serum was

given, it resulted in an increase in symptoms of serum sickness equivalent to the effect of the total dose of serum if given within the first 6 days, as had been so clearly shown by v. Pirquet & Schick and described in their monograph published in 1905 (translation, 1951). If the second dose was given after sensitization had

TABLE 24.1

Anti-diphtheritic serum reactions
(From the Report on the City of Birmingham Infectious Diseases Hospitals for 1935 by Dr John McGarrity, Medical Superintendent)

Dose given	Up to 16,000 units I.M.	24,000 units or more I.M.	I.V. or I.M.+I.V.	Total
Patients	603	284	258	1145
Serum reaction	60 (9.95%)	83 (29.22%)	122 (47.29%)	265 (23.14%)

Note: Serum used contained 1500 units per ml.

TABLE 24.2

Anti-diphtheritic serum (1500 units/ml)
(From the Report on the City of Birmingham Infectious Diseases Hospitals for 1936 by Dr John McGarrity, Medical Superintendent)

Dose	Up to 8000 units I.M.	Over 8000 units I.M.	I.V. or I.V.+I.M.	Total
No. treated	702	237	144	1083
Urticaria	19	30	38	87
Rigor	—	—	1	1
Morbilliform rash	1	—	—	1
Total	20 (2.85%)	30 (12.66%)	39 (27.08%)	89 (8.23%)

Anti-streptococcal serum

No. treated	477	100%
Urticaria	122	27.29%
Arthritis	10	2.24%
Pyrexia	2	0.45%
Erythema	1	0.22%

occurred, an accelerated reaction occurred in 4–6 days instead of the usual 6–10 days, and this was likely to be accompanied by a local or general immediate reaction suggesting that after sensitization two types of serum sickness occur, an anaphylactic (Type I) reaction, and a Type III, more slowly developing, serum

TABLE 24.3
Incidence of reactions following anti-rabies serum
(From KARLINER & BELAVAL 1965)

Age	Number	Serum sickness
0– 5	309	38 (12.3%)
6–10	134	20 (14.9%)
11–15	42	9 (21.4%)
Over 15	41	19 (46.3%)
Total	526	86 (16.3%)

Dose usually 6–10 ml for a child and up to 30 ml for an adult.

sickness like that occurring after only one injection of serum, but accelerated and usually more severe.

CLINICAL MANIFESTATIONS

IMMEDIATE ANAPHYLACTIC REACTIONS

The immediate anaphylactic reaction is by far the more important and dangerous reaction due to serum, but its occurrence in persons not previously given horse serum more than a week before is very rare indeed. The clinical importance is that it may be rapidly fatal, but even if it is not fatal, it will be very alarming.

It is characterized by faintness, itching of the skin or urticaria, tightness in the chest, wheezing, collapse, and in severe cases cardiac arrest and possibly death. The reaction usually comes on within half an hour and the sooner it does so, the more severe are the manifestations likely to be. There may or may not be local swelling, redness and itching at the site of injection, coming on in the same period of time. In many cases, only the local manifestations occur. It was common practice in fever hospitals to give 0.1 ml of serum subcutaneously in the first place and watch for a local reaction occurring within 20–30 min before giving the remainder of the therapeutic dose. In urgent cases of diphtheria, the rest of the dose was usually given in small divided amounts if a local anaphylactic reaction occurred. This procedure was often described as 'desensitization', but it was not truly this. Results, however, over the years when many thousands of patients received treatment, were good and only very rarely did a general anaphylactic reaction occur and then it was extremely rare for death to occur. (Dr R. Fothergill assures us that no such death occurred in the Birmingham Fever Hospitals in the past 30 years.)

'Delayed Serum Sickness' Reactions

It is essential not to confuse the syndrome which is usually called 'delayed serum sickness' with delayed (Type IV) allergy, which is not, or not likely to be, involved. The allocation of the symptoms in man to types of allergic reaction (cf. Chapter 20) is discussed later.

No better account of this condition is available than that of v. Pirquet & Schick (1905: 1951) and there is nothing of importance to add to their account of the condition, its clinical manifestations and pathogenesis.

There is an interval of 8–12 days usually following the injection of serum before the manifestations appear. Some local swelling at the site of injection and enlargement of the related lymphatic glands usually appears first, followed by an urticarial rash spreading from the site of injection to involve part or all of the skin surface elsewhere. An urticarial rash is the most frequent type and is by far the most common single manifestation of this condition. Other types of rash, however, occur such as morbilliform and scarlatiniform rashes, but these are usually associated with constitutional upset of which pyrexia and rigors are frequent signs.

Pain and swelling of the joints occur less commonly, probably in only about 2–3% of all cases, but the careful studies of v. Pirquet and Schick showed that oedema of nephritic type and leucopenia usually occur. The oedema is usually only evident if daily weights of the patients are recorded and the leucopenia may be very short-lived so that daily leucocytic counts are required to show it. Eosinophilia is not a feature of this condition. Most of the leucopenia is due to a fall in polymorphonuclear leucocytes. Albuminuria is rare and there is no increase in cells or casts in the urine. Hildreth (1965), however, commenting on the report of De la Pava *et al* (1962) which described three fatal cases of glomerulonephritis following administration of horse serum, does suggest that the renal lesions of serum sickness may be more serious than has previously been thought. He suggests that renal biopsy might show whether or not patients who have had serum sickness reactions are in danger of developing chronic glomerulonephritis. He feels that the occurrence of subtle chronic renal complications of serum sickness is possible and a deliberate study of such a series as that reported by Karliner & Belaval (1965) by renal biopsy would clarify the position.

With reinjection of serum after the first week, a smaller dose will cause similar symptoms and the onset may be within 24 hours, but usually by the fourth day. In the case of reinjection, up to 90% of patients will give an accelerated reaction and nearly all will at least have a local reaction. With reinjection, immediate anaphylactic reactions will, of course, also be more common.

In rare cases, diarrhoea, abdominal pain, pericarditis, myocarditis, meningitis, meningo-encephalitis, peripheral neuritis and myelitis have been described. Even in such cases, death is rare and the symptoms usually clear up in 3–5 days, leaving no sequelae.

PROPHYLAXIS OF SERUM SICKNESS

Immediate Anaphylactic Reactions

It should be emphasized that serious immediate reactions were rare even in the days when large doses of horse serum were given therapeutically to thousands of patients each year. Von Pirquet and Schick note that they had heard of one fatal case in a patient given serum for the first time, but had no personal knowledge of a fatal reaction although they mention one nearly fatal case and advise against giving serum intravenously. The patients at risk are those who have previously had any serum treatment and also those people who have a particular liability to develop Type I reactions and who have a personal or family history of asthma, eczema or hay fever. Some of these may have been sensitized by contact with horses or horsehair or even by prophylactic inoculations. It should be noted, however, that for the majority of the population, contact with horses is now uncommon and horsehair is no longer in common use in upholstery and bedding, but it is likely that sensitization of some subjects to an exquisite degree may well occur by inhalation of extremely small quantities of antigen. Sensitization has been noted in nurses sometimes to an extremely high degree by inhalation of fine droplets of antibiotics when giving injections.

Sensitization to horse serum as a result of ingestion of horse meat is probably very rare. Trinca & Reid (1967) report a virtual absence of reactions to bovine antitetanus serum which may in part be due to treatment of the serum by ethanol fractionation but may be also partly due to an inherent lesser reactivity of bovine serum compared with horse serum. Their results certainly do not suggest that previous sensitization by ingestion is likely.

Precautions which have proved adequate in practice have consisted of giving 0.1 ml of serum undiluted subcutaneously as an initial dose and waiting half an hour before giving the rest of the dose. Adrenalin should be kept ready in a separate syringe with a long fine needle so that it can be given intracardially if necessary, and the patient should not be left during the half-hour. If a severe reaction occurs, resuscitation may be required immediately and at the first complaint of tightness in the chest or faintness, adrenalin 0.5 ml should be given, followed by a further 0.5 ml if symptoms are not rapidly relieved, and repeated if required.

In the patient in whom the risk of an immediate reaction is serious, either due to a history of asthma, eczema or hay fever or previous serum treatment, the procedure advised by Smith (1965) may be used. This takes 1½–2 hr, a delay which can have little effect on the therapeutic outcome of a case of diphtheria, and the procedure, although a little tedious, is not at all difficult. He advises intradermal injection of 0.1 ml of 1 in 100 million, 1 in 1 million, 1 in 100,000, 1 in 10,000, 1 in 1000, 1 in 100 and 1 in 10 dilutions of serum at 15 min intervals. A marked local reaction will give warning of possible general reaction with the

next dose. It may be preferable to give the injections subcutaneously, however, since the size of the local reaction with intradermal injections may be misleading. In many cases it will be found that the history of previous reactions has been inaccurate and the therapeutic dose of serum can be safely given.

This procedure does not, however, constitute true desensitization, and in persons found to be acutely sensitive desensitization would be extremely difficult and likely to take several weeks, so that the therapeutic indication for serum would probably be over. In such cases even massive doses of steroid drugs might not protect adequately against severe immediate reactions and might well be therapeutically undesirable. Antihistamines are of some value in preventing reactions in highly sensitive subjects, but as treatment of acute reactions they are of little or no value, since their action, even if given intravenously, is too slow in these circumstances. Adrenalin is the only drug of real value in the treatment of acute anaphylaxis.

The only safe way to deal with the rare individual requiring prophylaxis or treatment with antidiphtheria or antitetanus serum is to use human immune globulin, but recently it has been reported that serum produced in the cow is safer than horse serum and may be used in some of these cases.

'Delayed Serum Sickness' Reactions

As already noted, the manifestations of this condition are rarely serious and should not prevent the use of serum therapeutically when required, except that intravenous dosage should be avoided and the dose should not be repeated especially after the first week, when sensitization may have occurred. Concentrated serum preparations giving the therapeutic effect in the smallest amount of horse serum antigen are desirable. Steroids can be used in moderate dosages such as 20–30 mg of prednisolone daily with effect to control delayed serum sickness, and will be required for only a few days. Adrenalin has been used in the past, given four-hourly, and is helpful. Antihistamines may help to relieve and prevent milder manifestations, but have on the whole a disappointing value and have only a minor place in treatment. In urticarial rashes they are of value and will give symptomatic relief if given by mouth or injection in adequate dosage.

SERUM SICKNESS DUE TO DRUG ALLERGY

Although this subject is more properly dealt with in Chapter 26, serum-sickness-like syndromes are produced by drugs, especially by penicillin, and something may be said about them here. Most drugs probably combine with protein but it is unlikely that mere unstable adsorption to protein gives rise to antigenicity; Parker (1963, 1964) considers that it may be necessary for the drug to combine by stable covalent binding to protein before it becomes antigenic. It has been shown in the case of penicillin by de Weck & Eisen (1960) and by Levine and his

colleagues (1960, 1961, 1962, 1964) that binding to protein is usually with one of the active degradation products of penicillin such as penicilloic or penicillenic acid. The degradation products concerned may form only a very small part of the amount of the therapeutic dose of the drug. It is, however, only in the case of penicillin that this process has been investigated. The protein combinations with active substances have an antigenic specificity dependent upon the drug element in the combination. It is not surprising if they can cause clinical manifestations resembling serum sickness. Clearly we have an ideal situation for the development of antigen-excess complexes as a result of continual dosage with the drug, and its steady breakdown in the tissues to produce antigenic conjugates with tissue protein. Since no more than three or four molecules of attached hapten are sufficient to render homologous protein antigenic, a drug like penicillin will potentially have an antigenicity of about 100 times that of the same weight of protein. Owing to this continual parenteral production of antigen, and especially if dosage with the drug is continued, it is not surprising if serum sickness is produced and occasionally proceeds to chronic arteritic inflammation resembling polyarteritis nodosa and similar to that described by Rich & Gregory (1943) resulting from sulphonamides.

Reactions may either be of an acute anaphylactic nature, presumably a Type I (reaginic) sensitivity or a less acute, predominantly Type III sensitivity, resembling ordinary serum sickness. The anaphylactic reactions are clinically most important and account for most of the deaths, but are less frequent than those so often associated with urticarial rashes. Smith and his colleagues (1966) put the incidence of penicillin reactions occurring in Johns Hopkins Hospital at 7.8%. This figure is very similar to that given by Smith & Zirk (1961) for both PAS and streptomycin. Anaphylactic reactions to PAS and streptomycin are very rare. Most of the reactions seen clinically occur after 1–3 weeks of treatment and the common manifestations are rashes which may be urticarial, scarlatiniform or of other types, fever, hepatitis (Smith & Springett 1966), and occasionally asthma, joint swelling and glandular enlargement are seen. When test doses are given to establish the cause of the reaction it is usually safe to give 10 mg as an initial dose followed by 100 mg then 1 g on subsequent days and finally 10 g in the case of PAS if no reaction occurs after earlier doses. Owing to the greater frequency of anaphylaxis associated with penicillin, test doses are very rarely used.

'Desensitization' is usually quite easily accomplished to PAS or streptomycin in 2–3 weeks by giving daily doses starting from one-tenth of the dose which caused the recurrence of reaction and increasing daily. Thus if 100 mg of streptomycin caused recurrence of the reaction and 10 mg did not the 'desensitization' is started with 10 mg increasing by 10 mg daily to 100 mg and then by 100 mg daily to the full therapeutic dose of 1 g.

Such a procedure is seldom attempted with penicillin because good alternative antibiotics are available and because the danger of anaphylaxis is much greater.

Although it is usually stated that drug reactions occur more frequently in atopic individuals or in those with an atopic family history this is probably only true of the anaphylactic (Type I) reactions. There is no evidence that the majority of the reactions which occur in the less severe form and seem to be of serum-sickness type are more common in atopic individuals (Samter & Berryman 1964; Smith 1962). This in itself suggests that two different mechanisms occur in allergy to these drugs. It is possible that there may be fundamental differences in the manifestations of drug serum sickness depending upon the extent to which any drug is bound in a particular tissue.

Levine (1964) states that most patients treated with penicillin have detectable serum antibodies but clinically allergic patients have much higher titres of antibodies of several types. He considers that penicillin allergy may be mediated by both 'fixed' reagin-type antibodies and by toxic soluble complexes. A report of the Food and Drug Administration of the United States of America on penicillin allergy occurring in 800 hospitals in a period of 4 years notes 793 cases of anaphylactic shock with 72 deaths, all from intramuscular injection. There were also 1616 cases of less severe reaction comprising urticaria and oedema in 1224 cases (4 deaths), other rashes in 319 cases (6 deaths), 'serum sickness' in 65 cases, diarrhoea in 2 cases and local moniliasis in 6 cases. It seems possible that the cases of urticaria and of other rashes were largely of the same immunological type as those classified as 'serum sickness'.

Clinical syndromes resembling erythema nodosum, Stevens-Johnson syndrome, Henock-Schönlein purpura, periarteritis nodosa and systemic lupus erythematosus have also been attributed to penicillin allergy.

Direct skin testing with drugs has been unsatisfactory but much work has been done to develop a test for penicillin allergy which would be reliable at least in identifying subjects likely to react with anaphylaxis. These efforts have not been entirely successful (British Medical Journal 1964). A protein combination with penicilloyl might sensitize subjects not previously sensitive if used clinically, but penicilloyl-polylysine has been reported by Parker (1964) to give up to 90% of positive immediate reactions in subjects clinically sensitive. It has not, however, been so successful in other hands and this again may be due to failure to separate patients with a Type I sensitivity who could be expected to give an immediate skin reaction from patients with a Type III sensitivity who would not.

PATHOGENESIS OF SERUM SICKNESS

Experimental Work on Animals

Dixon *et al* (1959) and Weigle (1961) have fully reviewed the experimental work on animals which has led to much clarification of our ideas on the pathogenesis of serum sickness in man. This has entailed the production of an analogous

disease by means of the intravenous injection of rather large doses of foreign proteins into animals, usually rabbits.

An intravenously injected foreign protein, say bovine serum albumin (BSA) in the rabbit, is removed at much the same rate as the animal's own autologous albumin until about the eighth day: the rate of removal then rapidly increases until none is detectable by about the twelfth day: from this time onwards antibodies specific to BSA begin to be detectable in the serum in rising titre. It can be shown that this sudden inflexion of the antigen-disappearance curve occurs as a result of the secretion of antibody into the circulation which is immediately combined with the excess circulating antigen: the complexes so formed are therefore soluble, as in the 'antigen-excess' zone of an *in vitro* antigen-antibody titration. A valuable advance was made when it was pointed out (Hawn & Janeway 1947; Germuth 1953; Dixon *et al* 1958) that under these circumstances lesions were demonstrable in the animal's tissues from the eighth day onwards, consisting of a generalized arteritis affecting especially the renal glomeruli and the endocardium. About the fourteenth day these lesions began to heal, and the tissues had returned to complete normality 2 or 3 weeks later. An essential point made early in this work was that the timing of the disappearance of the antigen, the appearance of antibody, and the development of the lesions was a function of the particular pure foreign protein; thus if γ-globulin was used instead of albumin, the inflexion of the disappearance curve occurred earlier, about the fifth day instead of the eighth, free antibody began to be demonstrable on the seventh to eighth days, and lesions first appeared on the sixth or seventh. It could be shown directly by appropriate techniques that during the stage of accelerating disappearance of antigen, complexes of antigen and antibody were present in the circulation (cf. Weigle & Dixon 1958) and, since free antigen was also demonstrable, these were naturally soluble, antigen-excess complexes. This and other work showed that complexes in a ratio of around three antigen to two antibody molecules (the molar equivalence ratio for BSA being of the order of one to five), are not readily filtered out by the liver and lungs, but are readily taken up by polymorphs and deposited in the intima of blood vessels, where they cause inflammation. Complexes formed *in vitro* behave in a similar way, and can produce an acute anaphylactic-like symptom in the guinea-pig (Germuth & McKinnon 1959) and characteristic cutaneous reactions as studied by Ishizaka & Campbell (1958). Cochrane & Weigle (1958) produced in the rabbit a lesion similar in all respects to a local passive Arthus reaction by the injection of BSA-anti-BSA complexes at 3 × equivalence with respect to antigen. It was shown by McCluskey & Benacerraf (1959) that intravenous injection of complexes made *in vitro* into mice could provoke the lesions of experimental serum sickness, although it has not been easy to reproduce this in the rabbit; in the latter species it appears that the complexes must be built up serially in the circulation before they can provoke the characteristic lesions in the deeper tissues.

Pathogenesis in Man

Two essential points have been brought out by the work: (i) It is very little use talking about the effects of antibodies to 'horse serum'. Horse serum, like any other mammalian serum, contains at least thirty antigenic components; and the amount of antibody produced against any one of them (which is entirely specific to that one particular component) and especially the timing of its appearance, is a function both of the concentration and of the intrinsic 'antigenicity' of that protein. (ii) Complexes between antigen and antibody in a particular ratio, of moderate antigen excess, can be demonstrated as being peculiarly noxious to the tissues in a variety of ways, while complexes in gross antigen excess, at equivalence, or in antibody excess, do not have this property. Taking these two points together, it is clear that damage may still be due to complexes in antigen excess, even though antibody to 'horse serum' is demonstrable in excess in the circulation—because the complexes are with a different serum protein from that against which the antibody is directed. This caveat is applicable also to 'purified' γ-globulin preparations, since they are unlikely to be wholly free of trace components of high antigenicity, such as the α_2-macroglobulin.

It is probable that at least two types of hypersensitivity are involved in the symptoms and signs of serum sickness in man.

Type I (*anaphylactic*) reactions may be involved when reagins are produced to, for example, trace components of crude or partially purified serum, or to very small amounts of conjugates of a particular kind with drugs. There is no reason to suppose that normal non-'atopic' subjects cannot produce typical reaginic-type antibodies, to judge from the animal experiments (see Chapter 24); the only difference may be that these are less regularly produced and last for a far shorter time. On the other hand it is still an open question whether under some circumstances 'ordinary' antibodies may be able to fix to cells in the same way that proper reagins do. The essential difference is, however, a quantitative one; if the amount of free antigen is at some time in considerable excess of the amount of antibody, either because antibody production is sluggish or because the dose of antigen is very large, a *Type III* reaction will result from the formation of toxic, antigen-excess complexes. Such complexes will form both in the blood stream, (as has been shown in experimental animals) and be localized in the walls of small blood vessels, and also at sites in the tissues where antigen is fixed in high concentration, in particular the initial injection site.

Though it is difficult to sort out the complexities of the lesions produced in man, one may postulate that the urticaria is probably due to Type I, the joint pains and general malaise, and also the lymphadenopathy, to Type III. Of course, if the total amount both of antigen and of antibody is small, or the phase of antigen excess over antibody is very short, damage and symptoms will be negligible or absent; and in any case as soon as antigen is eliminated either by

phagocytosis in complex with antibody or by normal metabolism, the damage heals completely. Variations in these quantitative, temporal and constitutional factors can, however, rationally explain the protean manifestations of human serum sickness and related syndromes.

REFERENCES

BRITISH MEDICAL JOURNAL (1964) Testing sensitivity to penicillin. *Brit. Med. J.* **1,** 1329.

COCHRANE C.G. & WIEGLE W.O. (1958) The cutaneous reaction to soluble antigen–antibody complexes: a comparison with the Arthus phenomenon. *J. exp. Med.* **108,** 591.

DE LA PAVA S., NIGOGOSYAN G. & PICKREN J.W. (1962) Fatal glomerulo-nephritis after receiving horse anti-human cancer serum. *Arch. Inter. Med.* **109,** 391.

DIXON F.J., VAZQUEZ J.J., WEIGLE W.O. & COCHRANE C.G. (1958) Pathogenesis of serum sickness. *Arch. Path.* **65,** 18.

DIXON F.J., VAZQUEZ J.J., WEIGLE W.O. & COCHRANE C.G. (1959) Forms and mechanisms of experimental serum sickness. In *Immunopathology*, ed. GRABAR P. & MIESCHER P. Basel/Stuttgart: Benno Schwabe.

GERMUTH F.G. (1953) A comparative histologic and immunologic study on rabbits of induced hypersensitivity of serum sickness type. *J. exp. Med.* **97,** 257.

GERMUTH F.G. & MCKINNON G.E. (1957) Anaphylactic shock induced by soluble antigen–antibody complexes in unsensitized normal guinea-pigs. *Fed. Proc.* **16,** 413.

HAWN C.V. & JANEWAY C.A. (1947) Histological and serological sequences in experimental hypersensitivity. *J. exp. Med.* **85,** 571.

HILDRETH, E.A. (1965) Editorial. *J.A.M.A.* **193,** 386.

ISHIZAKA K. & CAMPBELL D.H. (1958) Biological activity of soluble antigen–antibody complexes. I. Skin reactive properties. *Proc. Soc. exp. Biol. (N.Y.)* **97,** 635.

KARLINER J.S. & BELAVAL G.S. (1965) Incidence of reactions following administration of anti-rabies serum. *J.A.M.A.* **193,** 359.

LEVINE B.B. (1960) Studies on the mechanism of the formation of the penicillin antigen. *J. exp. Med.* **112,** 1131.

LEVINE B.B. (1961) Some reactions of D-benzyl penicillenic acid in aqueous solution at pH 7.5. *Arch. Biochem.* **93,** 50.

LEVINE B.B. & OVARY Z. (1962) The N (D α benzyl) penicilloyl group as an antigenic determinant responsible for hypersensitivity to penicillin G. *J. exp. Med.* **114,** 875.

LEVINE B.B. (1964) Studies on the immunological mechanism of penicillin allergy. I. Antigenic specificities of guinea-pig skin sensitizing rabbit anti-benzylpenicillin antibodies. *Immunology* **7,** 527.

LEVINE B.B. & PRICE V.H. (1964) Studies on the immunological mechanism of penicillin allergy. II. Antigenic specificities of allergic wheal-and-flare skin responses in patients with histories of penicillin allergy. *Immunology* **7,** 542.

LEVINE B.B. (1964) Immunological mechanisms involved in penicillin hypersensitivity in experimental animals and in human beings. *Postgrad. Med. J.* **40,** Suppl. 146.

MCCLUSKEY R.J. & BENACERRAF B. (1959) Localization of colloidal substances in vascular endothelium: a mechanism of tissue damage. *Amer. J. Path.* **35,** 275.

PARKER C.W. (1963) Penicillin allergy. *Am. J. of Med.* **34,** 747.

PARKER C.W. (1964) The immunochemical basis for penicillin allergy. *Postgrad. Med. J.* **34,** 747.

VON PIRQUET C. & SCHICK B. (1905) *Die Serum Krankheit.* English trans. (1951) *Serum Sickness.* Baltimore: Williams & Wilkins Co.

RICH A.R. & GREGORY J.E. (1943) The experimental demonstration that periarteritis nodosa is a manifestation of hypersensitivity. *Bull. Johns Hopk. Hosp.* **72,** 65.

SAMTER M. & BERRYMAN C.H. (1964) Drug allergy. *Annual Review of Pharmacology* **4,** 265.

SMITH J.M. (1962) Symposium on Drug Sensitivity. *Proc. R. Soc. Med.* **55,** 19.

SMITH J.M. & ZIRK M.H. (1961) Toxic and allergic drug reactions during the treatment of tuberculosis. *Tubercle, Lond.* **42,** 287.

SMITH J.M. & SPRINGETT V.H. (1966) Serum transaminase levels during treatment with isoniazid, PAS and streptomycin. *Tubercle, Lond.* **47,** 245.

SMITH J. W., JOHNSON J.E., CLUFF L.E. (1966) Studies on the epidemiology of adverse drug reactions. II. An evaluation of penicillin allergy. *New Engl. J. Med.* **274,** 998.

SMITH R.E. (1965) Allergic reactions to immunization materials in children. *Ann. Allerg.* **23,** 600.

TRINCA J.C. & REID J.C. (1967) Prevention of tetanus by anti-toxin of bovine origin. *Lancet, i,* 76.

VAISHNAVA H., GOYAL R.K., NEOGY C.H. & MATHEW G.P. (1966) A controlled trial and anti-serum in the treatment of tetanus. *Lancet ii,* 1371.

DE WECK A.L. & EISEN H.N. (1960) Some immunochemical properties of penicillenic acid as an antigenic determinant derived from penicillin. *J. exp. Med.* **112,** 1227.

WEIGLE W.O. (1961) Fate and biological action of antigen–antibody complexes (review). In *Advances in Immunology,* vol. 1, p. 28. New York and London: Academic Press.

WEIGLE W.O. & DIXON F.J. (1958) Relationship of circulating antigen-antibody complexes, antigen elimination and complement in serum sickness. *Proc. Soc. exp. Biol. (N.Y.)* **99,** 226.

CHAPTER 25

HORMONE RESISTANCE AND HYPERSENSITIVITY

J.G.DEVLIN

INTRODUCTION

The fundamental concept of distinguishing between self and non-self forms the basis for the immunologic problems associated with protein and polypeptide hormone administration. The intensity of the allergic response varies with the size and molecular weight of the hormones, ACTH m.w. 4500 being very weakly antigenic, even when heterologous hormone is used, while GH (growth hormone) m.w. 21,500 (Andrews 1966), used only as the homologous hormone, may induce antibody production in a very high proportion of cases (five out of six subjects, Roth 1964) to whom it is administered. Insulin, m.w. 6000, is intermediate in competence as an antigen, although antibody response to both heterologous and homologous administration is known (Brunfeldt & Deckert 1964; Renold *et al* 1964). The nature and extent of the antibody response to hormones will be influenced by the same factors which influence the immune response in general, some of which, however, require general consideration before proceeding to a more detailed study of the particular issues involved with the individual hormones.

GENERAL IMMUNOLOGICAL PROBLEMS

Size and Structure of Antigen

Immunological potency is determined by a number of factors which are as yet not clearly understood but include in addition to the number of amino-acids the variety of the particular antigenic amino-acids, and their internal structural relations (Borek *et al* 1965; Edsall 1966). The size of the antigen is also important, as polypeptides below 5000 (Humphrey 1962) are unlikely to be antigenic although some very low molecular weight antigens (1000 and less) have been described (Dietrich 1966). In addition to these factors consideration must also be given to the degree of similarity between the hormone acting as antigen and the existing endogenous hormone, and also to the possible difference in antigenicity resulting from total absence of the endogenous hormone. The latter has been postulated as one possible cause of antibody response to exogenous HGH (Prader 1964). ACTH (m.w. 4500) is now virtually non-antigenic (West 1962, Buytendijk & Maesen 1964), but a third special consideration must be taken into account in defining the antigenicity of this hormone, i.e. the effects of the increased steroid output in inhibiting the allergic response. Indeed, the effects of the varied altered metabolic states for which hormones have been administered on the allergic response in man have not been studied in detail. The specific significance of structure in relation to hormone antigenicity is exemplified by the different immunological cross-reactions observed by Berson (1963) between sperm whale and pork insulins which have a similar amino-acid structure with presumably different tertiary structure.

Impurities

The whole question of the antigenicity of polypeptide and protein hormones has been clouded and continues to be clouded by the possible presence of small traces of protein 'impurities' in the preparations used. Local and systemic hypersensitivity phenomena to insulin and ACTH are decreasing, with improvements in extraction procedures. However, with reference to insulin some authorities (Brunfeldt 1966) believe that it much more difficult to extract beef insulin without associated impurities than pork insulin.

Species Source

As the differences in structure between hormones from heterologous species increases, their antigenicity increases also. It follows that the ideal hormone for man is human or the closest related heterologous hormone. With the small molecular weight hormones (ACTH 4500, glucagon 3500, parathormone 8500 and insulin 6000) which are relatively weak antigens, resistance problems are not as serious as with the larger hormones such as growth hormone (21,500).

Clinical resistance when it does occur to the human preparations of these hormones is a more serious problem as the limited availability renders adequate therapy impossible.

Physicochemical Characteristics of Administered Hormone

Antigenicity is increased by the necessity to administer hormones as relatively insoluble preparations. Solubility of the extracted preparation has been a limiting factor in permitting administration at physiological pH, although with improvement in extraction techniques, preparations of insulin, for example, at neutral pH are becoming available. At the lower pH (3.0, Prader 1964) at which growth hormone is administered, aggregation is known to occur (Andrews, 1966). Not only does polymerization improve antigenic potency, but it can also render non-antigenic preparations potently antigenic (Dresser 1962; Edsall 1966; Claman 1966). Some such mechanism is probably responsible for rendering the homologous hormone antigenic, although other possibilities, viz. alteration of tertiary structure during extraction, or the inclusion of minute traces of non-hormone protein from the parent gland must be considered. The differences in solubility between beef and pork insulin (Schlichtkrull 1958) resulting in the administered beef insulin more frequently existing in an insoluble form at the injection area (e.g. ultra lente *v.* semi-lente) probably accounts for some of the described greater antigenicity of the beef preparation.

Mode of Administration

Three factors known to influence the allergic response must be considered here: (a) the administered dose, (b) the route of administration, and (c) the frequency of administration.

The dose administered

This is determined by the pathophysiological needs of the patient. With the increase in dose of insulin required by stress, e.g. infection, increase in antibody response has been noted, in accordance with the general principles described by Stevens (1956, 1957). Accurate estimations of antibody concentration in relation to antigen are not available, however.

Route of administration

Most hormones are administered by the subcutaneous route. It is known that the antibody response to many protein antigens in adjuvant in experimental animals can be drastically reduced, or the animal may even be rendered tolerant by the simultaneous or prior administration of the antigen in an aqueous solution (Weigle 1966; Dresser 1962). Insulin is frequently administered initially in a highly soluble form intravenously which could theoretically reduce the allergic

response. Unfortunately no studies are available relating the intensity of the allergic response to the initial mode of administration of the antigen. Such a study, if it should indicate a reduction in antibody titre or be associated with the induction of a state of tolerance as a result of initial intravenous therapy in a proportion of cases would serve as a directive to future hormone therapy. Practical application of the Liacopoulos (1962) phenomenon (induction of non-specific immune suppression with large doses of unrelated antigen), has yet to be made in this field.

Hormones administered by inhalation (see under Vasopressin) can induce an allergic response, and the antibodies so produced can cross-react with the endogenous hormones. A retrospective study carried out by me some time ago, attempting to relate diabetes to ingestion of pancreas, yielded negative results (unpublished obs.).

The frequency of administration

Although precise data are lacking the most advantageous time interval between first and second exposure to antigen in man to induce maximum antibody response is considered to be 4 weeks (Edsall 1966). With most protein and polypeptide hormones, the rate of administration varies from two to three times daily (insulin in some cases) to once or twice weekly (growth hormone). In some cases the latter hormone is administered once monthly. No definite relationship between frequency of administration for therapeutic purposes and the allergic response in man has been established, beyond the enhancement of the allergic response which has been noted on reinstitution of therapy following cessation. The more frequent administration demanded by therapeutic requirements has probably diminished the possible maximal antibody response.

Individual Variations

As with the guinea-pig (Arquilla & Stavitsky 1956; Wright 1966) many workers have demonstrated (Berson 1959b; Devlin & Brien 1965; Feldman 1963) not only a varying allergic response in terms of overall antibody concentration but also marked individual variations in the cross-species-specific reactivity of the antibody produced. Presumably such differences are based on genetic considerations (Arquilla & Finn 1965) although the influence of slight variations in the other factors mentioned already would have to be considered, in addition to variations in the species homogeneity of the commercial hormone preparations. The allergic response to the individual hormones will now be discussed in greater detail. With the exception of insulin, information is scanty. The greater part of the discussion, therefore, is taken up with an account of resistance and hypersensitivity problems pertaining to insulin, and it is assumed that the general principles relating to techniques of investigation, binding capacity and hypersensitivity phenomena apply also to the other protein and polypeptide hormones in greater or lesser degree depending on their antigenicity.

INSULIN

Shortly after the introduction of insulin therapy, reports of local and systemic hypersensitivity reactions were reported in addition to insulin resistance. It was noted that changing the brand of insulin frequently effected a cure, thus suggesting an allergic response to either the species source of insulin or else to associated impurities in the preparation (Lawrence 1925). Although the significance of the role of 'impurities' in the insulin preparation in the aetiology of the local hypersensitivity reactions has not yet been finally solved, the immunologic basis for resistance and systemic hypersensitivity has been firmly established. Lerman's (1944) hypothesis of an immunologic basis for insulin resistance was confirmed by Lowell's demonstration of an insulin-neutralizing factor in the serum of an insulin-resistant diabetic patient. By means of parallel determinations of the titre of the passive transfer reaction (Prausnitz-Küstner) and of the serum quantities required to protect mice from insulin hypoglycaemia he differentiated between the 'reaginic' and neutralizing antibody. Loveless (1945) subsequently fractionated two antibodies to insulin from human serum, and de Filippis *et al* (1952) localized the neutralizing antibody in the γ-globulin fraction. Before describing the allergic response in greater detail and discussing its clinical significance, it is necessary to describe some of the laboratory procedures used and their limitations in addition to giving an account of the immunoglobulins to insulin which have been observed.

Radio-immunoelectrophoresis

This technique has been used successfully by a number of authors (Morse & Heremans 1962; Yagi *et al.* 1963; Devlin & O'Donovan 1966) to characterize insulin immunoglobulin patterns. The technique can detect less than 1 μg of reacting antibody, and has been found to be roughly quantitative. Briefly the technique consists in reacting the serum under examination with labelled insulin (I-131 or I-125) and effecting separation of the immunoglobulins by electrophoresis on microslides and precipitation of the individual immunoglobulins with immunoglobulin-specific antiserum. The presence or absence of insulin binding by the precipitated immunoglobulin is then detected with sensitive film.

Insulin binding by immunoglobulins which are themselves present in low titre, e.g. IgM, can be difficult to detect, due to failure to obtain adequate precipitin lines. Frequently more than one batch of specific IgM immunoglobulin antiserum must be used therefore before a satisfactory result is obtained. Adequate purification of the labelled insulin ($< 5\%$ damaged products) and the introduction of normal sera as controls are essential as some non-specific adsorption of the labelled material by serum proteins always occurs (Merrimee *et al* 1965).

Total Binding Capacity

Following the procedures established by Berson & Yalow (1959a) many authors have estimated total or maximum binding capacity of hormones by sera, using the same basic technique with minor modifications. Basically the concept is to determine the percentage binding by the test serum of different concentrations of the hormone, using a fixed quantity of isotopically labelled hormone to trace the percentage bound (B) and the percentage unbound or free (F). A straightforward calculation gives the amount of hormone bound at each concentration of added hormone. Then by plotting the B/F (tracer hormone) on the ordinate against the total hormone bound on the abscissa, and extrapolating the curve so obtained until it intercepts the abscissa when the B/F = 0, a figure for the total binding capacity is obtained (see Fig. 25.1). Alternatively the reciprocal of the

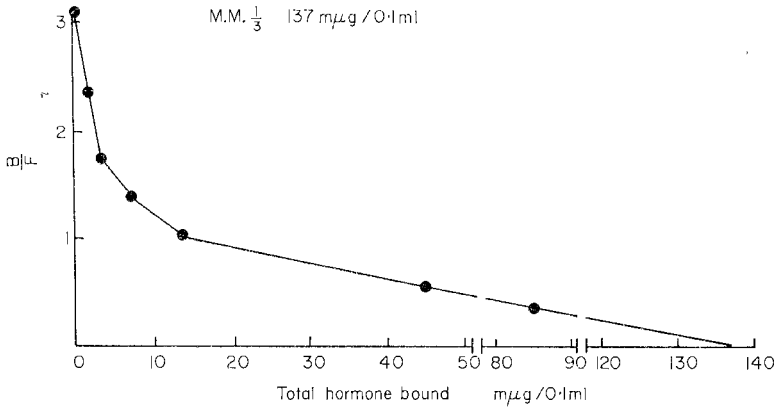


FIG. 25.1. Determination of total binding capacity; serum diluted 1/3; total binding capacity 137 mμg/0.1 ml.

bound hormone on the ordinate may be plotted against the reciprocal of the total hormone on the abscissa when a straight-line relationship obtains, which can be extrapolated back to the ordinate from which the total binding capacity may be estimated. The basic laboratory procedures are identical, i.e. determination of the concentration of hormone bound by antibody at varying concentrations of added hormone. The separation of bound and free hormone may be accomplished by a variety of techniques (Chapter 10). Some technical problems must be appreciated before results obtained by this technique from different centres are compared.

1. Damaged isotope fractions and non-specific adsorption of labelled hormone to plasma protein fractions. As is seen in Fig. 25.1 the Ag/Ab reaction is biphasic, with two equilibrium constants. Accuracy in determining the points on the steep first part of the curve where highly significant alterations in the B/F for

relatively small changes in total hormone bound occur, is easy to achieve, but in the second phase where quite large alterations in hormone bound for relatively small changes in B/F occur difficulties arise. From 4 to 6% of the determined total activity bound is non-specific due to adsorption by proteins of the damaged fraction and possibly some of the intact hormone. As the B/F approaches zero the alteration produced in percentage bound (tracer) approaches and becomes less than the total non-specific 'bound', thus introducing the possibility of additional error. A variation of at least 10–15% between replicate experiments occurs as a result of this problem (Berson 1959a).

2. Alteration in antigenic reactivity of the hormone with labelling. Inherent in this technique is the assumption that the unlabelled and labelled hormone are 100% immunologically similar. Berson has found that whether he uses labelled hormone alone or tracer amounts of labelled hormone plus unlabelled hormone, identical results are obtained. However, Brunfeldt (1966) has shown that the addition of only one atom of iodine/molecule insulin can result in a 20% drop in immunologic competence, hence all determinations of total binding capacity are subject to error, an error which may vary from laboratory to laboratory, and within the same laboratory to a lesser extent depending on the degree of iodination (however, see Glover *et al* 1967).

3. Antigen-antibody equilibrium. The experimental conditions at which equilibrium is established must be rigidly controlled as the antigen-antibody reaction is known to be temperature dependent. With insulin and growth hormone at least 5 days and 10 days respectively are required at 4°C to establish equilibrium, while the glucagon-antibody reaction is extremely unstable at room temperature (Heding 1966). The age and method of storage of the labelled hormone can also alter its antigenic reactivity significantly, e.g. Jorgensen (1966) finds that storage of labelled insulin at room temperature for 3 days results in a 20% drop in immunologic reactivity. Protein and anion concentration can also influence the insulin-antibody reaction significantly (Heding 1966: Devlin & Stephenson 1967).

Insulin-Binding Immunoglobulins

Although precipitating antibodies to insulin are well documented in experimental animals (Birkinshaw *et al* 1962—sheep and guinea-pig: Jones & Cunliffe 1961—guinea-pig; Moloney & Aprile 1959—horse; Steigerwald 1960—dog) most workers agree with the observations of Skom & Talmage (1958) and Berson (1959a, b) that the human insulin-antibody complex is non-precipitating. Some early publications (Tuft 1928; Karr *et al* 1931) contained accounts of precipitation in capillary tubes, but the possibility of antigen contamination with impurities seems the most likely explanation for these observations in view of subsequent experience. Recently Penchev *et al* (1966) demonstrated precipitin antibodies in untreated diabetics, an observation at variance with the experience

of all studies using tracer insulin to detect antibody; the sensitivity of the latter technique is such that less than 1 mμg antibody can be detected.

IgG immunoglobulin

IgG insulin-binding antibody has been detected as early as the 14th day of insulin therapy (Devlin 1967) and is well established by the 4th week. It has been characterized by chromatographic, ultracentrifugation and radio-immunoelectrophoretic techniques (Chao *et al* 1965; Toro-Goyco *et al* 1966; Morse & Heremans 1962; Yagi *et al* 1963; Devlin & O'Donovan 1966). It is the antibody associated with insulin resistance, and in the vast majority of insulin-treated patients it is the only insulin-binding immunoglobulin present. Fast- and slow-moving components which can be readily separated by ion-exchange chromatography, and which have similar equilibrium constants, have been identified. Both fast- and slow-moving components appear simultaneously (Devlin 1967).

IgM immunoglobulin

Insulin-binding immunoglobulins of this class have been found as the antibody of the primary response (Devlin 1966). It may be present as early as the 2nd day of insulin therapy and can persist for up to 43 days. It has also been found, although rarely, in patients on long-term insulin therapy and Yagi *et al* (1963) have noted its presence in addition to IgA insulin-binding immunoglobulin in one patient with systemic hypersensitivity.

IgA immunoglobulin

With the exception of the patient mentioned above by Yagi no definite evidence for the existence of insulin binding in this immunoglobulin class exists. It has been presumed hitherto that the antibody responsible for passive transfer reactions and which was also described earlier by Loveless (1945) as a heat-labile antibody was the 'reagin' antibody associated with systemic hypersensitivity. However, the recent observations of Ishizaka and his co-workers (see Ishizaka *et al* 1966c), in which a new immunoglobulin class, IgE, has been reported in association with reaginic activity, would indicate that previous reports on the incidence of IgA reaginic antibody be considered with caution and that a fresh reappraisal of reaginic antibody in insulin therapy is indicated.

Insulin Resistance

Incidence

Absolute figures on the incidence of insulin resistance (i.e. insulin requirements in excess of 200 u/day in the absence of aggravating factors, e.g. infection (Martin *et al* 1941), are not available, but the suggested figure from literature surveys is less than 0.1% (Shipp *et al* 1965). There are no figures on the incidence of anti-

body-related resistance amongst resistant cases, due to the inadequate technical procedures available for such studies until recently, although the available evidence suggests that it comprises at least 60% of cases studied (see Shipp *et al* 1965). However, as the basic physiological insulin requirement of the depancreatectomized subject lies in the region of 40 units/day, a state of relative insulin resistance is much more common, as the mean insulin requirement of a juvenile diabetic is closer to 70 units/day. Studies of the maximum insulin-binding capacity in insulin-resistant cases yield figures in excess of 60 units/litre, and usually in excess of 200 units/litre (i.e. 2.6–7.3 μg/ml, or higher) (Berson & Yalow 1959a; Morse 1961; Feldman *et al* 1963). There is only a very approximate relationship between binding capacity and insulin dosage from patient to patient (Berson & Yalow 1959a; Prout and Katins 1959) although there is a definite relationship between alterations in total binding capacity and insulin requirements within the same patient (Morse 1961). The failure to demonstrate an exact relationship between binding capacity and insulin dosage arises from a number of factors.

1. Total binding capacity is an *in vitro* figure established by extrapolation under steady-state conditions which cannot obtain *in vivo*. The free hormone concentration and therefore the available biologically active hormone concentration is determined by the equilibrium constants of the antigen-antibody reaction, and the concentrations of antigen and antibody according to the general formula

$$K = \frac{(\text{antigen:antibody})}{(\text{antigen})\,(\text{antibody})}.$$

As the rate of destruction of free insulin is independent of concentration over a very wide range, the permitted available concentration of free hormone is determined by the biological requirements. Adequate hormone must be administered, therefore, to satisfy the antibody characteristics at the required biological concentration of free hormone. This is determined by the rate constants of the reaction in addition to the total binding capacity. Antibody with quite different affinity for antigen can have similar total binding capacity; for example, the total binding capacity for different species of insulin by the same serum, assuming there is immunologic cross-reaction, is similar, although the equilibrium constants are quite different (Berson 1959a). Hence the antigen-antibody affinity must also be considered in any quantitative study of the effect of antibody on hormone requirements. That the affinity of antigen for antibody relates to the hormone requirement even in the relatively resistant diabetic patient, was demonstrated by the observation that there is a straight-line relationship between the clinical requirements of beef and pork insulin and the relative affinity of the antibody for beef and pork insulins as determined *in vitro* (Devlin *et al* 1967), see Fig. 25.2.

2. The pathophysiological insulin requirements of the diabetic state vary from patient to patient depending on a number of known factors, e.g. infection, and on the presence or absence of inhibitors or antagonists. Thus the biologically determined concentration of available hormone is independent of the immunological factors. It seems unlikely that complexed hormone is biologically active.
3. Earlier studies in which insulin haemagglutination antibody titre studies failed to correlate with insulin dose (Moinet 1958) can be partly explained by the relatively greater effect of insulin-binding IgM antibody on this reaction (Devlin & Laher 1966). The 'blocking' antibody of insulin resistance as was mentioned earlier has been shown to belong to the IgG immunoglobulin class.

Insulin Hypersensitivity

This may be local or systemic. Local reactions may be immediate, i.e. developing within $\frac{1}{2}$–2 hr after injection and persisting for 12–24 hr, or delayed, i.e. developing within 6–24 hr after injection and persisting for 1–4 days. They never occur with the first insulin injection, usually develop within the first week and persist for 2–4 weeks, and frequently recur on resumption of insulin therapy when the latter has been omitted. They can appear for the first time during the course of insulin treatment, however, without any specific relationship to onset of insulin therapy or to alteration (Kreines 1965). Local reactions may persist and progress to systemic reactions. The systemic reactions may take the form of anaphylaxis, serum sickness, gastro-intestinal symptoms and very rarely thrombocytopenic purpura (Constam 1956). Hypersensitivity phenomena occur in a high percentage of cases of true insulin resistance (from 7 to 30% of cases, Burkart *et al* 1963; Daweke 1966; Davidson *et al* 1950; Shipp *et al* 1965).

Systemic Insulin Hypersensitivity

The overall incidence of systemic hypersensitivity is probably less than that of insulin resistance, i.e. 0.1%, with which it is frequently associated. Positive Prausnitz-Küstner reactions, and insulin-binding IgA immunoglobulin have been found in this condition (Yagi *et al* 1963). No specific study with reference to IgE has been completed. With the possible exception of the reports by Harris-Jones *et al* (1963) gamma A antibody to insulin has not been found except in the presence of systemic insulin hypersensitivity (Morse 1962; Devlin & O'Donovan 1966). Intradermal testing with crystalline insulin results in a positive weal and flare reaction (Kreines 1965; Pfeiffer 1966), and can be used to distinguish species-specific hypersensitivity.

Local Insulin Reactions

As with systemic reactions the incidence of local reactions is decreasing with the improvement in the purity of insulin preparations. Severe local reactions, immediate in type, described as being Arthus phenomena (Type III reactions, see

Chapter 20), were a much more prominent feature of earlier publications although sporadic case histories continue to be published (Schirren 1953). Three types of local reactions are discussed—weal and flare, Arthus reaction and delayed hypersensitivity.

Weal and Flare Reactions

Studies of the weal and flare reactions to insulin have produced fruitful but confusing results. Earlier studies in this field (see Paley & Tunbridge 1950, 1952) have highlighted the importance of outruling false positive reactions due to either the physicochemical state of the test substance or to 'secondary' proteins. With the single exception of the observation that it is positive in patients with systemic hypersensitivity reactions, i.e. with urticarial reactions and eosinophilia, universal agreement on the remaining aspects of the reaction is lacking. There is good evidence that the reaction is positive in a high percentage of insulin-treated patients (53%) compared with normal controls (10%) (Arkin *et al* 1962). These studies are supported by the report that 50% of a beef insulin only treated diabetic population show positive reactions to beef insulin while only 6% show similar reactions to pork insulin (Devlin & O'Donovan 1966).

There is good evidence, therefore, to indicate that the reaction is due to an antibody to insulin presumably tissue fixed as the weal and flare reaction in a local variety of a Type I reaction. The nature of the antibody in this reaction is obscure. In the presence of a systemic hypersensitivity state with eosinophilia, etc., presumably a specific reagin antibody is involved. However, in the absence of systemic hypersensitivity it must be postulated that either a reagin antibody is present, but is rendered ineffective by excess blocking antibody, or else that reaginic activity can be associated with more than one immunoglobulin class. While no specific studies with reference to IgE have been reported, it is known that neither IgA nor IgM is essential for the reaction, and there is evidence from radioimmunoelectrophoretic studies (Devlin & O'Donovan 1966) suggesting a relative increase of the fast-moving (γ_1) component of the IgG complex. IgG is certainly widely distributed in the human dermis (Allansmith *et al* 1964) and it is possible that in some circumstances it may have reaginic activity (Terr & Bentz 1965). Heat aggregation of human γ-globulins which are otherwise non-toxic, can result in the production of dermal toxicity (Ishizaka 1963), and perhaps the interaction of antigen with the skin-fixed IgG antibodies can be associated with the development of 'reaginic' activity. On the other hand, perhaps the newly discovered reaginic IgE antibody is widely distributed.

Arthus Reaction

There is some doubt as to whether these reactions are due to an allergic response to insulin alone or to insulin plus an associated 'secondary' protein. The case described by Schirren, in which careful histological studies are reported, was

certainly due to an associated protein in the depot insulin preparation. Also Poulsen (1966) reports that with the increasing purity of Nordisk insulin preparations no local reactions have been observed at the Steno Memorial Hospital for over 10 years. It is possible, therefore, that the reaction between crystalline and IgG antibody may not be associated with an Arthus-type reaction in man especially as (1) the reaction is not complement dependent (see Chapter 10), (2) there is a relatively low incidence of local reactions despite the presence of IgG antibodies, in varying titres, both high (Oakley *et al* 1959, etc.) and low in insulin-treated patients, (3) the reported absence of local reactions in 106 cases treated only with crystalline insulin (Altshuler 1937) and (4) the extreme solubility of the crystalline insulin-antibody complex (see Berson & Yalow 1959, etc.). Of some significance, maybe, is the observation that local reactions are more frequent in the early stages of insulin therapy, during the time of the primary antibody response. They are associated with a demonstrable IgM insulin-binding antibody (Devlin & O'Donovan 1965; Devlin 1966). IgM antibody is capable of inducing reversed Arthus-type reactions in guinea-pigs (Tada & Ishizaka 1965), and theoretically would be more likely to be associated with the aggregation which is probably necessary for Arthus-type reactions (Levenson & Cochrane 1964). The fact that precipitation with this antibody and insulin has not been demonstrated using whole sera may be a question of titre and time of testing. Further study, both histologic and experimental, is required before the true incidence of Arthus-type reactions can be stated, and the antigens involved described.

Delayed Hypersensitivity

Although local reactions to insulin have been subdivided into immediate and delayed, the clinical descriptions do not permit a distinction between a delayed immediate response and a true delayed Type IV reaction. There are very few accounts of detailed sequential histological studies. Recently, however, Federlin *et al* (1966) have demonstrated the existence of true delayed hypersensitivity to insulin mediated by lymphocytes. The reaction was species specific. In some of the cases studied there were also humoral antibodies (immunoglobulin class not determined).

Theoretical Implications of the Allergic Response

Apart from the problems of resistance and hypersensitivity there are two further theoretical possibilities which require brief mention.

Allergic response and diabetic complications

The possible role of antigen-antibody reaction in the mechanism of diabetic glomerulosclerosis and retinopathy has been investigated by Berns *et al* (1962) and Coleman *et al* (1962), who observed that fluorescein-labelled insulin will fix

to these lesions. However, as the glomerular and vascular lesions associated with diabetes can apparently be observed both in the experimental animal and in the man, in the absence of any exogenous insulin therapy (see MacDonald & Ireland 1964; Rees *et al* 1964), an allergic basis for these diabetic complications seems unlikely. Also fixation of fluorescein insulin to hyalin material can be relatively non-specific (Berns *et al* 1963).

Islitis of islets of Langerhans in insulin-treated animals

Round-cell infiltration of the islet tissue has been reported by Renold (1964) and Grodsky (1965) in the cow and rabbit respectively which have been treated with insulin and in whom there has been a detectable allergic response. In some of the rabbits diabetes has occurred. The incidence of diabetes is, however, quite low, despite high levels of antibody which can cross-react with the endogenous insulin. However, the fact that proven diabetes has occurred in the experimental animal, as a result of an allergic reaction, certainly suggests that a similar reaction can take place in treated human subjects. There is no documentary evidence as yet of such a reaction. The histologic nature of the lesion in the islet tissue of the diabetic animals was closely related to the histology of auto-allergic disease in general. It has been considered, therefore (Grodsky 1966; Federlin 1966), that the reaction is a manifestation of delayed hypersensitivity (Type IV). The ultimate prognosis of the reaction, if it does occur in man, may be good, as one of the two diabetic rabbits described by Grodsky *et al* improved spontaneously.

Therapy of Insulin Resistance and Systemic Hypersensitivity

This problem, occurring in less than 0.1% of diabetic patients, is not frequently encountered. Resistance also frequently regresses spontaneously, and often quite rapidly, resulting in serious hypoglycaemia (Michel 1965; Shipp *et al* 1965) and death.

Resistance

By determining the effectiveness of insulin from different species to displace a labelled insulin, a preferential binding factor of the serum for one species of insulin with reference to another is obtained. Assuming, for example, that the patient is resistant to beef but sensitive to pork insulin the preferential binding of the serum for beef insulin is obtained in the following manner. Serum aliquots are incubated with labelled beef insulin. Three groups of sera are incubated; to one (control) group buffer alone is added, to another group a fixed aliquot of cold beef insulin and to the third group a similar aliquot of cold pork insulin is added. The preferential binding of the serum for beef with reference to pork insulin is then obtained from the formula:

$$\frac{\%\text{ binding (control)} - \%\text{ binding (beef)}}{\%\text{ binding (control)} - \%\text{ binding (pork)}}.$$

This factor has been shown to be directly related to the subsequent clinical requirements of the test insulin (see Fig. 25.2). If an obvious preference is detected then appropriate therapy can be instituted. In this manner a number of patients have been satisfactorily treated with pork insulin. Where mixtures of beef/pork insulin have been used, species-specific resistance is apparently always

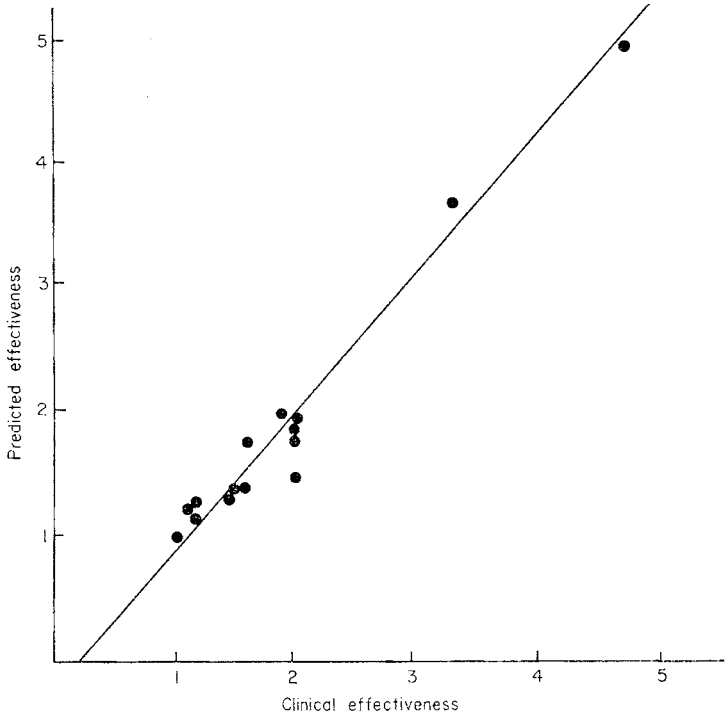


FIG. 25.2. Relationship between predicted and clinical effectiveness of pork insulin in a beef-insulin-treated diabetic population (Devlin, Brien & Stephenson 1967).

developed against the beef insulin. If no species specificity can be demonstrated, then steroid therapy can reduce insulin requirements. Immunosuppressive agents may be of some value, but available evidence is insufficient.

The prognosis following alteration from beef to pork insulin is good, as no gross change in either the preferential binding factor or insulin-binding capacity was detected in patients up to 18 months after such a changeover (Devlin *et al* 1966) nor was there any detectable evidence of a primary antibody response to the pork insulin.

Hypersensitivity

Hypersensitivity may exist to all available species of insulin or may be limited to insulin from one species. The intensity of the weal and flare reaction to the intradermal inoculation of insulins either from different species or modified in some other way (viz. dealanineated porcine insulin) can be used to determine whether the hypersensitivity reaction is restricted to one species or type of insulin. In this way porcine, dealanineated porcine (Kreines 1965), sulfated insulin (Menczel *et al* 1966) and human insulin can be used to overcome specific hypersensitivity states. Should the patient be hypersensitive to all types of insulin then, if insulin therapy must be continued, the patient must be desensitized (Corcoran 1938). The report on the value of heating the insulin (Dolger 1952) has not been confirmed (Loveless 1958). With some patients (Jorpes 1949) crystalline insulin has been of some use in overcoming both local and systemic hypersensitivity states. The latter report, however, of 300 cases of insulin hypersensitivity treated with crystalline insulin, arose directly from the difficulties in obtaining highly purified preparations for therapeutic purposes during the last war.

OTHER HORMONES

Growth Hormone

The allergic response to non-human growth hormone precludes its use in man. Human growth hormone, m.w. 21,500 (Andrews) would be expected to be antigenic in man on the basis of the experience above with homologous insulin. Antibody formation has been reported from numerous centres (Prader 1964; Roth 1964; Frasier 1966; Parker 1964), and has been associated with resistance to the hormone, although this is not invariable. Significant resistance in 50% of cases treated with human growth hormone has been reported by Parker & Daughaday (see Melick *et al* 1967). This has not been observed in all centres (Cerasi & Luft 1966). The variable incidence of serious resistance relates in part to the purity of the preparation (Prader *et al* (1964) used a preparation at pH 3 which was not always a clear solution). Other factors such as biological variation may also be involved. The question of pH and of aggregation has already been discussed.

Growth hormone binding-capacity studies carried out on resistant patients have revealed binding capacities varying from 1.9 to 100 μg/ml. There is a rapid fall in antibody titre on cessation of therapy which rises rapidly on reinstitution. Trials of steroid or other immunosuppressive therapy in resistance have not been reported. Hypersensitivity phenomena, of either a local or systemic nature, have not so far been reported. The antibody involved in resistance has been localized to the IgG immunoglobulin class.

Adrenocorticotrophic Hormone

Local and systemic hypersensitivity reactions resulting occasionally in anaphylactic reactions causing death occur in 5% of cases treated with ACTH (Buytendijk *et al* 1964). The local reactions are both acute and delayed in type. The incidence of these reactions is decreasing, a decrease which is attributed to the increasing 'purity' of the preparations (West 1962). Both bovine and porcine ACTH are used.

Therapy

The synthetic polypeptide containing the first twenty-four amino-acids of the ACTH molecule can be used in hypersensitive patients.

Follicle-Stimulating Hormone and Human Chorionic Gonadotrophins

Human FSH and chorionic gonadotrophins have been used intermittently for periods of up to 2 years in some patients and after a lapse of 3 to 4 years in others without evidence of either resistance or hypersensitivity phenomena (Gemzell 1964, 1967). Crookes (1966) from a group of eighteen patients reported the development of local reaction in one patient to FSH but not to HCG. The patient also became resistant to FSH.

Vasopressin

Antibodies capable of reacting with lysine vasopressin (m.w. 1056) and presumably also with arginine vasopressin have been reported by Pepys *et al* (1966), as a result of inhalation therapy of crude bovine and porcine pituitary extracts as snuff. Reaginic antibody resulting in positive weal and flare reactions and passive transfer reactions with the crude extract was noted. Also precipitin antibodies to antigens of both heterologous and homologous pituitary glands were noted. Positive immunofluorescence by two sera with human pituitary, adrenal medulla and brain tissue was also observed. The latter appeared to be due to an antibody response to the heterologous pituitary extract and not to be a true auto-antibody. The precipitin antibodies were associated with pulmonary mottling similar to farmer's lung.

The unusual mode of entry of the antigen in this case suggests that a breakdown of tolerance as a result of exposure to cross-reacting antigenic material by other routes, e.g. gastro-intestinal tract, could occur and, if so, could conceivably form a theoretical basis for the demonstration of auto-antibodies to insulin found by Penchev (1966) and suggested by the complement-fixing insulin:serum reaction in untreated diabetic patients reported by Pav (1963).

Parathormone

Melick *et al* (1967) reported the association of clinical resistance to parathyroid hormone with antibody formation in one patient. The preparation used was

relatively crude. Antibody formation in man to highly purified parathormone has not been reported. Clinical resistance to parathormone is not a serious clinical problem as therapy of hypoparathyroidism with vitamin D is satisfactory. Idiopathic hypoparathyroidism is not associated with auto-antibodies (Melick *et al*).

REFERENCES

ALLANSMITH M., GOIHMAN-YAHR M. & BUELL D.N. (1964) Demonstration of gamma-2 globulin in human skin. *J. Allergy* **35,** 313.

ALTSHULER S.S. (1937) The clinical use of crystalline insulin. *Ann. Int. med.* **11,** 901.

ANDREWS P. (1966) Molecular weights of prolactins and pituitary growth hormones estimated by gel filtration. *Nature* **5019,** 155.

ARKIN J.A., ENGBRING N.H. & LENNON E.J. (1962) The incidence of skin reactivity to insulin in diabetic patients. *J. Allergy* **33,** 69.

ARQUILLA E.R. & FINN J. (1965) Genetic control of combining sites of insulin antibodies produced by guinea pigs. *J. Exp. Med.* **122,** 771.

ARQUILLA E.R. & STAVITSKY A.B. (1956) The production and identification of antibodies to insulin and their use in assaying insulin. *J. Clin. Invest.* **35,** 458.

BERNS A.W., OWENS C.T. & BLUMENTHAL H.T. (1963) A histo and immunopathologic study of the vessels and islets of Langerhans of the pancreas in diabetes mellitus. *J. Geron.* **19,** 176.

BERNS A.W., OWENS C.T., HIRATA Y. & BLUMENTHAL H.T. (1962) The pathogenesis of diabetic glomerulosclerosis. II. A demonstration of insulin binding capacity of the various histopathological components of the disease by fluorescence microscopy. *Diabetes* **11,** 308.

BERSON S.A. & YALOW R.S. (1959a) Quantitative aspects of the reaction between insulin and insulin-binding antibody. *J. Clin. Invest.* **38,** 1996.

BERSON S.A. & YALOW R.S. (1959b) Species-specificity of human antibeef, pork insulin serum. *J. Clin. Invest.* **38,** 2017.

BERSON S.A. & YALOW R.S. (1963) Antigens in insulin: determinants of specificity of porcine insulin in man. *Science* **139,** 844.

BIRKINSHAW V.J., RANDALL S.S. & RISDALL P.C. (1962) Formation of precipitin lines between insulin and anti-insulin serum produced in sheep and guinea pigs. *Nature* **193,** 1089.

BOREK F., STUPP Y. & SELA M. (1965) Immunogenicity and role of size: response of guinea pigs to olygotyrosine and tyrosine derivatives. *Science* **150,** 1177.

BRUNFELDT K. (1966) Personal communication.

BRUNFELDT K. & DECKERT T. (1964) Antibodies in the pig against pig insulin. *Acta endocr. (Kbh.)* **47,** 367.

BURKART F., HARTMANN G., FANKHAUSER S. & KOLLER F. (1963) Insulinresistenz und insulinallergie. *Schweiz. Med., Wschr.* **93,** 1247.

BUYTENDIJK H.J. & MAESEN FR. (1964) Comparative skin tests with animal and synthetic corticotrophin in patients hypersensitive to animal corticotrophin. *Acta endocr. (Kbh.)* **47,** 613.

CERASI E. & LUFT R. (1966) Personal communication.

CHAO P.Y., KARAM J.H. & GRODSKY G.M. (1965) Insulin I-131 binding in serum from normal and diabetic subjects by ultracentrifugation and gel-filtration. *Diabetes* **14,** 27.

CLAMAN H.N., BRENSKY E.A. & BAUGHMAN J. (1966). Production of tolerance in immunized mice and the effects of immunosuppression. *J. Allergy* **38,** 208.

COLEMAN S.L., BECKER B., CANAAN S. & ROSENBAUM L. (1962) Fluorescent insulin staining of the diabetic eye. *Diabetes* **11,** 375.

CONSTAM S.R. (1956) Thrombocytopenic purpura as a probable manifestation of insulin allergy. Report of a case. *Diabetes* **5,** 121.

CORCORAN A.C. (1938) Note on rapid desensitization in case of hypersensitiveness to insulin. *Am. J. Med. Sci.* **196,** 359.

CROOKES A.C., BUTT W.R. & BERTRAND P.V. (1966) Clinical trial of human gonadotrophins. *Acta endocr. Suppl.* **111,** 3–26.

DAVIDSON J.K. & EDDLEMAN E.E. (1950) Insulin resistance. *Arch. Int. Med.* **86,** 727.

DAWEKE H. (1966) Klinik der Insulinresistenz. *Dtsch. Med. Wschr.* **21,** 973.

DE FILIPPIS V. & IANNACCONE A. (1952) Insulin neutralizing activity of gamma globulin derived from the serum of an insulin resistant patient. *Lancet i,* 1192.

DEVLIN J.G. (1966) Evidence for the existence of an IgM immunoglobulin to insulin. *Ir. J. Med. Sci* Sixth series, No. 491, 507.

DEVLIN J.G. (1967) Unpublished observations.

DEVLIN J.G. & BRIEN T.G. (1965) Relationship between differential antibody binding capacity and clinical requirements of beef and pork insulin. *Metabolism* **14,** 1034.

DEVLIN J.G., BRIEN T.G. & STEPHENSON N. (1966) Effect of alteration of species source of insulin-antibody levels. *Lancet* ***ii,*** 883.

DEVLIN J.G., BRIEN T.G. & STEPHENSON N. (1967). Relation between antibody and insulin dose. *B.M.J.* **1,** 542.

DEVLIN J.G. & LAHER M. (1966) Dependence of insulin haemagglutination antibody titres on IgM antibody. *Nature* **212,** 84.

DEVLIN J.G. & O'DONOVAN D.K. (1965) Association of acute local reactions to insulin with an insulin-binding IgM antibody. *J. Clin. Path.* **18,** 356.

DEVLIN J.G. & O'DONOVAN D.K. (1966). Preferential beef/pork insulin-binding capacity, radioimmunoelectrophoretic and chromatographic data in patients with dermal reactions to insulin. *Diabetes* **15,** 790.

DEVLIN J.G. & STEPHENSON N. (1967) Unpublished observations.

DIETRICH, F.M. (1966) Immunogenicity of synthetic angiotensin II. *Int. Arch. Allergy* **30**: 497

DOLGER H. (1952) Denatured insulin: a simplified, rapid means of treatment of allergy to insulin complicating diabetic ketosis. *New York State J. Med.* **52,** 2023.

DRESSER W.D. (1962) Specific inhibition of antibody production. *Immunology* **5,** 378.

EDSALL G. (1966) Principles of active immunization. *Annu. Rev. Med.* **17,** 39.

FEDERLIN K. (1966) Personal communication.

FEDERLIN K., HEINEMANN G., GIGLI I. & DITSCHUNEIT H. (1966) Antigenbindung durch zirkulierende Leukozyten bei der verzögerten lokalen Insulin-allergie. *Dtsch. med. Wschr.* **17,** 814.

FELDMAN R., GRODSKY G.M., KOHOUT F.W. & MCWILLIAMS N.B. (1963) Immunologic studies in a diabetic subject resistant to bovine insulin but sensitive to porcine insulin. *Am. J. Med.* **35,** 411.

FRASIER S.D. & SMITH F.G. (1966) Antibodies to human growth hormone. *Am. J. Dis. Child.* **112,** 383.

GEMZELL C. (1964) Induction of ovulation with human gonadotrophins. *Proceedings of the Second International Congress of Endocrinology,* Part II, p. 805. Excerpta Medica Foundation.

GEMZELL C. (1967) Personal communication.

GLOVER, J.S., SLATER, D.N. and SHEPHERD, B.P. (1967) A study of some factors that influence the iodination of ox insulin. *Biochem. J.* **103**: 120

GRODSKY G.M. (1965) Production of autoantibodies to insulin in man and rabbits. *Diabetes* **14**, 396.

GRODSKY G.M., FELDMAN R., TORESON W.E. & LEE J.C. (1966) Diabetes mellitus in rabbits immunized with insulin. *Diabetes* **15**, 579.

HARRIS-JONES J.N., MILLER H. & OWEN G. (1963) Insulin-binding antibodies in relation to insulin therapy. *J. Clin. Path.* **16**, 120.

HEDING L. (1966) Personal communication.

HUMPHREY J.H. (1962) Basic immunological considerations. *Ciba Foundation Colloquia on Endocrinology*, vol. 14, p. 6. J. & A. Churchill Ltd.

ISHIZAKA K. (1963) Gamma globulin and molecular mechanisms in hypersensitivity reactions. *Progr. Allergy* **7**, 32.

ISHIZAKA K. & ISHIZAKA T. (1966a) Physicochemical properties of reaginic antibody. *J. Allergy* **37**, 169.

ISHIZAKA K., ISHIZAKA T. & BANOVITZ J. (1965) Biological activities of aggregated γ-globulin. *J. Immunol.* **94**, 824.

ISHIZAKA K., ISHIZAKA T. & HORNBROOK M.M. (1966c) Physicochemical properties of reaginic antibody. II. Correlation of reaginic activity with γE globulin antibody. *J. Immunol.* **97**, 840.

ISHIZAKA K., ISHIZAKA T. & RICHTER M. (1966b) Effect of reduction and alkylation on allergen combining properties of reaginic antibody. *J. Allergy* **37**, 135.

JONES V.E. & CUNLIFFE A.C. (1961) A precipitating antibody to insulin. *Nature* **192**, 136.

JORGENSEN K. (1966) Personal communication.

JORPES J.E. (1949) Recrystallized insulin for diabetic patients with insulin allergy. *Arch. Int. Med.* **83**, 363.

KARR W.G., KREIDLER W.A., SCULL C.W. & PETTY O.H. (1931) Certain immunologic studies in insulin sensitivity. *Am. J. Med. Sci.* **181**, 293.

KREINES K. (1965) The use of various insulins in insulin allergy. *Arch. Int. Med.* **116**, 167.

LAWRENCE R.D. (1925) Local insulin reactions. *Lancet* **1**, 1125.

LERMAN J. (1944) Insulin resistance. The role of immunity in its production. *Am. J. Med. Sci.* **207**, 354.

LEVENSON H. & COCHRANE C.G. (1964) Nonprecipitating antibody and the Arthus vasculites. *J. Immunol.* **92**, 118.

LIACOPOULOS P., HALPEM B. & PERRAMENT F. (1962) Unresponsiveness to unrelated antigens induced by paralysing doses of bovine serum albumin. *Nature* **195**, 1112.

LOVELESS M.H. (1945) Coexistence of two antibodies for crystalline insulin in human serum. *Fed. Proc.* Part II, 5:250.

LOVELESS M.H. (1958) Immunologic studies of insulin in man. Effect of heat on allergenicity. *Diabetes* **7**, 278.

LOWELL F.C. (1944) Immunologic studies in insulin resistance. II. The presence of a neutralizing factor in the blood exhibiting some characteristics of an antibody. *J. Clin. Invest.* **23**, 233.

LOWELL F.C. (1942) Evidence for the existence of two antibodies for crystalline insulin. *Proc. Soc. Exp. Biol. Med.* **50**, 167.

MACDONALD M.K. & IRELAND J.T. (1964) The glomerular lesion in idiopathic and secondary diabetes. *Ciba Foundation Colloquia on Endocrinology*, vol. 15, p. 301.

MARTIN P.M., MARTIN H.E., LYSTER R.W. & STROUSE S. (1941) Insulin resistance. *J. Clin. Endocr.* **1**, 387.

MELICK R.A., GILL J.R., BERSON S.A., YALOW R.S., BARTTER F.C., POTTS J.T. & AURBACH G.D. (1967) Antibodies and clinical resistance to parathyroid hormone. *New Engl. J. Med.* **276,** 144.

MENCZEL J., LEVEY M. & BENTWICH Z. (1966) Insulin resistant diabetes treated with sulfated insulin. *Israel Journal of Medical Science* **2,** 764.

MERIMEE Y.J., LOCKWOOD D.H. & PROUT T.E. (1965) The relationship of insulin I-131 to serum protein fractions. *Bulletin of the Johns Hopkins Hospital* **116,** 191.

MICHEL H. (1965) Behandlung des diabetes mellitus bei Insulin-Resistenz und Insulin-Allergie. *Dtsch. Med. Wschr.* **49,** 2211.

MILLER H. & OWEN G. (1960) Immunoelectrophoresis of insulin-binding antibodies. *Nature* **4744,** 67.

MOINET P. (1958) A quantitative estimation of antibodies to exogenous insulin in diabetic subjects. *Diabetes* **7,** 462.

MOLONEY P.J. & APRILE M.A. (1959) On the antigenicity of insulin: flocculation of insulin-anti-insulin. *Canad. J. Biochem. Physiol.* **37,** 793.

MORSE J.H. (1961) Correlation of insulin requirements with the concentration of insulin-binding antibody in two cases of insulin resistance. *J. Clin. Endocr.* **21,** 533.

MORSE J.H. & HEREMANS J.F. (1962) Immunoelectrophoretic analysis of human insulin-binding antibody and its papain-produced fragments. *J. Lab. Clin. Med.* **59,** 892.

OAKLEY W., FIELD J.B., SOWTON G.E. & RIGBY B. (1959) Action of prednisone in insulin resistant diabetes. *B.M.J.* **1,** 1601.

PALEY R.G. (1950) Analysis of the accessory factors in the causation of dermal reactions to insulin. *J. Pharm. Pharmacol.* **2,** 304.

PALEY R.G. & TUNBRIDGE R.E. (1952) Dermal reactions to insulin therapy. *Diabetes* **1,** 22.

PARKER M.L., MARIZ J.K. & DAUGHADAY W.H. (1964) Resistance to human growth hormone in pituitary dwarfism: clinical and immunologic studies. *J. Clin. Endocr.* **24,** 997.

PAV J., JEZKOVA Z. & SKRHA F. (1963) Insulin antibodies. *Lancet ii* 221.

PENCHEV I., ANDREEV D. & DITZOV ST. (1966) Insulin-precipitating antibodies in insulin-treated and untreated diabetic patients (Abs.). *Diabetologia* **2,** 214.

PEPYS J., JENKINS P.A., LACHMANN P.J. & MAHON W.E. (1966) An iatrogenic autoantibody: immunological responses to 'Pituitary snuff' in patients with diabetes insipidus. *Clin. exp. Immunol.* **1,** 377.

POULSEN J.E. (1966) Personal communication.

PRADER A., WAGNER H., SZEKY J., ILLIG R., TOUBER J.L. & MAINGAY D. (1964) Acquired resistance to human growth hormone caused by specific antibodies. *Lancet ii* 378.

PROUT T.E. & KATIMS R.B. (1959) The effect of insulin binding serum globulin on insulin requirement. *Diabetes* **8,** 425.

REES J.B., CAMERINI-DAVALOS R.A., CAULFIELD J.B., LOZANO-CASTENADA O., CERVANTES-AMEZCUS A., TATON J., POMETTA D., KRAUTHAMMER J.P. & MARBLE A. (1964) Pathophysiology of microangiopathy in diabetes mellitus. *Ciba Foundation Colloquia on Endocrinology*, vol. 15, p. 315.

RENOLD A.E., SOELDNER J.S. & STEINKE J. (1964) Immunological studies with homologous and heterologous pancreatic insulin in the cow. *Ciba Foundation Colloquia on Endocrinology*, vol. 15, p. 122.

ROTH J., GLICK S.M., YALOW R.S. & BERSON S.A. (1964) Antibodies to human growth hormone (H.G.H.) in human subjects treated with H.G.H. *J. Clin. Invest.* **43,** 1056.

SAXENA B.B. & HENNEMAN P.H. (1966) Isolation and properties of the electrophoretic components of human growth hormone by Sephadex-gel filtration and preparative polyacrylamide-gel electrophoresis. *Biochem. J.* **100,** 711.

SCHIRREN C.G. (1953) Ein ungewöhnlicher Fall von lokaler Insulin-Anaphylaxie. *Hautarzt* **4,** 531.

SCHLICHTKRULL J. (1953) *Insulin Crystals.* Copenhagen: Munksgaard.

SHIPP J.C., CUNNINGHAM R.W., RUSSELL R.O. & MARBLE A. (1965) Insulin resistance: clinical features, natural course and effects of adrenal steroid treatment. *Medicine* **44,** 165.

SKOM J.H. & TALMAGE D.W. (1958) The role of non-precipitating insulin antibodies in diabetes. *J. Clin. Invest.* **37,** 789.

SMELO L.S. (1947) Lack of response to insulin. *Southern Medical Journal* **40,** 333.

STANWORTH D.R. (1965) The structure of reagins. *Int. Arch. Allergy* **28,** 71.

STEIGERWALD H., SPIELMANN W., FRIES H. & GREBE S.F. (1960) Neuere untersuchungen uber die antigenwirkung des insulins. *Klin. Wschr.* **38,** 973.

STEVENS K.M. (1956) Some considerations of the antigen dose-antibody response relationship. *J. Immunol.* **76,** 187.

STEVENS K.M. (1957) Poliovirus as an antigen. *Hyg.* **55,** 489.

TADA T. & ISHIZAKA K. (1965) Arthus type inflammation with rabbit γM antibody. *J. Immunol.* **96,** 112.

TASHJIAN A.H., LEVINE L. & WILHELMI A.E. (1965) Immunochemical relatedness of porcine, bovine, ovine and primate pituitary growth hormones. *Endocrinology* **77,** 563.

TERR A.I. & BENTZ J.D. (1965) Skin-sensitizing antibodies in serum sickness. *J. Allergy* **36,** 433.

TERRY W.D. (1965) Skin sensitizing activity related to γ-polypeptide chain characteristics of human IgG. *J. Immunol.* **95,** 1041.

TORO-GOYCO E., MARTINEZ-MALDONADO M. & MATOS M. (1966) Insulin antibodies: partial characterization by gel-filtration. *Proc. Soc. Exp. Biol. & Med.* **122,** 301.

TUFT L. (1928) Insulin hypersensitiveness: immunologic considerations and case reports. *Am. J. Med. Sci.* **176,** 707.

WEIGLE W.O. (1966) The suppression of antibody production to serum protein antigens in guinea pigs. *Int. Arch. Allergy* **29,** 254.

WEST H.F. (1962) Ten years of ACTH therapy. *Ann. Rheum. Dis.* **21,** 263.

WRIGHT P.H. & NORMAN L.L. (1966) Some factors affecting insulin antibody production in guinea-pigs. *Diabetes* **15,** 668.

YAGI Y., MAIER P. & PRESSMAN D. (1962) Immunoelectrophoretic identification of guinea pig anti-insulin antibodies. *J. Immunol.* **89,** 736.

YAGI Y., MAIER P., PRESSMAN D., ARBESMAN C.E., REISMAN R.E. & LENZNER A.R. (1963) Multiplicity of insulin binding antibodies in human sera. *J. Immunol.* **90,** 760.

CHAPTER 26

ALLERGIC DRUG REACTIONS

J.F.Ackroyd and A.J.Rook

Introduction

Classification of Drug Reactions

The differentiation of idiosyncrasy and hypersensitivity

Factors Determining the development of Hypersensitivity

The Patient: Age and sex: Genetic factors: Occurrence of drug reactions in patients with a previous history of asthma, hay fever or infantile eczema. Disease. The Drug: Chemical structure and sensitizing capacity: Cross-sensitization: The route of administration and the vehicle: Dosage

Clinical Patterns

Cutaneous Reactions: Pruritus: Urticaria: Exanthematic eruptions: Exfoliative dermatitis: Bullae: Erythema multiforme: Epidermal necrolysis: Lichenoid eruptions: Fixed eruptions: Purpuric eruptions: Eczema: Photosensitization: The Association of Cutaneous Reactions with Blood Dyscrasias Systemic Reactions: Fever: The serum sickness type of reaction: Reactions characterized by the development of a state of shock: Bronchial asthma: Polyarteritis: Syndrome resembling disseminated lupus erythematosus: Liver damage: Nephropathy: Retroperitoneal fibrosis: Lymph gland enlargement simulating malignant lymphoma: Blood dyscrasias

Immunological Mechanisms

Mechanisms of Cutaneous Reactions. Mechanisms of Systemic Reactions: Fever: Asthma, urticaria, the serum sickness type of reaction and reactions characterized by a state of shock: Blood Dyscrasias: Thrombocytopenic purpura: Haemolytic anaemia: Agranulocytosis

Diagnosis

Skin Tests: The technique of patch testing. In Vitro Tests: Reactions involving the release of histamine: Demonstration of haemagglutinating antibodies to penicillin: *In vitro* stimulation of peripheral blood lymphocytes: Thrombocytopenic durpura: Haemolytic anaemia: Agranulocytosis: The significance of positive serological findings: Test Dosing: The technique of test dosing

TREATMENT
Prophylaxis: Desensitization

INTRODUCTION

Untoward reactions to drugs, their early recognition, their prevention and treatment, and the investigation of the mechanisms which underlie them, constitute one of the most urgent problems of modern medicine. Although rare, fatal reactions to drugs are more common than is generally recognized. It has, for instance, been estimated that up to 1956 penicillin had probably caused one thousand deaths in the United States (Feinberg & Feinberg 1956). More indirectly, by precluding the use of an essential therapeutic agent, or by the diagnostic confusion for which they are often responsible, drug reactions are a serious hazard. Of several hundred new pharmaceutical products made available each year, some 10–15% are new chemical compounds. No effective measures have yet been devised for screening drugs by animal experiments for their sensitizing capacity in man.

CLASSIFICATION OF DRUG REACTIONS

The following classification is based on that of Brown (1955).

1. *Overdosage*. The toxic effects are directly related to the total amount of the drug in the body. The overdosage may be due to excessive intake or to cumulation as a result of some abnormality in the patient, which prevents the normal breakdown or excretion of the drug.

2. *Intolerance*. The effects of the drug are qualitatively normal but quantitatively increased.

3. *Idiosyncrasy*. The reaction to the drug is qualitatively abnormal and, in this way, idiosyncrasy resembles hypersensitivity (see below). However, unlike hypersensitivity, the reaction does not depend on an immunological mechanism.

4. *Side effects*. This term, often employed more loosely, should be reserved for the undesirable but unavoidable pharmacological actions of a drug.

5. *Secondary effects*. The secondary effects are an indirect and not inevitable consequence of the primary drug action. The disturbance of the normal balance of the bacterial flora of the bowel in patients receiving long-term antibiotic therapy is an important example.

6. *Hypersensitivity reactions*. Hypersensitivity reactions are the result of allergic sensitization to a drug by previous exposure to the same drug or to a chemically related substance. They are mediated by antigen-antibody reactions.

Frequently it is not possible to state with certainty that a symptom or syndrome is due to drug hypersensitivity. In fact it may not be possible even to be certain

that the reaction is due to the drug at all. It may be due, for instance, not to the drug but to the disease for which the drug is being given. Thus, although aplastic anaemia may develop as a reaction to the drug itself in a patient receiving chloramphenicol, it may occasionally occur because the patient's bone marrow has been damaged by the overwhelming infection for which the drug is being given. Furthermore, a syndrome apparently due to drug hypersensitivity may be due to the development, by the patient, of an entirely different disease. As an example of this may be quoted the condition now known as homologous serum jaundice. This was at one time thought to be due to a reaction to arsphenobenzol compounds and other drugs given by injection but is now known to be due to contamination of syringes with a hepatotoxic agent.

The Differentiation of Idiosyncrasy and Hypersensitivity

Recent advances in the investigation of drug reactions have made it clear that one of the major difficulties is in the differentiation of reactions due to idiosyncrasy from those due to hypersensitivity. This is shown by recent studies of cases of haemolytic anaemia caused by therapeutic doses of primaquine (Beutler 1959). It has been found that this drug causes haemolysis in patients whose red cells are deficient in the enzyme glucose-6-phosphate dehydrogenase. It seems probable that other drugs, including nitrofurantoin, sulphonamides, *para*-amino-salicylic acid, naphthalene and phenacetin can also cause haemolytic anaemia in such individuals. Also, the condition favism—an acute haemolytic anaemia which results when the broad bean, *Vicia faba*, is eaten by a susceptible individual—has been shown to be associated with a deficiency of this same enzyme (Zinkham, Lenhard & Childs 1958), although favism has been widely believed in the past to be due to an immunological mechanism. Sideroblastic anaemia occasionally develops in the course of treatment with certain antituberculous drugs. This appears to be due not to an immunological mechanism, but to interference by the drugs with pyridoxine metabolism (Mollin 1965; Verwilghen, Reybrouk, Callens & Cosemans 1965). The drugs with which these patients have been treated—and almost all of them have been receiving more than one drug—include isoniazid, cycloserine and pyrazinamide. As further examples of drug reactions with no immunological basis may be mentioned the prolonged apnoea occasionally produced by suxamethonium compounds, and the megaloblastic anaemia occasionally seen during treatment with anticonvulsant drugs. The prolonged apnoea has been shown to be due to a genetically determined abnormality of the enzyme pseudocholinesterase. The megaloblastic anaemia is thought to be due to interference by the drug with the metabolism of folic acid. A further example of a drug reaction in the genesis of which a genetically determined factor probably plays an important role is the development of peripheral neuritis by patients being treated with *iso*-nicotinic acid hydrazide.

Some individuals are able to acetylate, and so inactivate, this drug much more rapidly than others and this difference is genetically determined (Evans & White 1964). Patients who inactivate the drug slowly develop peripheral neuritis much more ferquently than those who inactivate it rapidly (Devadatta *et al* 1960; Evans & Clarke 1961). The acetylation of hydrallazine seems to be similarly genetically determined (Evans & White 1964). However, no correlation has been demonstrated between the rate of acetylation of this drug and the development of a syndrome resembling disseminated lupus erythematosus that patients being treated with this drug apparently sometimes develop (see below). Another interesting example of a drug reaction closely resembling hypersensitivity but not due to an immune mechanism is the precipitation by barbiturates and other drugs of the symptoms of acute intermittent porphyria. Not all patients with this disease develop symptoms in response to the same drug, although a patient who has once developed symptoms in response to one drug apparently always does so in response to the same drug. The mechanism is unknown but the drug is certainly not the only factor involved, for attacks of acute porphyria may occur in patients who have not recently taken drugs. However, a history of symptoms repeatedly precipitated by the same drug may strongly suggest a hypersensitivity reaction and the true nature of the condition may not be recognized if porphyria is overlooked as a cause.

The following criteria have commonly been quoted as characteristic of drug hypersensitivity and necessary for its diagnosis. However, none of these excludes idiosyncrasy. These criteria are:

1. The reaction does not resemble the pharmacological action of the drug. This is, of course, also true of idiosyncratic reactions.
2. There is a latent interval before the reaction occurs, during which the drug has been taken with no untoward result. This can vary from a few days to many years. Although the existence of such a symptom-free period suggests that the condition is due to drug hypersensitivity the latent interval will, of course, be exactly mimicked if the patient develops an intercurrent illness unconnected with the drug during a course of treatment with that drug. Furthermore, in glucose-6-phosphate dehydrogenase deficiency, drugs such as primaquine do not cause haemolysis immediately; haemolysis occurs only after 2–3 days of treatment.
3. Identical reactions may be provoked by chemically dissimilar drugs. This, also, is true of idiosyncrasy. There is, for example, no obvious similarity between the drugs which can cause haemolytic anaemia in patients with glucose-6-phosphate dehydrogenase deficiency.
4. The reaction may be reproduced by minute doses even after long intervals. This phenomenon is also observed in idiosyncrasy. Perhaps the best example is favism in which attacks of haemolytic anaemia may occur at long intervals in the same patient and in which haemolysis has been reported in response to the eating of a single bean and has even been described as having resulted when a

susceptible patient has walked near a field of beans in flower, the reaction presumably resulting from inhalation of pollen.

5. The pathological changes resemble those present in reactions known to be due to drug hypersensitivity. Although this is clearly true, the histological changes in many hypersensitivity reactions are not specific. For example, there are few, if any, significant differences between the morbid anatomical changes seen in cases of haemolytic anaemia, agranulocytosis or thrombocytopenic purpura due to drug hypersensitivity, as compared with cases in which the cause is not known. Furthermore, this type of argument has often been used uncritically; for example, the facts that polyarteritis may occur in patients taking drugs, and that lesions resembling polyarteritis in man can be produced in rabbits by the repeated intravenous administration of very large doses of horse serum have been construed as demonstrating that polyarteritis in man may be due to drug hypersensitivity. Evidence from human studies is, however, by no means conclusive (see Rose & Spencer 1957).

6. Administration of the same drug to the patient after recovery causes recurrence of the same reaction. As already indicated, this is also true of reactions due to idiosyncrasy. Thus, except during the refractory period immediately following haemolysis due to primaquine, the administration of the drug always causes haemolysis. Similarly, once a patient has developed the symptoms of acute porphyria in response to a drug, he always develops these symptoms in response to the same drug.

A diagnosis of drug hypersensitivity can be made with certainty only when an antibody can be demonstrated either *in vitro* or by means of the passive transfer test *in vivo*. In the vast majority of cases of reactions occurring in the course of drug therapy, no antibody can be demonstrated. It is clear, therefore, that a diagnosis of drug hypersensitivity can often not be made with certainty and it may well be that increasing knowledge will show that many drug reactions now believed to be due to an immune mechanism are due to enzymatic or other abnormalities in the patient.

FACTORS DETERMINING THE DEVELOPMENT OF HYPERSENSITIVITY

The factors which determine the development of hypersensitivity by a particular patient to a particular drug are largely unknown, and too few facts are available to permit any general conclusions to be drawn.

The Patient

Age and Sex

Drug hypersensitivity may occur at any age and in either sex. It is commonly stated that children are less susceptible than adults. Smith & Zirk (1961) found

that reactions to antituberculous drugs were uncommon below the age of 15 years. On the other hand, Huguley, Erslev & Bergsagel (1961) have reported that in a series of thirty-three cases of blood dyscrasias due to chloramphenicol, twenty-seven were children 1–10 years old. Furthermore, Sulzberger and Baer found that sensitivity to picryl chloride was more readily induced in the skin of young than of elderly individuals (Baer & Schwarzschild 1955). It is generally believed that drug reactions are commoner in females than in males. Smith & Zirk (1961) found this to be true for streptomycin; and of the thirty-three cases of blood dyscrasias due to chloramphenicol (Huguley *et al* 1961) mentioned above, twenty-eight were females.

Genetic Factors

The possible importance of genetic susceptibility in the development of hypersensitivity to specific chemicals or groups of chemicals is suggested by experiments with guinea-pigs (Chase 1958), but has not been demonstrated in man.

Occurrence of Drug Reactions in Patients With a Previous History of Asthma, Hay Fever or Infantile Eczema

The statement has frequently been made that patients who have a history of asthma, hay fever or infantile eczema develop drug reactions more frequently than do normal individuals (Cooke 1919; Prickman & Buckstein 1937; Berkowitz, Glaser & Johnstone 1953; Brown 1959). This opinion is, however, based on inconclusive evidence. Smith & Zirk (1961) were unable to confirm this in a study of reactions to antituberculous drugs. Wayne (1958), however, considers that it may be true for those drug reactions characterized by the development of asthma, urticaria or a state of shock. It does appear that there is a group of patients with asthma and nasal polypi in whom aspirin tends to precipitate severe and sometimes even fatal asthma (Samter 1959). There is also evidence which suggests that penicillin is more liable to produce severe reactions in asthmatics than in normal individuals. Thus Corr & Wellman (1956) reported that sixty of 250 patients who had experienced severe reactions to penicillin gave a history of asthma. In endeavouring to assess the significance of such findings it should, however, be borne in mind that patients with chronic asthma have often received a great deal of drug therapy and may therefore have had much more opportunity than healthy individuals of becoming sensitized.

Disease

The frequency of reactions to many anti-infectious drugs has led to the suggestion that certain infections may increase susceptibility to sensitization, but this has not been proved. A very high incidence of photosensitization to sulphonamides in recently vaccinated subjects (Watkinson & Hillis 1947) lends some

support to this suggestion. The findings of Harvey and his colleagues (1954) suggest that susceptibility to drug reactions is increased in patients with disseminated lupus erythematosus. However, during the long course of this disease, these patients often receive a great deal of drug therapy which will inevitably increase their risk of becoming sensitized. It has also been said that drug reactions occur less commonly in patients with sarcoidosis than in normal individuals. Certainly sensitivity to tuberculin is depressed in this condition and it has recently been shown that there is a reduced susceptibility to contact dermatitis which is also mediated by a cellular rather than a humoral mechanism (Epstein & Mayock 1957). In hypogammaglobulinaemia, delayed-type allergic responsiveness is often unimpaired and contact sensitivity to dinitrofluorobenzene can be readily induced (Good, Kelly, Rötstein & Varco 1962). However, other forms of drug reactions are rare in hypogammaglobulinaemia, although reactions to penicillin have occasionally been reported.

In the course of a study of antithyroid antibodies Blizzard, Hamwi, Skillman & Wheeler (1959) formed the clinical impression that patients with thyroid disease had an abnormal tendency to develop hypersensitivity reactions to penicillin. They then showed that there was a high incidence of haemagglutinating antibodies to penicillin in the sera of patients with antithyroid antibodies. Although the significance of these findings is uncertain, they do raise the possibilities either that endocrine factors may have some influence on the development of drug hypersensitivity or that there may be some association between the mechanisms underlying autoimmunity and drug hypersensitivity. Both these possibilities are, however, entirely conjectural.

The Drug

Chemical Structure and Sensitizing Capacity

Some drugs rarely if ever provoke reactions, whereas others will do so in the majority of those exposed. Attempts to correlate sensitizing capacity with chemical structure have been unsuccessful because our knowledge of the mechanisms of sensitization is fragmentary and because many variables, such as the rate of metabolism and excretion of the drug, may be concerned in addition to the chemical structure of the drug itself or its metabolic products. The problem has been reviewed by Davies (1958). Landsteiner & Jacobs (1936) were able to correlate the capacity of a series of halogenated nitrobenzene compounds to sensitize guinea-pig skin with their reactivity with aniline which is a measure of their ability to form stable compounds with protein. However, although the ability of the drug itself or its metabolic products to combine with protein may be a factor influencing sensitizing capacity, some of the drugs commonly causing reactions are not known to react directly with protein.

The possibility that the patient has become sensitized, not to the drug itself, but to an impurity must always be considered. This has recently been shown to

be of particular importance in some cases of reactions apparently caused by the penicillins (Batchelor *et al* 1967; Knudsen *et al* 1967; Stewart 1967).

Cross-Sensitization

Cross-sensitization may be said to occur when allergic symptoms induced by one compound are subsequently also produced in the same patient by one or more related compounds. The compound originally inducing sensitivity is referred to as the primary allergen; other compounds to which the patient reacts are called secondary allergens. Most secondary allergens can function as primary allergens but some can only cause symptoms of hypersensitivity after primary sensitization with a more potent allergen. Theoretically, cross-sensitization may be due to a close immunochemical relationship between primary and secondary allergens or to metabolic conversion into immunochemically similar or identical compounds (Baer 1954). The range of cross-sensitization is not predictable in the individual patient. It may be specific for a single compound or may extend to many chemically related compounds. The patient reported by Meltzer & Baer (1949) provides an excellent example. He was clinically sensitive to benzocaine, sulphaguanidine and monoglycerol *para*-aminobenzoate. To these three substances he gave strongly positive patch tests. He was also patch tested with twenty other chemically related substances. Some of the positive reactions are listed in Table 26.1. In general the stronger the sensitivity to the primary allergen—in this case benzocaine—the wider the range of secondary sensitivities. However, so great is the variation in individual response that not all patients sensitized to the same primary allergen show cross-sensitivity to the same secondary allergens.

The clinical importance of cross-sensitization is obvious. Exposure, perhaps by a different route, and sometimes unsuspected, may have induced sensitization to a drug. The failure to appreciate the chemical relationship between different drugs, a danger increased by the widespread use of proprietary names, may lead to the inadvertent substitution of a secondary allergen in a patient with a severe drug reaction. A few of many examples may be cited. In a patient sensitized by topical applications of *para*phenylenediamine or benzocaine the systemic administration of sulphanilamide or certain other aromatic amines (Mayer 1950) may provoke a reaction. Neomycin may cross-sensitize to streptomycin (Sidi, Hincky & Longueville 1958). Cross-sensitization within the expanding group of phenothiazine derivatives (promethazine, chlorpromazine and related compounds) is of common occurrence. The possibility of cross-sensitization, now recorded for drugs of many types, must be considered in all reactions whose origin or persistence is not readily explained.

The Route of Administration and the Vehicle

Apart from contact dermatitis which is rarely, if ever, produced in man except

TABLE 26.1

Cross-sensitization with chemicals causing eczema (from Baer 1954)

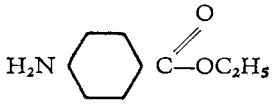
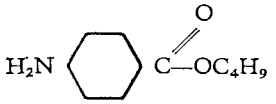
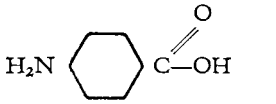
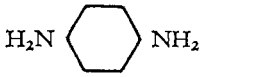
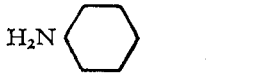
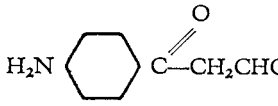
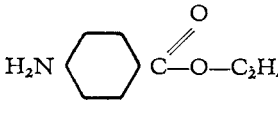
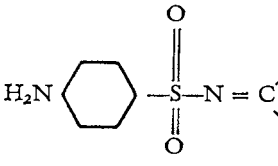
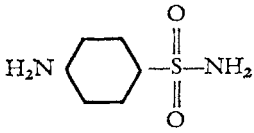
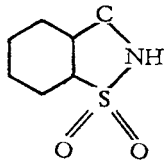
Substance	Chemical structure	Reaction
Benzocaine		++++
Butesin		++++
Para-aminobenzoic acid		++++
Para-phenylenediamine		++++
Aniline		++++
Monoglycerol para-aminobenzoate		++++
Procaine		++++
Sulphaguanidine		+++

TABLE 26.1—*contd.*

Substance	Chemical structure	Reaction
Sulphanilamide		+++
Saccharin		++

by the repeated application of the drug to the skin, the route of administration of a drug is of little importance in determining the type of hypersensitivity reaction.

Vehicles which provoke local inflammatory changes appear to facilitate sensitization. Beeswax and oils enhance the sensitizing capacity of penicillin (Lepper *et al* 1949). Their action is probably similar to that of Freund's and other adjuvants which are used experimentally to enhance antibody response to antigenic stimuli. It is not altogether clear how they produce this effect (see Munoz 1964). When mixed with antigen, they probably delay absorption and so prolong antigenic stimulation. They may also increase the number of inflammatory cells attracted to the area.

DOSAGE

In general, the higher the dose and the longer the period of administration, the greater is the possibility of sensitization. Once the patient has become sensitized, the reaction may sometimes be provoked by extremely small doses. As examples of this, a severe penicillin reaction has followed the intradermal injection of 3×10^{-6} of a unit of procaine penicillin G (Bierlein 1956) and a mild attack of generalized purpura with thrombocytopenia has been produced by the intradermal injection into a highly sensitized patient of 1.4×10^{-6}g of Sedormid (Ackroyd 1949). However, in the course of time, if no further drug is given, the concentration of antibody may fall to a level at which even a therapeutic dose of

the drug given on a single occasion may fail to precipitate a reaction. Further administration of the drug, however, invariably stimulates rapid antibody formation, and thereafter the drug again causes the reaction. The fall in antibody concentration may occur quite rapidly over the course of a few weeks or it may occur so slowly that a reaction can be provoked by a single small dose after an interval of several years during which the patient has not taken the drug (see Ackroyd 1964).

CLINICAL PATTERNS

In patients who have not previously taken the drug, the onset of the reaction seldom occurs after less than a week of continuous treatment. It may develop at any time thereafter, even after years of continuous or intermittent administration. If the drug forms a depot from which it is slowly liberated, the reaction may develop weeks or even months after the drug has been discontinued. As an example of this may be quoted a patient who developed thrombocytopenic purpura 6 months after his last injection of a gold salt. This responded to treatment with dimercaprol (BAL) but relapsed when the treatment was discontinued (Hazlett &Yendt 1958). In such cases, unless a careful history is taken, the true cause of the condition may be overlooked. In highly sensitized patients symptoms appear rapidly—within seconds or a few hours—after administration of the drug itself or of a chemically closely related compound. This may happen in response to the first dose taken after a long interval but, as already stated, if the degree of sensitivity has become less with the passage of time, the patient may tolerate a single therapeutic dose with no untoward reaction. Eventually, usually within a few days, or at most within 2 or 3 weeks, if the administration of the drug is continued, symptoms of hypersensitivity will reappear.

Certain drugs provoke certain reactions sufficiently frequently for the type of reaction to be of value in diagnosis; for example, the fixed eruption due to phenolphthalein and the itching purpuric rash caused by carbromal. However, some drugs are capable of provoking almost any reaction. The extraordinary variety of drug reactions defies logical classification in the present state of our knowledge. In the following section, only a relatively small number of reactions are considered but many others have been attributed to drugs, some on very questionable evidence. It must be emphasized that, although the different reactions are discussed separately, more than one reaction is commonly seen in the same patient. Systemic and cutaneous reactions are often associated, although in published case reports so much emphasis is placed on the most conspicuous feature that reliable information on the frequency of systemic manifestations in drug eruptions, and of skin rashes in blood dyscrasias and other systemic reactions, is largely lacking. The drugs mentioned below are chosen only as examples of familiar drugs known to provoke the reaction described. For detailed lists,

the textbooks of Alexander (1955) and Meyler (1952) and the latter author's periodical reviews of the world literature (1955 onwards) should be consulted.

Cutaneous Reactions

Pruritus

Pruritus is a conspicuous feature of many drug eruptions but may occasionally be the only symptom, or may precede other cutaneous or systemic reactions, particularly to sulphonamides or gold salts.

Urticaria

Urticaria has been the commonest cutaneous drug reaction since the widespread use of penicillin. It frequently occurs alone. It is a troublesome feature of the serum sickness type of reaction. It usually subsides in a few days if treatment is discontinued but, when due to penicillin, it sometimes persists for 2–3 months, or even longer. It increases in severity with continued administration of the drug but rarely evolves into other types of reaction. Other common causes are sera, pollen extracts and salicylates.

Exanthematic Eruptions

The exanthematic eruptions vary greatly in their morphology and extent. There may be a simple or scarlatiniform erythema, macules, or a combination of macules and papules closely simulating an infective exanthem. The papular eruptions sometimes have a violaceous or cyanotic tint, which should suggest a drug rather than an infective origin. The exanthematic eruptions may be confined to the extensor aspects of the hands, forearms and legs, or may involve predominantly the trunk, or they may be almost universal. If the offending drug is not discontinued exfoliative dermatitis may develop. They are amongst the commonest reactions caused by drugs. They have been produced by a very large number of chemically unrelated drugs; in particular the barbiturates, pyrazolones (phenazone and related compounds), and the sulphonamides. They are rarely due to penicillin.

Exfoliative Dermatitis

The term exfoliative dermatitis should be strictly confined to a universal or widespread persistent erythema and oedema with continued exfoliation. It is often wrongly applied to the secondary exfoliation which so often complicates exanthematic eruptions. Exfoliative dermatitis is a very serious reaction. The grossly increased heat loss, the prolonged loss of protein in exudate and scale and the added circulatory burden of the continued peripheral vasodilation may prove fatal in weak or elderly patients. Chills and fever are frequent. Exfoliative dermatitis may be of rapid onset, or may develop by gradual extension of

flexural erythema and scaling, or may follow an exanthematic eruption. The compounds of the heavy metals, the barbiturates and the sulphonamides are important causes.

Bullae

Purely bullous eruptions due to drugs are relatively uncommon and are classically associated with iodides and bromides, although many other drugs may be concerned. Bullae are a conspicuous feature of eruptions more correctly classified as erythema multiforme or as fixed eruptions.

Erythema Multiforme

Dull red oedematous papules and plaques, often with central bullae, develop symmetrically on the extensor aspects of the extremities. Fever and malaise are common and occasionally there may be joint pains and abdominal pain. Sulphonamides, barbiturates and pyrazolones (phenazone and related compounds) are perhaps the commonest of many drugs incriminated. A serious, predominantly bullous variant, with severe involvement of ocular, oral and genital mucous membranes, is indistinguishable from the Stevens-Johnson syndrome. It is usually attributable to phenobarbitone.

Epidermal Necrolysis

Tender plaques of erythema cover large areas of the body, usually reaching their greatest extent within a few hours. The lesions closely resemble scalds. After 12–48 hr vast flaccid bullae form quite suddenly on the erythematous skin. The necrotic epidermis is shed and healing takes place in 10–14 days. Severe toxaemia and death may occur when a large proportion of the skin surface is involved. Phenylbutazone, sulphonamides and many other drugs have been incriminated (Beare 1962; Braun-Falco & Geissler 1962). It is interesting that staphylococci also provoke this reaction, especially in infants (Jefferson 1967).

Lichenoid Eruptions

Pink or lilac flat shining papules, simulating lichen planus clinically and histologically are produced by many common drugs. The eruption, which may be extensive, often persists for weeks after the drug is stopped, usually leaves pigmentation and may leave scarring. Gold and bismuth, mepacrin, chloroquine and quinine, thiazides and amiphenazole are known offenders. The phenothiazines (promethazine, chloropromazine and related compounds) have also been incriminated, (Groth 1961) but much more often cause other types of eruption.

Fixed Eruptions

This term is applied to eruptions which recur in the same site or sites each time the drug is administered. Characteristically the lesion is a dull red oedematous

plaque or a bulla, which heals to leave persistent pigmentation. The lesions may be single or multiple, and additional lesions may appear each time the drug is given. Involvement of buccal or genital mucous membranes may accompany skin lesions or occur alone. Fever and malaise are sometimes present but are less common than in erythema multiforme. Phenolphthalein, phenazone, barbiturates and sulphonamides are most often incriminated. As with most drug reactions, cross-sensitivity to chemically related drugs is often observed, but occasionally chemically apparently unrelated drugs will also provoke a recurrence (Alexander 1955).

Purpuric Eruptions

Nonthrombocytopenic purpura

By far the commonest cause of nonthrombocytopenic purpura due to a drug is the hypnotic carbromal (diethyl-bromo-acetyl carbamide). This causes a very characteristic picture in which the skin haemorrhages are associated with a scaling, itching dermatitis. In long-standing cases, in addition to fresh haemorrhages, the skin is stained with numerous punctate haemosiderin deposits—the sites of previous purpuric haemorrhages. Patch tests are often positive (see Ackroyd 1960), but the causative mechanism is unknown. The condition often persists for weeks and, occasionally, for months after the drug is withdrawn; an observation which suggests that a purely immunological mechanism is perhaps unlikely.

Simple purpura unaccompanied by other skin lesions is not a common manifestation of drug hypersensitivity. It has been described as a result of treatment with iodides, gold, sulphonamides, barbiturates and quinine. No immunological mechanism has been demonstrated.

Anaphylactoid purpura (Henoch-Schönlein syndrome)

Anaphylactoid purpura is rarely attributable to any cause (Ackroyd 1953, 1960). Occasionally foods have been implicated. Although some cases have been attributed to drugs, very few of these reports will bear critical analysis. Most have been cases of nonthrombocytopenic purpura (see above). However, the case reported by Symmers (1958) does appear probably to have been a true example of anaphylactoid purpura due to aspirin. The case described by Creger & Houseworth (1954) resembled anaphylactoid purpura in many ways although the rash was not typical. The attacks were precipitated by quinine. An interesting finding in this case was that the patient's serum could sometimes be shown to contain a factor which caused erythrophagocytosis in his own blood, and on one occasion also in normal blood in the presence of the drug.

Eczema

Eczema is the characteristic response to a drug applied topically, of skin which has been sensitized by the same drug previously applied to it. In patients thus

sensitized, the systemic administration of the drug often, but not invariably, provokes a recurrence of the eczema. Eczema, however, rarely occurs as a manifestation of hypersensitivity to drugs, which have only been administered systemically. Antibiotics, phenothiazines (promethazine, chlorpromazine and related compounds), mercurials, sulphonamides, antihistamines and quinine are sometimes incriminated. Occasionally a substance topically applied may cross-sensitize to an immunochemically related drug administered systemically (see cross-sensitization above). The subject of eczematous reactions to drugs has recently been well reviewed by Fisher (1966).

Photosensitization

Drugs may increase the sensitivity of the skin to light by two distinct mechanisms: phototoxic and photoallergic. In phototoxic reactions the drug potentiates the effects of those wavelengths of light which it absorbs. The reaction, which clinically resembles an exaggerated sunburn, develops within a few hours of the administration of the drug. It occurs in all those in whom the dose of the drug and the exposure to light are adequate. The reaction sometimes provoked by dimethylchlortetracycline is of this type (Harber *et al* 1961).

In photoallergic sensitization there is a latent period which may last for days or even months during which the patient takes the drug but reacts normally to light. Then, whilst still taking the drug, the patient develops an abnormal reaction to light in areas of skin exposed to it. This type of reaction occurs only in a small proportion of those taking the drug. It may be urticarial, papular, eczematous, bullous, purpuric or lichenoid and may recur over a period of days or months after every further exposure to light, with or even without further administration of the drug. The fact that there is a latent period has suggested to several workers that the condition has an immunological basis (Epstein & Mayock 1957; Jillson & Curwen 1959). The drugs most commonly implicated are phenothiazines (promethazine, chlorpromazine and related compounds), thiazides, sulphonylurea derivatives (tolbutamide, etc.) and the sulphonamides (Knox 1961).

It should be noted that some drugs which can cause a phototoxic reaction may, in the same patient, also produce a photoallergic reaction (Jillson & Curwen 1959).

The subject of photosensitization due to drugs has been extensively reviewed by Kirshbaum & Beerman (1964).

The association of Cutaneous Reactions with Blood Dyscrasias

The drugs which are most liable to cause exanthematic and fixed eruptions, erythema multiforme and exfoliative dermatitis include some of the principal causes of blood dyscrasias, yet the association of any of these reactions with blood dyscrasias is sufficiently unusual to suggest that essentially different immuno-

logical mechanisms may be concerned in their production. However, the association does sometimes occur, and in patients with these eruptions the blood picture should be kept under careful observation.

Systemic Reactions

Fever

Fever is one of the commonest manifestations of drug hypersensitivity. It may occur alone. More commonly, it accompanies other reactions, either cutaneous or systemic.

Drug fever is particularly characteristic of reactions to sulphonamides, antibiotics, antithyroid drugs, *para*-aminosalicylic acid and mercurial diuretics.

The Serum Sickness Type of Reaction

A considerable number of apparently unrelated drugs, of which penicillin is by far the most commonly implicated, occasionally produce a reaction which closely resembles serum sickness. The essential features are fever, joint pains and urticaria. Whereas urticaria, when it occurs alone, commonly develops during treatment, the serum sickness type of reaction almost invariably develops a few days after treatment has been discontinued.

Reactions Characterized by the Development of a State of Shock

Within seconds or minutes of the administration of the drug, the patient complains of malaise and a feeling of extreme apprehension. The face may be flushed although more commonly the patient will be pale. He may begin to cough and usually complains of pain and a sense of tightness in the chest. There is increasing difficulty in breathing and the patient's mucous membranes become cyanosed. As a result of the combination of pallor and cyanosis, the skin now becomes ashen grey. Unconsciousness and death may ensue within minutes. If the patient does not die within the first few minutes, erythema and urticaria may be noted and this may be important in distinguishing the condition from other causes of acute collapse. The patient commonly develops asthma and characteristically complains of severe upper abdominal pain. With adequate treatment, the majority of patients recover. Penicillin, local anaesthetics, sera and pollen extracts are the most frequent causes.

Bronchial Asthma

Bronchial asthma due to drug hypersensitivity occasionally occurs alone, but is more commonly associated with urticaria. The patient usually has a past history of asthma and often has nasal polypi (Samter 1959). Although many drugs have occasionally been reported as having caused bronchial asthma, aspirin does so more frequently than any other drug.

POLYARTERITIS

It is widely believed that polyarteritis may be a manifestation of drug hypersensitivity. This view appears to be based on the following evidence:

1. There is often a history of drug administration before the diagnosis of polyarteritis was made. In many cases, however, the drug might well have been given for symptoms which were early manifestations of the polyarteritis itself. Occasionally, readministration appears to have caused an exacerbation of the polyarteritis. Although it is impossible to exclude coincidence, this is probably the best evidence that polyarteritis may sometimes be a manifestation of drug hypersensitivity.

2. Rabbits repeatedly injected intravenously with large doses of heterologous serum develop lesions histologically similar to those of polyarteritis nodosa in man (Rich & Gregory 1943). This suggests that polyarteritis may be a reaction to a state of hypersensitivity. It should, however, be noted that there is no experimental evidence that drug hypersensitivity can cause arteritis. It has been stated that a single injection of the compound 4 fluoro-10-methyl-1,2-benzanthracene causes polyarteritis in rats (Hartmann, Miller & Miller 1959). This was observed in all rats killed 6 weeks or more after the injection, a finding which suggests that the polyarteritis was probably a primary toxic effect rather than a manifestation of hypersensitivity. G.E.Davies (personal communication) has failed to produce any arterial lesions by administering a wide variety of different drugs in large doses to rats. This is, perhaps, not surprising because if polyarteritis is a hypersensitivity reaction then it might be necessary to give one drug to several thousands of animals before a single case of polyarteritis, due to that drug, was produced.

3. It has been claimed that polyarteritis may develop in patients in whom a drug has caused the serum sickness type of reaction, i.e. fever, joint pains, urticaria, etc., if treatment has been continued or, if discontinued, has been reinstituted later with recurrence of the same symptoms on one or more occasions. By analogy with the observations on rabbits injected intravenously with heterologous sera, quoted above, it has been argued that the drug caused the serum sickness type of reaction and that continued administration of the drug intensified the hypersensitivity reaction and so caused the development of the arteritis. Such a history is, however, rarely obtained in cases of polyarteritis nodosa and as fever and joint pains are common manifestations of this disease, the supposed drug reaction might well have been an early symptom of the polyarteritis itself.

In conclusion, it may be said that the evidence that polyarteritis can result from drug hypersensitivity is circumstantial only. The best evidence comes from the occasional case in which readministration of the drug appears, sometimes on more than one occasion, to have caused exacerbation of the disease. The subject of the aetiology of polyarteritis has been reviewed by Rose & Spencer (1957).

Syndrome Resembling Disseminated Lupus Erytematosus

As long ago as 1953 (Morrow *et al*), it became clear that patients being treated with the antihypertensive drug hydrallazine occasionally developed a condition that mimicked extraordinarily closely the clinical syndrome of disseminated lupus erythematosus. In the fully developed condition the patient might have arthritis of the rheumatoid type, fever, skin rashes and enlargement of the liver, spleen and lymph glands. In addition, the lupus erythematosus cell phenomenon could be demonstrated in the blood of a small proportion of the more severely affected of these patients (Dustan *et al* 1954). Some elements of this syndrome were seen in 7–10% of those taking large doses of the drug regularly for several months. Withdrawal of the drug usually resulted in rapid clinical improvement (Dustan *et al* 1954; Moser 1956), but in a few cases some elements of the syndrome persisted for years after cessation of therapy (Hildreth *et al* 1960; Alarcón-Segovia *et al* 1965). Comens (1956) succeeded in producing a syndrome that fairly closely resembled that seen in man by administering hydrallazine to dogs. Gardner (1957), who repeated this work, was only able to confirm some of Comens's (1956) findings and Dubois and his collaborators (1957), who also repeated it, obtained entirely negative results. The reason for these conflicting reports is not clear. Since then many workers have tried to produce the syndrome in other laboratory animals, and a few have claimed success (see Cruickshank 1966). Alarcón-Segovia and his colleagues (1965), in a retrospective study of patients who had developed this condition (referred to below as the hydrallazine syndrome) reported that, even before treatment was started, many of the patients who developed this syndrome had had clinical or laboratory signs which could have been early manifestations of disseminated lupus erythematosus. They therefore suggested that such patients 'have an underlying lupus diathesis that is uncovered by the drug.' This would imply that 7–10% of all hypertensive patients have an occult lupus diathesis. Although this seems improbable, the findings of these workers do strongly suggest that some patients who have been reported to have developed the hydrallazine syndrome were actually suffering from disseminated lupus erythematosus before treatment was started. These are probably the patients who develop the severest forms of the syndrome and in whom the condition persists after the drug has been withdrawn. The hydrallazine syndrome may therefore comprise two groups of patients: those who already have disseminated lupus erythematosus, and those who have not but in whom hydrallazine produces one or more of the classical signs or symptoms of this disease.

Since the hydrallazine syndrome was first described, many claims have been made that a condition resembling disseminated lupus erythematosus can be caused by a wide variety of other drugs (Holley 1961). Lupus erythematosus cells have been reported in patients sensitive to penicillin, and antinuclear

factors have been demonstrated in the sera of patients being treated with *iso*nicotinic acid hydrazide. However, the most convincing evidence has come from studies of patients being treated with procaine amide (Ladd 1962; Kaplan *et al* 1965) and with various anticonvulsants, particularly hydantoin compounds (Jacobs 1963; Shulman L.E. 1963).

The frequency with which treatment with some drugs has been accompanied by the development of a syndrome resembling disseminated lupus erythematosus suggests a causal relationship. Although such a relationship cannot be proved in any individual case, strong circumstantial evidence is provided by those cases in which withdrawal of the drug has been followed by remission and readministration by recurrence of the syndrome. However, such evidence has only rarely been produced. The relationship of the syndrome caused by drugs to the idiopathic disease, disseminated lupus erythematosus, is uncertain, as is the mechanism by which drugs cause this type of reaction. The finding of Schroeder (1959) that three years after recovery 'the peripheral blood of a patient has been found loaded with LE cells a week or two after readministering small doses of hydrallazine', suggests that it may be a hypersensitivity reaction and the occurrence of the lupus erythematosus cell phenomenon makes it probable that it is immunologically determined.

Liver Damage

It has been known for a long time that some drugs such as chloroform and carbon tetrachloride are potent liver poisons and will cause irreversible liver damage if given in sufficient quantities. More recently other forms of liver injury due to drugs have been described. These fall into two groups. In the first, intrahepatic biliary obstruction dominates the picture, whereas the second is characterized by hepatocellular degeneration and necrosis. The clinical picture closely resembles that of infective hepatitis, either of the cholestaic type or of the commoner type in which hepatocellular damage predominates. Unless irreversible cellular damage has been caused, recovery is usual once the drug is withdrawn. Occasionally in the cholestatic form, jaundice may persist for many weeks or months.

Cholestatic jaundice due to drugs

This group can be subdivided into two types.

Type I. In this type, if given over a long enough period, the drug invariably causes intrahepatic obstruction as shown by bromsulphalein retention or by frank jaundice. This type of liver damage has been caused by a number of C17-α-alkyl substituted testosterones including methyl testosterone, methandienone (dianabol), and constituents of some oral contraceptive ovulation inhibitors such as norethisterone and norethynodrel. Certain non-steroid compounds including sulphadiazine have also been implicated. This subject has recently

been reviewed by Sherlock (1963). The mechanism is obscure, but it is interesting that this one group of testosterone compounds invariably causes intrahepatic obstruction, whereas other testosterone derivatives apparently do not have this effect.

Type II. In this type, only a small proportion of those taking the causative drug are affected. The drugs most frequently involved are the phenothiazines (promethazine, chlorpromazine and related compounds). Other commonly used drugs which have caused this type of jaundice are nitrofurantoin and chlorpropamide. The subject has been reviewed by Gutman (1957), Hollister (1957) and by Sherlock (1963). The underlying mechanism is unknown. Skin testing with chlorpromazine has proved uninformative (Hollister 1957). In twenty-two cases due to chlorpromazine reported by Werther & Korelitz (1957) jaundice appeared after 11–29 days' treatment and in sixteen of Hollister's (1957) seventeen cases, jaundice appeared on the seventh to the thirtieth day of treatment. However, readministration of the drug after recovery frequently produced jaundice in under 2 days (Hollister 1957). This observation suggests that the condition may be a manifestation of drug hypersensitivity.

Jaundice resulting from hepatocellular damage due to drugs

This type of liver damage also affects only a very small proportion of those taking any one of the many drugs which can cause it. Prominent amongst these are a number of hydrazine derivatives which are mono-amine oxidase inhibitors. These include iproniazid (marsilid) and phenelzine (nardil). *Iso*nicotinic acid hydrazide, another hydrazine derivative, has also been implicated, as have certain non-hydrazine compounds including sulphonamides, phenindione, cincophen and *para*-aminosalicylic acid. The subject is reviewed by Sherlock (1963). As in cases with intrahepatic biliary obstruction, the causative mechanism is unknown. In cases of jaundice due to mono-amine oxidase inhibitors it appears that after recovery from jaundice due to one drug in this group, substitution of another drug in the same group may produce recurrence of the hepatitis (Holdsworth, Atkinson & Goldie 1961). The reported cases of jaundice due to *para*-aminosalicylic acid have often developed other disturbances including fever, arthralgia, skin rashes, lymph gland enlargement and changes in the peripheral blood: eosinophilia and a picture resembling infectious mononucleosis (Lichtenstein & Cannemeyer 1953; Sleeper, Tyor & Smith 1960; Simpson & Walker 1960). Lichtenstein & Cannemeyer (1953) obtained positive reactions to patch testing with *para*-aminosalicylic acid in some of their patients, but most authors have found skin testing uninformative (Simpson & Walker 1960). In cases due to this drug, recurrence of the syndrome described above may occur rapidly if, after recovery, the drug is given again, even in relatively small doses (Lichtenstein & Cannemeyer 1953; Simpson & Walker 1960), a finding which suggests that the hepatitis in these cases may be due to hyper-

sensitivity to the drug. Further evidence that some cases of hepatitis are probably due to drug hypersensitivity is strongly suggested by a fascinating case reported by Himsworth (1950). The patient was a woman who, after 5 weeks' treatment with aminothiazole for thyrotoxicosis, developed an illness indistinguishable from infective hepatitis. The drug was withdrawn and she recovered after a month. She was then given 100 mg of aminothiazole and within a few hours she developed a brief illness with fever and biliuria. Ten days later she was given a further single dose of 100 mg of the drug. She promptly developed fever and jaundice and her liver became enlarged. She made a slow but complete recovery.

Nephropathy

It is commonly stated that certain drugs, particularly the sulphonamides, can produce renal lesions as a result of drug hypersensitivity, and that these lesions may be entirely independent of the precipitation of crystals of the drug in the renal tubules. The evidence is, however, circumstantial. In many cases the renal lesion could well have been due to the condition for which the drug was given, but in some it does appear that the drug was probably responsible. Perhaps the most convincing evidence of a hypersensitivity mechanism is provided by the occurrence of tubular necrosis in patients with haemolytic anaemia in which the anaemia has been shown by immunological methods to have been due to drug hypersensitivity. Muirhead, Halden & Groves (1958) have described one case of this type in which the anaemia was due to quinine, and MacGibbon and her colleagues (1960) have described two further cases, one due to phenacetin, and one to *para*-aminosalicylic acid. Even here, however, the evidence is incomplete, for it is clearly possible that the renal damage might have been secondary to the acute haemolysis.

Attention has recently been drawn to the occurrence of renal failure, which is often accompanied by a shortened red cell survival time, in patients who have taken grossly excessive doses of analgesics over long periods. This subject has been reviewed by Prescott (1965). Although the renal lesion has generally been referred to as phenacetin nephropathy it has almost always occurred in patients taking mixtures of analgesics, and the possibility that other analgesics in these mixtures may be important has not been excluded. Prescott (1965) observed the effect on renal tubular cell excretion of giving large doses of analgesics to volunteers over a 5 day period. Cell excretion was markedly increased in all those taking aspirin and in some taking phenacetin, but was only slightly increased in those taking paracetamol, which is a metabolite of phenacetin. However, Angervall, Lehmann & Bengtsson (1964) who studied the renal concentrating power of rats given large doses of analgesics over long periods found that phenacetin and paracetamol caused a significant decrease in concentrating power, whereas aspirin had little effect. It is clear, therefore, that there is still a lot to be learnt about this interesting condition. Because not all patients taking large doses

of analgesics are affected, the possibility that the condition is due to a form of drug hypersensitivity has been considered but, in fact, the causative mechanism is unknown.

The very occasional occurrence of a nephrotic syndrome in patients taking the anti-convulsant drug troxidone (tridione) seems to be well established (see Bergstrand *et al* 1962). The condition usually recovers if the drug is withdrawn. A similar condition has been observed in rats given large doses of this drug over long periods (Heymann *et al* 1960). Half the rats developed a nephrotic syndrome and the remainder, although they did not have proteinuria, developed histological renal lesions similar to those seen in the nephrotic rats. As the rats did not recover when treatment was discontinued, the condition seen in these animals may be significantly different from that seen in man. This is also suggested by the fact that renal changes occurred in all the treated rats, whereas the nephrotic syndrome is only very rarely seen in patients taking the drug. This latter observation suggests that in man the condition may possibly be due to drug hypersensitivity.

Retroperitoneal Fibrosis

Methysergide, a drug used in the treatment of migraine, seems to have the remarkable effect of producing retroperitoneal fibrosis in a small proportion of patients taking it (Graham 1964; Graham, Suby, Le Compte & Sadowsky 1966). In the series of cases reported by Graham and his colleagues (1966) partial or complete regression of the disease followed withdrawal of the drug in all the cases not treated surgically. The mechanism by which methysergide produces this reaction is unknown. The facts that it occurs in such a small proportion of those taking the drug and that the condition is reversible suggest that it may be due to hypersensitivity to the drug.

Lymph Gland Enlargement Simulating Malignant Lymphoma

Treatment with phenytoin (epanutin) or troxidone (tridione) or their derivatives occasionally causes fever and enlargement of one or more groups of lymph glands. There may also be skin rashes, and occasionally the liver and spleen may be enlarged. Histologically the picture sometimes resembles that of malignant lymphoma (Salzstein & Ackerman 1959). The patient recovers rapidly if the drug is withdrawn. In view of the closeness with which this condition may simulate malignant lymphoma, it is clearly essential, before starting treatment for malignant lymphoma, to make sure that the patient has not been taking drugs of this type. Attempts to demonstrate drug-dependent antibodies have generally been unsuccessful. Robinson and his colleagues (1965) have reported one case in which the addition of sodium diphenyl hydantoin to the patient's serum caused degranulation of rabbit basophil leucocytes. They interpreted this as indicating

an immune mechanism. Recently it has been shown that if peripheral blood lymphocytes from a patient who has been sensitized to an antigen are cultured in the presence of the antigen, they undergo transformation to 'blast' forms (Elves, Roath & Israëls 1963). Holland & Mauer (1964) have applied this technique to the lymphocytes of a patient who had recovered from the syndrome of lymph gland enlargement due to phenytoin. The drug caused lymphocyte transformation and an increase in the uptake of tritiated thymidine by these cells. The drug had no comparable effect on the lymphocytes of controls. These findings, if confirmed, provide strong evidence that this syndrome is a hypersensitivity reaction.

Blood Dyscrasias

A considerable number of cases of thrombocytopenic purpura and a much smaller number of cases of haemolytic anaemia have been shown unequivocally by *in vitro* immunological methods to have been due to allergic drug hypersensitivity. That agranulocytosis may be caused by such a mechanism is reasonably well established, but the evidence is less complete. With regard to aplastic anaemia, there is good circumstantial evidence, but no immunological evidence, that this also may be due to an immune mechanism. These conditions are all indistinguishable clinically from cases which are not due to drugs. Aplastic anaemia generally persists long after treatment has been stopped but, unless the initial illness is fatal, or unless the drug forms a depot from which it is slowly released, cases of thrombocytopenic purpura, haemolytic anaemia and agranulocytosis usually recover rapidly once the drug is withdrawn. Bouma (1966) has, however, recently claimed that thrombocytopenic purpura due to aspirin sometimes persists after the patient has stopped taking the drug. He was able to show in some of his cases that the thrombocytopenia was probably due to a drug-dependent complement-fixing antibody, but he considered that the prolonged thrombocytopenia indicated that the drug had caused bone-marrow depression. Bouma (1966) also made the interesting alternative suggestion that salicylates in the patient's food might have been sufficient to cause persistence of the thrombocytopenia. This, however, implies a very high degree of sensitivity, as the salicylic acid content of the fruits thought by Bouma to be responsible is only of the order of 1 mg per kg (Lancet 1903). The concept that acetylsalicylic acid may cause bone-marrow depression is supported by a recent report from Wijnja and his colleagues (1966) on the occurrence of pancytopenia in patients taking very large doses of acetylsalicyclic acid over long periods. Recovery occurred on withdrawal of the drug and the condition recurred if the drug was readministered. In the two patients in whom they were investigated, platelet and red-cell survival times were normal during the period of pancytopenia, suggesting that the drug, if taken in excessive amounts over a long period, may act as a marrow depressant.

Thrombocytopenic purpura

An immunological basis has been demonstrated in cases due to many different drugs (Ackroyd 1964). Of these, the most commonly involved are allyl-isopropyl-acetyl-carbamide (Sedormid), quinidine, quinine, gold salts, the sulphonamides, antazoline, acetazoleamide and the thiazides.

Haemolytic anaemia

Haemolytic anaemia has been caused by a wide variety of different drugs (see Wintrobe 1961). Immunological mechanisms have been demonstrated in cases due to *para*-aminosalycylic acid, phenacetin, quinine, quinidine salicylazosulphapyridine, chlorpromazine, stibophen (see Ackroyd 1964), amidopyrine (Dausset *et al* 1963) and penicillin (Petz & Fudenberg 1966; Rosner *et al* 1966). The haemolytic anaemia caused by α-methyldopa has also been shown to have an immunological basis (Worlledge *et al* 1966).

Agranulocytosis

Agranulocytosis has been much less extensively investigated. It has been associated with treatment with many drugs (see Wintrobe 1961). By far the most frequently implicated have been amidopyrine, the sulphonamides and the antithyroid drugs, thiourea and thiouracil and their derivatives.

Aplastic anaemia

Of the many drugs which have caused aplastic anaemia, chloramphenicol is by far the most frequently implicated in current therapeutic practice. Other important drugs are the hydantoins, gold salts and the arsenobenzole compounds. Some cases in which an initial course of chloramphenicol has apparently been well tolerated have developed aplastic anaemia when given a relatively small dose in a subsequent course. A case of this type has been reported by Huguley, Erslev & Bergsagel (1961). Such cases suggest the possibility of an allergic mechanism. Recent studies, however, have shown that reversible functional (Rubin, Weisberger, Botti & Storaasli 1958; Saidi, Wallerstein & Aggeler 1961) and morphological (Krakoff, Karnofsky & Burchenal 1955; Saidi, Wallerstein & Aggeler 1961) changes are probably present in many patients receiving large doses of this drug. These changes may well be due to a disturbance of bone-marrow metabolism caused by the drug. This view receives some support from the observations of Amos (1964) on the effect of chloramphenicol on protein synthesis by chick fibroblasts in tissue culture. It seems possible, therefore, that it will ultimately be shown that chloramphenicol causes aplastic anaemia more frequently by an effect on the metabolism of the bone marrow than by an immunological mechanism.

IMMUNOLOGICAL MECHANISMS

Mechanisms of Cutaneous Reactions

The mechanism of eczematous contact dermatitis has been thoroughly investigated because it is readily induced in the guinea-pig and can be elicited experimentally in man with no danger and with little inconvenience to the patient. A full account is given in Chapter 27. Eczematous reactions to drugs administered systemically probably depend on a similar mechanism.

We also have some knowledge of the mechanisms causing urticarial reactions (see below), but of the mechanisms determining the wide variety of other cutaneous reactions little is known. They cannot be reproduced in laboratory animals and their experimental induction in man is usually unjustifiable, except perhaps in the case of the fixed eruptions. It is not known why one drug will produce the same reaction in all patients who become sensitized, while another drug may provoke different reactions in different patients or even in the same patient (Urbach & Gottlieb 1946).

Experiments with skin autografts in patients with fixed drug eruptions have given conflicting results. Wise & Sulzberger (1933) used full-thickness skin grafts. In a patient with a fixed eruption to phenolphthalein they transplanted skin from a normal area to a site previously affected by the eruption, and skin from this area to the normal area. When the grafts had healed (7 weeks after grafting) the drug was again administered to the patient. The eruption appeared in the normal skin transferred to the previously affected area, but not in the previously affected skin transferred to the normal area. Loveman (1934) confirmed this observation in a case due to aprobarbital. Urbach & Sidaravičius (1930) and Naegeli and colleagues (1930) repeated these experiments in cases due to phenolphthalein and antipyrine respectively. Unlike Wise & Sulzberger (1933) and Loveman (1934) they observed recurrence in the transplanted abnormal skin but not in the transplanted normal skin. However, these workers used superficial Thiersch grafts. Knowles and his colleagues (1936) repeated these experiments using full-thickness grafts in a patient with a fixed eruption due to phenolphthalein. Five weeks after grafting, readministration of the drug caused recurrence in the previously affected skin but not in the grafted normal skin. When the drug was again given to the patient 2 months later, the eruption appeared only in the previously normal skin, as in the experiment of Wise & Sulzberger (1933). Chargin & Leifer (1940), using full-thickness grafts in a patient with a fixed eruption due to arsphenamines, were unable to produce a recurrence in either transplant, although the patient continued to react in other previously affected sites. It could be assumed on the basis of these experiments that fixed eruptions involve predominantly the deeper vessels rather than the epidermis or upper dermis, and that differences in the thickness of the grafts account for the inconsistent experimental results. This, however, is difficult to

reconcile with the histological changes which show involvement predominantly of the vessels of the papillary and subpapillary dermis (Goodman & Arthur 1941).

Mechanisms of Systemic Reactions

Fever

Extraordinarily little work has been done on the mechanisms causing drug fever. The subject is, for instance, not mentioned in Atkins's (1960) exhaustive monograph on the pathogenesis of fever. An investigation of a single case of fever due to streptomycin has been reported by Snell (1961) who removed 420 ml of blood from the patient when his rectal temperature was 39°C. The blood was taken into acid citrate dextrose, centrifuged and the cell-free supernatant plasma was stored at 4°C. Four days later, when the patient's temperature had returned to normal as a result of withdrawing the drug, the plasma was reinjected into the patient. This caused no rise in the patient's rectal temperature. Apart from this failure to demonstrate a circulating pyrogenic factor in drug fever, no clinical investigations appear to have been made on this subject. Studies on fever produced by antigens in sensitized animals suggest that the interaction of antigen with humoral or cellular antibody *in vivo* causes release of circulating pyrogen. The latter can be demonstrated by observing the febrile reaction caused by passive transfer of serum from febrile to normal, unsensitized animals (see Atkins & Snell 1965). By analogy, fever in drug hypersensitivity would seem probably to be due to a reaction of an antibody with the drug itself or with a combination of the drug with some bodily constituent. This hypothesis, however, does not explain Snell's (1961) negative findings, referred to above.

Asthma, Urticaria, the Serum Sickness Type of Reaction and Reactions Characterized by a State of Shock

These four types of reaction can be considered together because they overlap to a considerable extent, so that more than one commonly occurs simultaneously in the same patient. Also, as all these reactions may be caused by penicillin, they have often been studied together in the course of investigations on reactions to this drug.

Various workers have tried to demonstrate immune precipitate formation by adding the appropriate drugs to the sera of patients who had developed these and other types of drug hypersensitivity (Hoigné 1958; Muelling *et al* 1958; Mendes *et al* 1959). The results have, in the main, been inconclusive and have not been generally confirmed. Definite floccular immune precipitates have, however, very rarely been produced by adding the appropriate drug, in solution, to sera from cases with thrombocytopenic purpura (Ackroyd 1958, 1960, 1964) or with both granulocytopenia and thrombocytopenia (Tullis 1961) due to drug

hypersensitivity. The phenomenon of immune precipitates caused by drugs will be considered below.

The sera of some patients who have been treated with penicillin will agglutinate penicillin-coated red cells. This phenomenon was first observed by Ley and his colleagues (1958). Its significance is obscure because it is not seen in the sera of all patients who are sensitive to penicillin and has been observed in the sera of many patients who, although they have been treated with the drug, are not apparently sensitive to it (Ley *et al* 1958; Watson, Joubert & Bennett 1959, 1960; de Weck 1964). The significance of this reaction has been discussed by van Arsdel and his collaborators (1963). Although it seems that the antibodies causing haemagglutination of penicillin-coated red cells may well not be those responsible for the type of penicillin hypersensitivity considered here, studies of the haemagglutination reaction have thrown considerable light on the antigenicity of penicillin. By discovering which penicillin derivatives can inhibit haemagglutination it has been shown that, in the majority of sera, the penicilloyl group is the most immunologically active. These sera usually also react strongly with the penamaldate and penicilloate derivatives of penicillin. Although this is the commonest pattern of reactivity a significant proportion of sera show different reaction patterns (Parker 1964). The significance of the haemagglutination reaction in the rare cases of haemolytic anaemia due to penicillin is considered below.

In 1961 Shelley and Juhlin introduced a test based on the assumption that some drug reactions involve release of histamine from tissue mast cells and from the basophils in the blood. They reported that penicillin caused 'degranulation' of basophils *in vitro* in the blood of sensitive patients. This test has the major disadvantage that it involves observation of a cell which is present in human blood in only very small numbers. In an attempt to overcome this difficulty Shelley (1963) later described an indirect test using rabbit blood which contains a much higher concentration of these cells. In this test a drop of the patient's serum is mixed with a drop of the drug in solution and a drop of rabbit buffy coat. Degranulation of the basophils in the rabbit buffy coat is then observed. Shelley (1963) has claimed that this test can be used to detect hypersensitivity to a wide range of different drugs. Although there seems to be no doubt that the addition of a drug to the serum of a sensitive patient does sometimes cause a striking degranulation of basophil leucocytes, these tests have not been generally accepted as reliable indicators of drug hypersensitivity. Even in Shelley's laboratory (Shelley 1963) the direct test gave ninety and the indirect test seventy-seven negative results on testing, respectively, 138 and 137 patients thought to be sensitive to penicillin. Katz and his colleagues (1964), working in collaboration with Shelley, and using the indirect test observed thirty-five negative and fifteen equivocal results on testing a total of 100 patients thought to be sensitive to penicillin.

Noah & Brand (1954, 1955), who have developed a method for demonstrating histamine release when pollen extracts are added to the blood of pollen-sensitive patients, have applied the same technique to the investigation of hypersensitivity to penicillin. They were unable to demonstrate any release of histamine on adding penicillin to the blood of sensitive patients (Noah 1964). Recently, however, because of his observations on basophil degranulation which he considers to be a manifestation of histamine release, Shelley, working in collaboration with Comaish, has tried to increase the sensitivity of Noah & Brand's (1954, 1955) technique. Because rabbit blood contains a much higher concentration of histamine than does human blood, these workers have estimated histamine released by penicillin in rabbit blood to which samples of serum from penicillin-sensitive patients had been added (Shelley & Comaish 1965). The reliability of this method remains to be determined, for three of twenty-one controls gave positive results and five of twenty-seven patients thought to be sensitive to penicillin gave negative results when their sera were tested in this way.

In a study of these types of reaction, whether due to penicillin or other drugs, Halpern and his colleagues (1967) have recently claimed that if the patient's lymphocytes in his own plasma are cultured in a suitable medium in the presence of the drug the cells undergo transformation to lymphoblastic forms. These results were highly specific. These workers observed similar findings in cases of contact dermatitis. As stated above, Holland & Mauer (1964) had previously made similar observations in a case of lymph gland enlargement due to phenytoin.

The results of skin testing penicillin-sensitive patients (Parker, Shapiro, Kern & Eisen 1962) and animals with experimentally induced contact sensitivity to penicillin (Levine 1964) have shown that positive skin reactions may be specific for a number of penicillin derivatives. As with positive haemagglutination reactions, the penicilloyl group is clearly important although some reactions are undoubtedly specific for other haptenic determinants. These findings led to attempts to discover a penicilloyl-containing reagent which could safely be used for skin testing in man. Penicillin itself is too dangerous as it has caused a number of severe and at least one fatal reaction (see below). Parker and his colleagues (1962) suggested that penicilloyl polylysine might be a suitable reagent and this has been used extensively for skin testing patients suspected of being sensitive to penicillin. The results have, however, by no means been clear cut. Rytel, Klion, Arlander & Miller (1963) gave 1,200,000 units of penicillin by injection to twenty-six patients who had just produced strongly positive wheal and flare reactions to penicilloyl polylysine. Two developed delayed cutaneous reactions, but the remainder showed no reaction of any kind. These workers also studied thirty-six patients whose skin reactions to penicilloyl polylysine had become strongly positive after they had been given penicillin. They gave each of these patients 1,200,000 units of penicillin: none experienced either an immediate or

a delayed reaction. In another series reported by Brown, Price & Moore (1964), 206 patients giving strongly positive skin reactions to penicilloyl polylysine were subsequently treated with penicillin. Only twenty-one developed reactions of any sort. Although reactions to penicillin in patients with negative skin tests to penicilloyl polylysine are rare, they do occur. Brown and colleagues (1964) observed reactions to penicillin treatment in sixty-three of 13,530 patients with negative penicilloyl polylysine skin tests. Perlman (1965) has recently reported four patients with negative skin tests to penicilloyl polylysine whose skin reacted strongly to scratch testing with penicillin G. In two of these patients the scratch test caused a generalized reaction; one of such severity as to show that a therapeutic dose of penicillin might well have been fatal. It is clear, therefore, that, in the individual case, no conclusions can be drawn from the results of skin testing with penicilloyl polylysine in man. Finally, consideration must be given to the safety of skin testing with this reagent. Although reactions are rare, Brown and his collaborators (1964) have observed five reactions to skin testing with penicilloyl polylysine of which three—possibly four—were generalized.

Attempts to transfer these types of sensitivity passively by means of the Prausnitz-Küstner reaction have sometimes, but not always, been successful (Mayer *et al* 1953; Swift 1954; Peters, Henderson & Prickman 1955; Mendes *et al* 1959; Siegel 1962). Using this technique, Halpern (1958a) has made a remarkably detailed immunological study of a single patient who was sensitive to amidopyrine and in whom the drug caused symptoms resembling anaphylactic shock. This investigation is worth considering in detail, although it must be borne in mind that the findings in a single case may not apply to all drug reactions characterized by symptoms of shock. If the patient's serum was injected into the skin of normal individuals, wheal formation occurred at the injection site if the recipients were subsequently given the drug by mouth or by intramuscular injection. When, in a further experiment, the drug was injected intradermally at the site of an intradermal serum injection there was no reaction, but when the recipient was later given the drug by mouth wheal formation did occur at the injection site, showing that the injected drug had not reacted with the patient's serum; for it did not cause wheal formation, nor did it inhibit the action of the ingested drug. If an excess of the drug was added to the patient's serum *in vitro* and the mixture was injected intradermally, wheal formation did not occur even when the recipient was subsequently given the drug (Halpern 1958b). Wheal formation did, however, occur if the mixture was dialysed against saline before being injected, and the recipient was subsequently given the drug (Halpern 1958a). These last two experiments suggest that the drug may combine with some component of the serum but that if it does, the union is so labile that it can be separated by dialysis. This is extraordinarily interesting in view of the facts that the union of drugs with platelets and the antibody in the sera of patients with thrombocytopenic purpura due to drug hypersensitivity can also be separated by

dialysis, and furthermore that the union of drugs with red cells and antibody in the sera of patients with some types of drug-induced haemolytic anaemia is equally labile. The possible significance of this lability is discussed below in the section on the immunological mechanisms causing thrombocytopenic purpura and haemolytic anaemia due to drug hypersensitivity.

An interesting finding—as yet unconfirmed—which may throw some light on the immunology of drug hypersensitivity has been reported by Torii & Kohoriuchi (1961). These workers found that rabbits could be immunized with a penicillin-egg albumin complex. If the antibody from the rabbit was injected into the skin of guinea-pigs which were later challenged with penicillin, there was no sign of passive cutaneous anaphylaxis; but if the animal was challenged with the penicillin-egg albumin conjugate or with normal human serum which had been incubated with penicillin at an acid pH, then passive cutaneous anaphylaxis did occur. Torii & Kohoriuchi (1961) then found that, whereas normal human serum had to be incubated with penicillin at an acid pH in order to produce cutaneous anaphylaxis, serum from patients with 'anaphylactoid reactions or angioneurotic oedema' due to penicillin did not need acidification. These workers concluded that the sera of patients hypersensitive to penicillin contain a peculiar protein which has a strong capacity for binding penicillin.

Blood Dyscrasias

Thrombocytopenic purpura (Ackroyd 1958, 1964)

An antiplatelet factor can often be readily demonstrated in the patient's serum and plasma. This causes agglutination of the patient's own platelets and of compatible normal platelets *in vitro* in the presence of the drug but not in its absence. The quantity of the drug used is not critical, and the reaction is not inhibited even by large concentrations of the drug. In the presence of complement, this factor may cause platelet lysis. The factor is found in the γ-globulin fraction of the patient's serum. The γ-globulin nature of this factor, the fact that it causes agglutination of platelets in the absence of complement and may cause platelet lysis in the presence of complement, all suggest that it is an antibody. The antigen cannot be the platelet alone, since platelets are not agglutinated by the antibody except in the presence of the drug. One possibility, therefore, is that the antigen is formed by union of the drug with the platelet, and it may be suggested that the drug acts as a hapten and so modifies the platelet that it becomes auto-antigenic. This implies that the drug, on the surface of the platelet, forms the determinant group with which the antibody combines. Evidence that this is so is provided by the following observations. First, if platelets are incubated with the serum of a sensitive patient, in the presence of the drug but in the absence of complement, and the platelets are then washed, both antibody and drug are removed from the platelets. (The complement has to be inactivated in order to prevent platelet

lysis.) Second, if the platelets in the above experiment are washed, not in saline, but in a saturated solution of the drug in saline, the antibody remains in contact with the platelets; but if the platelets are now dialysed against saline the drug dialyses away and the platelets separate from the antibody, which can be made to combine with platelets again if further drug is added. This seems clear evidence that the drug acts as a link between the platelets and the antibody, and strongly supports the concept that the drug acts as a hapten. On this hypothesis, when the drug is removed the platelets must separate from the antibody because they are no longer antigenic.

Occasionally, the addition of the drug to the patient's serum causes immune precipitate formation. This reaction may or may not fix complement. In a case due to antazoline it was shown that the precipitin was probably identical with the anti-platelet antibody. This suggested that while platelet lysis resulted from the action of the antibody on intact platelets, precipitate formation might result from the action of the same antibody on platelet breakdown products liberated into the serum during coagulation. This, however, was found not to be so, for precipitate formation occurred equally in sera prepared from platelet-rich plasma and from plasma from which the platelets had been removed by centrifugation. The antigen must therefore have been some other constituent of the serum, and since precipitate formation occurred only in the presence of the drug, it seems reasonable to suppose that the antigen was one of the serum proteins which had been rendered antigenic by union with the drug.

The capillary lesion appears to be independent of any effect of the drug on the platelets for, by patch testing, purpuric haemorrhages can be produced in the skin in the absence of any change in the platelet count. For this reason, and because the capillary lesion can be produced only in the presence of the drug, it seems possible that the drug may combine with the endothelial cells to form yet a further antigen which then reacts with the antibody which causes platelet lysis and sometimes, also, precipitate formation; this reaction being responsible for the capillary damage and resultant purpura. It should, however, be noted that no such anti-endothelial activity of the antiplatelet antibody has, as yet, been demonstrated.

This review of the immunology of thrombocytopenic purpura due to drug hypersensitivity has, so far, considered only the immunology of the hypersensitive patient; it has not considered how the patient becomes sensitized. One of the most striking facts about drug hypersensitivity in general is that, although hypersensitivity reactions have been described in response to treatment with a wide variety of different drugs, yet only very few of those taking any of these drugs develop such reactions. It has been shown that platelets from normal non-sensitive individuals combine with sedormid when in contact with serum (Ackroyd 1958) or plasma (Dausset 1958) from the same individual. If this combination occurs in the blood of all patients taking this drug and if the

combination is antigenic, then it must presumably be a very weak antigen since antibody formation, and consequently purpura, occur so rarely. Another observation which suggests that it may be a weak antigen is the lability of the drug-platelet combination mentioned above. A combination of drug and platelets which, even in the presence of antibody can be readily separated by dialysis, will presumably be only weakly antigenic because such a labile compound will not often remain in contact with the antibody-forming tissues long enough to stimulate antibody formation.

It may, therefore, be suggested as a tentative explanation for the development of thrombocytopenic purpura as a manifestation of drug hypersensitivity, that the drug forms a very labile compound with the platelets of most, if not all, of those taking the drug. This combination is antigenic, the drug acting as a hapten. Because it is so labile, it stimulates antibody formation very rarely. Once the antibody has formed and is present in the blood, the patient develops thrombocytopenia whenever he takes the drug because the drug combines with the platelets which consequently become antigenic and react with the antibody. In the presence of complement, the drug-platelet antigen undergoes lysis with resultant thrombocytopenia. Very occasionally, the drug combines also with plasma protein forming a further antigen which reacts with the same antibody. The significance of this reaction is unknown. Finally, it seems possible that the capillary lesion results from the action of the same antibody on vascular endothelial cells rendered antigenic by union with the drug. This hypothesis explains the two most striking features of drug-induced thrombocytopenic purpura: the infrequency with which purpura occurs in those taking the drug and its almost invariable occurrence whenever the drug is taken by those who have become sensitized.

The above hypothesis provides what is perhaps the simplest explanation of the observed facts. There are, however, certain objections to it, for if the drug in these cases does act as a hapten then it is unlike any other known hapten. Haptens are small molecules which are incapable by themselves of stimulating antibody formation but which can do so if firmly attached to protein. Free haptens unattached to protein will, however, react with antibody once this has been formed, and so block the interaction of antibody with the complete antigen (hapten plus carrier protein). If the above hypothesis concerning the immunology of drug-dependent thrombocytopenia is correct, then the hapten (drug) in this condition is so loosely attached to the carrier protein (platelet) that even after reaction of the drug-platelet antigen with antibody the drug can readily be removed by dialysis. Furthermore, the interaction of antibody with the complete antigen (platelet plus drug) is not inhibited by excess of hapten (drug). Because of these difficulties, Shulman (1963a, b; 1964) has suggested that the antibody is formed, not against a drug-platelet antigen, but against 'a stable complex of the drug with some soluble noncellular macromolecule' and that the resulting

antigen-antibody complex is then adsorbed on to the platelets. This causes their agglutination and lysis. Because the formation of this antigen-antibody complex might be expected to be inhibited by excess of the drug, Shulman has further suggested that, once formed, the antibody can combine with the drug itself and that the resulting drug-antibody complex may also be adsorbed on to platelets and cause their agglutination and lysis. According to this hypothesis, the platelet plays a purely passive role and is, to use Dameshek's (1965) vivid phrase, an 'innocent bystander'.

A major objection to this concept is that although antigen-antibody complexes can be taken up by platelets and cause them to become agglutinated, when such an effect is produced experimentally *in vivo* the condition bears almost no resemblance to the clinical syndrome of drug hypersensitivity. Agglutination of platelets and of leucocytes in the presence of antigen-antibody complexes can be demonstrated *in vitro* and this probably explains the thrombocytopenia and granulocytopenia seen in anaphylactic shock produced by the intravenous injection of protein antigens into sensitized animals. The counts often fall to levels quite as low as those seen in drug hypersensitivity, but whereas in the latter condition they usually remain low for a matter of days, in anaphylactic shock the counts have usually returned to normal within an hour or so. These experiments involved sensitization to protein antigens. In order to produce an experimental model more closely resembling that envisaged in Shulman's (1963a, b; 1964) hypothesis, Cronin (1965) and Hartl (1965) have immunized animals with antigens made by conjugating drugs to proteins by diazotization. Both workers produced evidence to show that the chemical manipulations had not altered the immunological specificity of the original drugs. They then investigated the effects of the antibodies produced in these animals on the blood cells in the presence of the appropriate drugs. Cronin (1965), who worked on thrombocytopenic purpura due to sedormid, could not demonstrate any effect of the animal antibody on animal or human platelets in the presence of sedormid *in vitro* and Hartl (1965) in a study of amidopyrine agranulocytosis failed to show any effect of the antibodies he produced on white cells in the presence of amidopyrne either *in vivo* or *in vitro*. These experiments, therefore, provide no support for the hypothesis that the antibody in this type of drug hypersensitivity is stimulated by a stable union of the drug with a soluble macromolecule and that adsorption of complexes of the antibody with the drug by cells or platelets causes their lysis.

Further evidence against this hypothesis derives from the fact that the dialysis experiments described above seem to show that although the drug acts as a link between platelets and antibody it does not form a stable union with a soluble macromolecule, for such a union would clearly prevent removal of the drug during dialysis.

According to Shulman's (1963a, b; 1964) hypothesis, the platelets play a

purely passive role in this type of drug hypersensitivity, but there are several observations which suggest that, in fact, they probably take an active part in the reaction which leads to their destruction. One such observation is that, except in the very rare cases with a drug-dependent precipitin, complement fixation only occurs in the presence of platelets. If the major immunological reaction was between the antibody and the drug or a drug-macromolecular complex then complement fixation would be expected to occur even in the absence of platelets.

Evidence has been produced (Dausset 1958) which shows that some constituent of normal serum or plasma is necessary in order for platelets to be able to take up sedormid. This, however, does not support the concept that the platelets are involved non-specifically but rather suggests that they play an active role since the plasma component fixes the drug specifically to platelets but not to white cells. It seems probable that the study of this component may provide an important key to the understanding of the mechanisms causing thrombocytopenic purpura and possibly, also, other conditions due to drug hypersensitivity.

Evidence that the platelets play more than a purely passive role is also provided by the observation that each individual drug causing a haematological hypersensitivity reaction tends to produce the same type of reaction in every patient who becomes sensitized to it. Thus, for instance, sedormid and quinidine almost invariably cause thrombocytopenic purpura whereas amidopyrine causes agranulocytosis. Although different types of cells are involved, the immunological findings in thrombocytopenic purpura, haemolytic anaemia and agranulocytosis are often very similar (see below), and according to Shulman (1964), not only is thrombocytopenia due to 'non-specific' adsorption by platelets of complexes of antibody with a drug-macromolecular antigen but haemolytic anaemia is due to adsorption by red cells of similar complexes. Consequently, if the affected cells in drug-dependent thrombocytopenic purpura, agranulocytosis and haemolytic anaemia are involved passively it would be expected that any drug capable of causing, for instance, thrombocytopenia would cause some destruction of all the formed elements in the blood in the same patient, since the complex of antibody with the drug-macromolecular antigen would be taken up passively by platelets, white cells and red cells. Similarly, complement fixation would be expected to occur when red or white cells are added to a solution of the appropriate drug in the serum of a case of drug-dependent thrombocytopenia. In fact, complement is fixed only in the presence of platelets.

Very rarely, a drug does combine with a constituent of serum to form an antigenic compound. When the drug is added to the cell-free serum of such a patient an immune precipitate is formed (Miescher & Miescher 1952; Ackroyd 1958, 1964; Tullis 1961). Shulman (1964) has quoted such cases in support of his concept that the drug or a drug-macromolecular complex reacts with the antibody and that the platelets are involved secondarily and non-specifically. However, the drug does not appear to behave like a classical hapten when it causes

immune precipitate formation any more than it does when it causes cell agglutination and lysis. In general, haptens do not cause immune precipitate formation; in fact, if the drugs behaved like classical haptens, the high concentrations used to cause precipitate formation in the sera of patients with drug hypersensitivity would be expected to inhibit it. The failure of high concentrations of the drug to inhibit precipitate formation suggests that the union of the drug with the carrier protein and antibody may be as labile as the union of the drug with cell and antibody which is also not inhibited by high concentrations of the drug. This suggestion receives considerable support from the observations that in the case due to antazoline (Ackroyd 1958) immune precipitate formation and platelet agglutination were almost certainly due to the same antibody. The rare occurrence of drug-dependent precipitins, therefore, does not necessarily provide evidence that antibody formation in thrombocytopenic purpura, haemolytic anaemia and agranulocytosis due to drug hypersensitivity is stimulated by antigens formed by stable complexes of drugs with soluble macromolecules.

It will be clear from this brief review that neither of the hypotheses considered here can be accepted without reservation. In a discussion on the concept that the drug confers antigenic properties on the cell, Coombs (1964) has pointed out that since the drug does not appear to behave like a classical hapten, it is worth considering whether this type of drug hypersensitivity may not represent a hitherto unrecognized phenomenon in immunology. An alternative hypothesis which is in accord with classical immunological theory is suggested by some recent speculations of Levine (1965). If the drug is not the hapten then the real hapten may be a metabolic product of the drug which makes a stable union with the cell and so stimulates antibody formation. When the whole drug is used in *in vitro* experiments it might 'fit' the cell and antibody less well than the real hapten and might therefore readily become dissociated from both. The failure of excess of the drug to inhibit the reaction might be explained if high concentrations were needed to overcome the tendency of the drug to dissociate from cell and antibody. Although this hypothesis would explain why the drug does not behave like a classical hapten it raises the problem why a stable antigen formed by union of a metabolite of the drug with the cells so rarely stimulates antibody formation. One of the attractions of the concept that the antigen is labile is that because of its lability it might only rarely stimulate antibody formation. Although this is pure speculation, the rarity of this type of drug reaction might be explained if it could be shown that those patients who become sensitized metabolize the drug in an unusual way and that it is only those who can form a metabolite capable of making a stable antigenic union with the cell who can become sensitized to the drug.

In conclusion, it is clear that the cause of platelet destruction in thrombocytopenic purpura due to drug hypersensitivity is still obscure. The explanation of this interesting group of disorders must await further investigation.

Haemolytic anaemia

The mechanisms by which drug hypersensitivity causes haemolytic anaemia have been studied in less detail than have those causing thrombocytopenic purpura, but in many of the reported cases the immunological findings have been very similar (Ackroyd 1964). This latter type of case will be considered first and those forms of haemolytic anaemia due to drugs which appear to be caused by different immunological mechanisms will then be discussed.

In the cases in which the immunological findings resemble those just described for thrombocytopenic purpura, the patient's serum causes agglutination of the patient's own, or of compatible normal red cells, only in the presence of the drug. Haemolysis of these cells in the presence of complement has sometimes, but not always, been observed. In some cases in which haemolysis has not occurred, the patient's serum has caused haemolysis of compatible normal trypsinized cells or of compatible cells from cases of paroxysmal nocturnal haemoglobinuria, in the presence of complement and the drug. The antiglobulin test performed by incubating red cells with the patient's serum and the drug, washing the cells and exposing them to antiglobulin serum has often been positive, suggesting that the union of drug and antibody with the red cell will withstand washing. However, the positive antiglobulin test in these cases appears to be due to complement which remains on the red cells after the drug and antibody have been removed. This was shown by Shulman (1964) in a case of haemolytic anaemia due to stibophen (fouadin). Further evidence that the drug and antibody are not firmly bound to the red cells is provided by Freedman, Barr & Brody's (1956) study of a case due to quinidine. These workers found that the union of the drug with the red cells in the presence of the patient's serum was so loose that neither the drug nor the serum antibody remained in contact with the red cells after washing, so that the antiglobulin test was negative. It appears, therefore, that in haemolytic anaemia, as in thrombocytopenic purpura due to drug hypersensitivity, the drug and antibody do not form a stable union with the cell.

Apart from cases of the kind just described, there are other types of haemolytic anaemia due to drugs which are apparently due to different mechanisms. Three of these, two of which have been clearly shown to have an immunological basis, are considered below.

It has recently been reported that penicillin can cause haemolytic anaemia. The direct antiglobulin (Coombs) test in these cases has been positive and the patient's serum has agglutinated penicillin-coated red cells. This latter finding does not, however, by itself prove that penicillin was the cause of the anaemia for, as mentioned above, an antibody capable of doing this can be demonstrated in the sera of some patients who have been treated with penicillin but who are not allergic to it. This antibody can also be demonstrated in the sera of some patients who have developed classical hypersensitivity reactions to penicillin but have not

developed haemolytic anaemia. Proof of the causative role of penicillin has recently been provided in two cases of haemolytic anaemia. In each, readministration of the drug after recovery caused recurrence of the anaemia (Petz & Fudenberg 1966; Rosner, Lai & Ritz 1966). The mechanism by which penicillin causes haemolytic anaemia is not clear. Although the haemagglutinating activity of the antibody found in penicillin-treated patients who have not developed haemolytic anaemia can be inhibited by prior treatment of the serum with penicillin, the drug has not always inhibited this reaction in the sera of patients who have developed haemolytic anaemia (see van Arsdel & Gilliland 1965). This suggests that penicillin may be capable of stimulating the formation of more than one antibody. Such a possibility is also implied by Parker's (1964) observations on haemagglutination inhibition which indicate that more than one derivative of penicillin can form the antigenic (hapten) determinant. Swanson and her colleagues (1966) have recently described a patient with penicillin haemolytic anaemia who had, in fact, developed two antipenicillin antibodies. The macromolecular (IgM) antibody agglutinated penicillin-coated red cells in saline and the reaction was inhibited by prior incubation of the patient's serum with penicillin. The other (IgG) antibody did not cause agglutination of such cells in in saline, but did sensitize them so that they were agglutinated by antiglobulin serum. This antibody was not inhibited by prior incubation with the drug. These workers considered that since it was responsible for the positive direct antiglobulin test in the patient's blood, this latter antibody was most probably the cause of the haemolytic anaemia.

As mentioned above, analgesics, when taken in excessive quantities over long periods, cause renal damage, and this is sometimes associated with a reduction in the survival time of the patient's red cells (Friis *et al* 1960). It also appears that a very slight shortening of red cell survival may occur in these patients even when they have not developed renal damage (Friis *et al* 1960). The syndrome is generally ascribed to phenacetin, but it is possible that other analgesics incorporated in analgesic mixtures may be responsible. Although phenacetin may cause haemolytic anaemia by an immunological process (Muirhead, Halden & Groves 1958; MacGibbon, Loughridge, Hourihane & Boyd 1960), no such mechanism has so far been demonstrated in cases of haemolysis associated with a prolonged and excessive intake of analgesics.

Recently, a number of reports have appeared of cases of haemolytic anaemia occurring in patients being treated with the hypotensive drug α-methyl-dopa (see Worlledge, Carstairs & Dacie 1966). Rapid recovery has followed withdrawal of the drug and treatment with steroids. This apparent association of the drug with haemolytic anaemia led to the fascinating observation that about 20% of patients who have been treated with the drug for over a year but who are not clinically anaemic have a positive direct antiglobulin test (Carstairs *et al* 1966). The erythrocyte survival time of one of four such patients in whom this

investigation was carried out was shorter than normal. The antibody in some of these cases and in nearly all of those with frank haemolytic anaemia has been shown to have specificity for one or other of the Rh antigens (Carstairs *et al* 1966; Worlledge *et al* 1966). Since the antibody reacts with the Rh antigens on the patient's own red cells it has the characteristics of an autoantibody. All attempts to demonstrate an anti-erythrocyte antibody dependent for its action on the presence of the drug *in vitro* have proved unsuccessful. A possible explanation for these extraordinary findings is that the drug so modifies the Rh antigens on the patient's red cells that they become antigenic and stimulate antibody formation.

Agranulocytosis

Relatively little is known about the immunological basis of agranulocytosis due to drug hypersensitivity. As stated above, the little that is known suggests that it may be produced by a mechanism similar to that which causes thrombocytopenic purpura (Ackroyd 1964).

In the acute stage of agranulocytosis, the patient's serum may cause agglutination of compatible normal leucocytes. The degree of agglutination can sometimes be increased by the addition of the drug to the suspension of white cells in the patient's serum. In almost every case, however, the leucoagglutinin has disappeared during the phase of recovery.

Very occasionally a case has been reported in which the leucoagglutinin has persisted after recovery and could be shown to cause leucocyte agglutination in the presence of the drug but not in its absence. This was first shown by Moeschlin (1954) in a case due to sulphapyridine. This patient's serum agglutinated compatible normal leucocytes for 3 days after the onset of the agranulocytosis but did not cause any agglutination after the 5th day. From then until the 15th day, however, leucocyte agglutination did occur if the drug was dissolved in the patient's serum. Thereafter, the agglutinin could not be demonstrated. For a long time, this was the only reported case of agranulocytosis in which a leucoagglutinin, dependent for its action on the presence of a drug, had been demonstrated. This phenomenon, which must be very rare, has, however, recently been described in cases of agranulocytosis due to sulphamethoxypyridazine (Johnson & Korst 1961), amidopyrine (Magis *et al* 1962; Thierfelder *et al* 1964) and chlorpromazine (Hoffman *et al* 1963). Agglutination and lysis of leucocytes by the drug in the patient's serum in the presence of complement has been observed in cases due to chloral hydrate and to promazine (Tullis 1961). Lysis of leucocytes occurring only in the presence of salicylazosulphapyridine has been observed in the serum of a patient who had recovered from agranulocytosis due to this drug (Ritz & Fisher, 1960).

The agglutination of leucocytes by the patient's serum in the absence of added drug during the acute stage of the agranulocytosis could be explained as being

due to the persistence, during this period, of the drug in the patient's serum although this would suggest that only very minute quantities of the drug are necessary. The reason why leucocyte agglutination can usually be demonstrated only for so short a time has not been explained. In cases of thrombocytopenic purpura due to drug hypersensitivity, the antibody may occasionally persist for only a very short time, and it seems probable that in agranulocytosis the antibody may be even more evanescent, in which case the shortness of the period during which leucocyte agglutination can be demonstrated may be due to rapid disappearance of both drug and antibody; in the early stages, during which the antibody is still present, agglutination may be increased by adding the drug; but after the antibody has disappeared no further agglutination can be demonstrated. This concept would bring the rare group of cases in which the leucoagglutinin can only be demonstrated after addition of the drug into line with those cases in which it is active in the absence of added drug or in which its activity can be enhanced by the addition of the drug; the only difference being that in cases in the first group the antibody persists after the drug has disappeared, in contrast with the remaining cases in which the antibody seems to have disappeared at or about the same time as the drug.

Reference has already been made to the difficulty of distinguishing between idiosyncrasy and hypersensitivity. The complexity of this problem is underlined by recent studies of agranulocytosis due to chlorpromazine. As mentioned above, chlorpromazine may apparently cause agranulocytosis by a purely immunological mechanism. More commonly, however, no antibody can be demonstrated in cases of agranulocytosis due to this drug (Pisciotta *et al* 1958) and in these the agranulocytosis appears to be due to a totally different mechanism. Pisciotta (1965) has recently claimed that chlorpromazine almost completely inhibits cell division of granulocyte precursors *in vitro* in bone-marrow cultures from patients who have recovered from agranulocytosis due to this drug. This effect was much greater than that seen in controls. It is apparently produced by interference with the synthesis of desoxyribonucleic acid. Since the patients who had recovered from agranulocytosis were haematologically normal at the time of investigation, Pisciotta considered that the action of the drug was possibly a manifestation of a constitutional abnormality in the patient. Such an abnormality could result from a genetically determined enzymatic defect.

The above review shows that there are large gaps in our knowledge of the immunological mechanisms involved in causing the wide variety of clinical syndromes which can result from drug hypersensitivity. Not only are many drugs and many tissues involved, but in different patients the same drug may not always produce the same reaction. It is clear that no single fundamental mechanism could possibly cause all these phenomena. Indeed, even in a field as small as haemolytic anaemia due to drug hypersensitivity (see above) it appears

that different immunological mechanisms may underlie the haemolysis caused by different drugs. It may be expected, therefore, that with increasing knowledge, many further mechanisms will be discovered.

DIAGNOSIS

The most important factor in diagnosis is that the physician should be aware of the enormously wide variety of clinical syndromes that may result from drug hypersensitivity and of the particular reactions to which the drug he is prescribing most frequently gives rise.

During treatment with a drug known to be particularly liable to cause hypersensitivity reactions, a careful watch should be kept for unexplained fever as this may be the first sign of developing hypersensitivity. If treatment is stopped as soon as the fever develops, a serious illness may be averted.

Once a reaction has developed, it is essential to inquire about all the drugs the patient has been receiving. One of the difficulties quite frequently encountered is that the patient may deny taking any drugs at all. Drugs popularly regarded as harmless and regularly consumed such as, for example, phenolphthalein, are often forgotten, or not regarded as 'drugs'. Not infrequently, when the cause is found the patient will say with astonishment: 'Oh! Doctor, I never thought you meant things like that!' Sometimes the patient is completely unaware that he is exposed to a drug or other sensitizing agent. This is particularly true when the drug is incorporated in a food. Antibiotics, for instance, are present in some dairy products, and some food dyes and preservatives are potential causes of hypersensitivity reactions. Quinine, which is an ingredient of certain drinks, is another example.

An attempt must be made to establish that the reaction is due to drug hypersensitivity. Careful correlation of the time of onset of the reaction with the dates of administration of each drug the patient has been taking may provide suggestive evidence. In general, a reaction is unlikely to be due to a drug if symptoms have appeared more than 4 or 5 days at the most after the last dose was taken. This clearly may not be true of drugs such as long-acting sulphonamides (e.g. sulphamethoxypyridazine) or drugs which are administered in a form in which they are slowly released into the gut. Even more important, sensitization to drugs administered in depot form may develop weeks or even months after the drug was given. It should also be borne in mind that cases of hypoplastic anaemia involving predominantly the red-cell precursors do not immediately become apparent, for even with complete aplasia, the haemoglobin level will not fall by more than about 1% per day, and patients rarely complain of symptoms of anaemia until the haemoglobin has fallen to about 70%. The survival of white cells and platelets is much shorter but with incomplete failure of production of these elements, symptoms may not appear for several weeks.

Whenever a drug reaction is suspected all therapy should, if possible, be stopped. If the reaction now subsides, this will strengthen the suspicion that it was due to one of the drugs the patient was taking. It does not, of course, confirm this, for the reaction may have been a naturally occurring self-limited event unrelated to drug therapy. Nor does it prove that the reaction was allergic. It may have been due to one of the other mechanisms discussed above. Furthermore, the failure of the reaction to subside when the drug is withdrawn does not necessarily exonerate it. The drug may be excreted only very slowly, or it may have been administered in depot form. Occasionally the reaction may not subside because an immunochemically related drug has been inadvisedly substituted for the one omitted. Finally, in some cases, the hypersensitivity reaction may have caused irreversible tissue damage before the drug was withdrawn, so that the reaction pursues a relentlessly progressive course.

A history of remission of symptoms when the drug was withdrawn, and recurrence when it was resumed, provides strong evidence that the drug was responsible for the reaction. However, it should be realized that the readministration of a single dose of the drug may not cause symptoms to recur even though, by other means, the condition can be shown to have been a hypersensitivity reaction to the drug being administered. Although minute doses sometimes cause recurrence of symptoms, increasing doses over a period of a fortnight or more may be required to provoke a recurrence. This subject is dealt with more fully below in the section on test dosing.

Because the readministration of a drug may give rise to a dangerous reaction in a highly sensitive patient, skin tests and *in vitro* tests (see below), if these are applicable to the particular reaction from which the patient is suffering, should be performed before a test dose is given.

Skin Tests

In general, skin tests are of little value in the diagnosis of drug hypersensitivity. A positive reaction of the wheal and flare type to an intradermal, or a scratch, or prick test, indicates only that the patient's skin is hypersensitive to the drug; it does not necessarily imply that the drug has caused or will cause a systemic reaction or, except when applied to the skin, a cutaneous reaction. In other words, a patient who gives a positive skin test to a drug may tolerate its administration by any but the percutaneous route. Furthermore, negative skin tests have not infrequently been reported in patients who have subsequently experienced systemic reactions.

Intradermal tests have produced severe reactions and Rose (1953) has reported a fatality as a result of intradermal testing with penicillin. The problems of skin testing in cases of suspected penicillin hypersensitivity have been considered above. Although the procedure using penicilloyl polylysine seems reasonably

safe, the results are not clear cut and the method cannot be recommended for routine use (Levine 1964). The antigen can be obtained from the Sigma Chemical Co. Ltd, 12 Lettice Lane, London, S.W.6, under the trade name of Cilligen. It is injected intradermally (0.02–0.04 ml) and the injection site is inspected at intervals for 20 min for a wheal and flare response. The maximum diameter of the wheal should be recorded (see Budd *et al* 1964).

Although no serious generalized reactions have been reported from patch testing, the dangers of percutaneous absorption should be borne in mind. Blanton & Blanton (1953) produced a mild generalized reaction by patch testing a patient who was highly sensitive to penicillin, and Donaldson & Scarborough (1945) observed a generalized increase in capillary fragility and a slight, but probably significant, fall in the platelet count on two occasions as a result of applying sulphathiazole for $1\frac{1}{2}$ hr to the intact skin of a patient who had recovered from thrombocytopenic purpura due to this drug. Had the drug been left in contact with the skin for a longer period, the patient would probably have developed a further attack of thrombocytopenic purpura.

Patch tests are of value in patients whose primary sensitization was of contact dermatitis type, even although the eruption under investigation may have been provoked by systemic administration of the drug. They are also of value in eczematous eruptions due to antibiotics, sulphonamides, antihistamines, phenothiazines (promethazine, chlorpromazine and related compounds), mercurials and quinine.

In photosensitization reactions, whether or not of eczematous type, patch tests are also of some value. The test may be positive after 48 hr, or may become positive only after subsequent exposure to light (see below). Further details are given in Chapter 7.

In fixed drug eruptions patch tests are negative, but Goncalves (1956) found a friction test useful. The suspected drug in a 3% concentration in propylene glycol was lightly rubbed into previously affected skin. A positive reaction developed in 3–11 hr. This test requires further evaluation.

In patients who have recovered from thrombocytopenic or non-thrombocytopenic purpura due to drug hypersensitivity, patch testing may cause a local increase in capillary fragility or even the appearance of purpuric haemorrhages in the area of skin which has been exposed to the drug. Such reactions occur only in highly sensitized patients. A negative result should not be taken as evidence that the patient is not sensitive to the drug.

The Technique of Patch Testing

If the drug is a liquid, it may be used undiluted or diluted with an inert solvent. If it is a solid, a vehicle should be used in which it is both stable and readily soluble. Both water and propylene glycol have proved satisfactory. The drug is added to a small volume of the solvent in a test tube which is heated at a tempera-

ture at which the drug will not decompose. Heating is continued until all the drug has dissolved. If a sufficient quantity of the drug has been added, crystals will separate out as the solution cools, and eventually the preparation should form a fairly fluid paste of crystals in a saturated solution in the solvent. Before use it should be established that the preparation does not cause changes in the skin of normal individuals. If it does, increasingly dilute preparations should be tried until a concentration is found which is non-irritant to normal skin. This preparation is spread over an area of the patient's skin which has previously been cleaned with ether or acetone. It is covered with a piece of filter paper backed with oiled silk, and the whole preparation is held in place with a wide strip of Elastoplast or other adhesive dressing. As a control, the solvent alone is applied in a similar manner to a corresponding area of skin on the other side of the body. The skin should be inspected at intervals. A positive result is indicated by the reproduction of the original skin condition in the area to which the drug has been applied. The skin exposed to the solvent alone should show no more than slight erythema and superficial epithelial desquamation. If no reaction has occurred in the test area after 48 hr, the test may be regarded as negative, except in the conditions mentioned below.

In photosensitization reactions, if the skin shows no change the test and control areas should be exposed to an erythema dose of ultra-violet light. Only if the skin test area still shows no change as compared with the control area should it be concluded that the test is negative.

If the patient has recovered from thrombocytopenic purpura, patch testing should be undertaken only if the platelet count is normal. Because of the dangers of percutaneous absorption of the drug, platelet counts should be performed at intervals whilst the drug is in contact with the skin. If the count should fall to 50% or less of the initial level, the drug must immediately be removed from the skin as completely as possible. A positive result of patch testing is indicated by the appearance of purpura which is confined to the area exposed to the drug. If patch testing fails to cause the appearance of purpura, the arm should be congested at a pressure of 80 mm of mercury for 5 min as in the Hess (1916) capillary fragility test, or the area of skin exposed to the drug should be subjected to a negative pressure as in the Dalldorf (1933) method of demonstrating increased capillary fragility. An increase in capillary fragility confined to the area to which the drug was applied, and not seen in the area of the control patch, indicates an effect of the drug on the capillaries.

In Vitro Tests

In the following section the principles underlying various tests which can be used as aids in the diagnosis of cases of drug hypersensitivity are described. References are also given to articles providing detailed instructions for the performance of these tests.

Reactions Involving the Release of Histamine

Basophil degranulation tests

Histamine is thought to be released from tissue mast cells and from the basophils in the blood, as a result of a reaction involving antibody and the drug to which the patient is sensitive. These tests attempt to demonstrate the loss of granules from the basophils during this reaction.

(*a*) *Direct test* (Shelley & Juhlin 1961, 1962)

A solution of the drug in saline is mixed with the patient's whole heparinized blood. After allowing time for any reaction to occur, the blood is fixed and the white cells concentrated and stained. The granules in the basophils are then observed microscopically and compared with those seen in control preparations.

(*b*) *Indirect test* (Shelley 1962, 1963)

The main difficulty in performing the direct test is due to the fact that basophils are present in only very small numbers in normal human blood. For this reason the indirect test was developed. This employs rabbit blood which contains much larger numbers of basophils. One drop of the patient's serum is placed on a slide which has been covered with dried stain and is mixed with one drop of a solution of the drug in saline and one drop of rabbit buffy coat. The preparation is covered with a cover glass and sealed with vaseline. The basophil granules are observed microscopically and compared with those seen in appropriate control preparations.

Histamine release (Shelley & Comaish 1965)

In this test, a solution of the drug in Tyrode's solution is mixed with diluted whole heparinized rabbit blood and diluted serum from the patient. After incubation at 37°C for 30 min, the histamine released is estimated chemically and compared with that released in control experiments in which the patient's serum has been used in the absence of the drug. Further control experiments using normal serum should also be performed.

Demonstration of Haemagglutinating Antibodies to Penicillin (Ley *et al* 1958; Thiel *et al* 1964)

In the original technique of Ley and his colleagues (1958) compatible human blood is diluted with Alzever's solution and then added to a vial containing powdered penicillin. After incubation, the cells are washed three times in saline. These cells are then put up against the patient's serum and against normal serum. If no agglutination is observed, the cells are washed and then suspended in antiglobulin serum and again inspected for red cell agglutination.

Hapten inhibition studies can be performed by incubating the serum with penicillin or one of its derivatives before performing the haemagglutination test.

Thiel and his colleagues (1964) wash the red cells and then incubate them with a solution of penicillin. After the incubation the cells are washed again and are then put up against several dilutions of the sera to be tested. These workers have found that with some sera much higher titres are obtained if the red cells are incubated with penicillin at pH 8.6 rather than at neutral pH.

In vitro STIMULATION OF PERIPHERAL BLOOD LYMPHOCYTES (Holland & Mauer 1964; Halpern *et al* 1967)

The patient's venous blood is heparinized and the lymphocytes are isolated in the patient's plasma. They are then cultured for 3–6 days in a tissue culture medium containing the drug thought to have caused the reaction. Stained films of the cultured cells are prepared at intervals and the proportion of lymphocytes showing 'blast' transformation is compared with the proportion seen in a similar preparation of the patient's cells cultured in the absence of the drug. The proportion of cells synthesizing desoxyribonucleic acid can be determined by adding tritiated thymidine to the cultures and making autoradiographic preparations 1 hr later (Holland & Mauer 1964).

THROMBOCYTOPENIC PURPURA

Platelet agglutination and lysis (Ackroyd 1964; Dausset 1958)

Platelet agglutination by the drug can be demonstrated in many cases if the drug is added to the patient's platelet-rich plasma taken within a few days of recovery. Platelet lysis by the drug occurs less commonly. It requires the presence of complement, whereas platelet agglutination does not. If the patient still has thrombocytopenia, platelets from compatible normal blood may be added to the patient's plasma.

The sedimentation test (Ackroyd 1964)

This measures platelet agglutination and lysis. The patient's blood, after recovery is mixed with a solution of the drug in an anticoagulant, and the preparation is allowed to stand, and the red and white cells to sediment. Agglutination and lysis of platelets by the drug cause a striking fall in the platelet count in the supernatant plasma as compared with that in the control preparation in which the patient's blood is mixed with a solution of the anticoagulant alone.

Inhibition of clot retraction (Ackroyd 1964)

The patient's blood is mixed with a solution of the drug in saline and is allowed to clot. The clot retraction is compared with that of the patient's blood diluted equally with saline alone. Since clot retraction is a function of the blood platelets,

a significant reduction in clot retraction caused by the drug may be taken as an index of the action of the drug on the patient's platelets.

Complement fixation tests (Ackroyd 1964)

(*a*) *Without antibody concentration.* The patient's blood is mixed with a solution of the drug in saline and is then allowed to clot. The serum is separated and its complement is titrated. If complement fixation has occurred, the titre will be significantly lower in the serum in this preparation than in the serum from a control preparation of the patient's blood diluted with saline alone.

This is the simplest way of demonstrating complement fixation. It is essential that there should be a normal number of platelets in the patient's blood, or the test will usually be negative. If the patient's platelet count is still low, washed platelets from compatible normal blood should be suspended in the patient's serum and a solution of the drug in saline added. If the patient's fresh serum is used, the patient's own complement may be titrated. Alternatively guinea-pig serum may be added as a source of complement. After allowing time for the reagents to interact, the preparation is centrifuged and the complement in the supernatant is titrated and compared with that in a control preparation of the platelet suspension in the patient's serum to which saline has been added in place of the solution of the drug.

(*b*) *With antibody concentration.* The antibody titre is sometimes so low that complement fixation cannot be demonstrated by the techniques just described. In this case, advantage is taken of the fact that antibody becomes attached to platelets in the presence of the drug. Compatible normal washed platelets are suspended in as large a volume as possible of the patient's serum, the complement of which has previously been inactivated by heating at 56°C for 20 min. To half this suspension a solution of the drug in saline is added, and to the remainder an equal volume of saline alone. After allowing time for the reagents to interact, each preparation is centrifuged and an equal quantity of the supernatant is removed from each, leaving the smallest amount required for complement titration. The platelets are then resuspended in the remaining serum. Equal volumes of guinea-pig serum are added to each preparation and the complement in each is titrated after allowing time for complement fixation to occur. Using this technique the antibody in the large volume of serum in the presence of the drug is concentrated on the platelets in the remaining smaller volume, and the degree of complement fixation is thereby greatly increased.

A simple method for demonstrating antibodies to platelets by complement fixation has been described by Aster and his colleagues (1964). This may well prove suitable for the investigation of cases of drug-dependent thrombocytopenic purpura.

Precipitate formation by the drug (Ackroyd, 1964)
This is very rare. It can be demonstrated by preparing doubling dilutions of the patient's serum in saline and adding to each tube an equal volume of a solution of the drug in saline. A control series of tubes is put up, in which saline alone is added to each dilution of the patient's serum. A further series of controls using normal sera are also observed. The preparations are mixed by shaking and incubated at 37°C. A floccular precipitate developing in one or more of the tubes containing the patient's serum and the drug, and not seen in the control tubes, indicates the presence of a precipitin.

Liberation of platelet factor 3 (Horowitz, Young & Nachman 1964; Horowitz, Rappaport, Young & Fujimoto 1965)
This test measures the release of platelet factor 3 from platelets damaged as a result of the addition of the drug which has caused the reaction, to the patient's platelet-rich plasma. Alternatively, the drug may be mixed with normal platelet-rich plasma to which the patient's inactivated serum has been added. In the latter type of test, a sample of normal platelet-rich plasma is incubated with the patient's inactivated serum and the drug, and the amount of platelet factor 3 liberated is estimated at intervals and compared with that liberated in a control mixture in which the patient's serum is replaced with normal serum. Although this may prove to be a simple and satisfactory technique, its specificity has not yet been established and Horowitz and his colleagues (1965) have seen one patient on long-term quinidine therapy whose serum repeatedly gave positive results with this test over a period of 6 months but who did not develop thrombocytopenia.

Amino-acid generation (Horowitz, Young & Nachman 1964; Horowitz & Nachman 1965)
In this test, a suspension of normal washed platelets is incubated with the patient's serum and the drug which has caused the reaction. The amount of α-amino nitrogen liberated in 1 hr is compared with that liberated in a mixture of normal washed platelets, normal serum and the drug. The sera used in these tests are adsorbed with barium sulphate before use (Horowitz, personal communication).

Choice of test
For reasons which are not clear, not all these tests are positive in every case of thrombocytopenic purpura which can be shown to be due to drug hypersensitivity. In consequence, ideally, all the tests should be performed on each patient. If this is impracticable, the complement fixation test with antibody concentration is probably the most reliable single test.

In vitro tests should be performed as early as possible in the course of the disease,

for the period during which the antibody remains detectable is very variable. It may persist virtually unchanged for years (Ackroyd 1958), or it may become rapidly less in successive samples of the patient's fresh blood. As an example of this, a case of thrombocytopenic purpura due to quinidine (Barkham & Tocantins 1954), may be quoted. In this case, 11 days after the patient had developed thrombocytopenia in response to a test dose of quinidine, *in vitro* tests performed on her freshly drawn blood were negative although, before the test dose was given, they had been positive.

Haemolytic Anaemia

The *in vitro* tests available for investigating cases of haemolytic anaemia due to drug hypersensitivity (see Ackroyd 1964) are essentially similar to those described for thrombocytopenic purpura.

Red cell agglutination and lysis

The addition of the drug to a suspension of the patient's red cells, or of compatible normal red cells in the patient's serum, causes red cell agglutination. Occasionally red cell lysis will occur if complement is present. Although lysis occurs infrequently, the lytic nature of the antibody can often be demonstrated if compatible trypsinized red cells or red cells from cases of paroxysmal nocturnal haemoglobinuria are used. Lysis of these cells also requires the presence of complement.

Techniques for coating red cells with penicillin in order to demonstrate haemagglutinating antibodies have already been described. As stated above, although such antibodies are found in cases of haemolytic anaemia due to penicillin they are also found in other types of penicillin hypersensitivity and in the sera of patients who have been treated with penicillin without developing any abnormal reactions to it.

The antiglobulin test. The initial steps in this test are the same as those just described for demonstrating red cell agglutination. If the drug does not cause red cell agglutination or lysis, in this test, the cells are washed and suspended in antiglobulin serum. This may cause them to become agglutinated, the agglutination being usually, if not always, due to components of complement on the red cell surface, the antibody having been removed during the washing procedure.

The exclusion of cases of haemolysis by drugs due to enzyme deficiency

As mentioned above, haemolytic anaemia due to drugs in patients with red cells deficient in the enzyme glucose-6-phosphate dehydrogenase may closely mimic that caused by drug hypersensitivity. It is for this reason desirable that the level of this enzyme in the patient's red cells should be investigated in every case

in which it appears that a drug has caused haemolytic anaemia. A low level of this enzyme can readily be demonstrated either by the screening test of Motulsky & Campbell (Doxiadis, Fessas, Valaes & Mastrokalos 1961) or that described by Brewer, Tarlov & Alving (1960). The concentration of this enzyme is higher in young cells than in old cells and, as it is the older cells which are destroyed during the stage of acute haemolysis, the enzyme content of the remaining cells is likely to be higher than the mean content of the cells before haemolysis occurred. It is possible, therefore, that if the enzyme is estimated immediately after a haemolytic crisis, the rise in the mean enzyme content caused by the destruction of the older and more enzyme-deficient cells might mask a deficiency of the enzyme. If, therefore, the first estimation indicates a normal enzyme level, the estimation should be repeated after the patient has recovered.

Agranulocytosis

In vitro tests have proved of relatively little value in the investigation of cases or agranulocytosis due to drug hypersensitivity. The following may be tried (see Ackroyd 1964).

Leucocyte agglutination

It has been shown in a number of cases that the serum of the patient taken during the acute stage of the agranulocytosis contains a leucoagglutinin which causes agglutination of leucocytes from compatible normal blood. Unlike the red cell or platelet agglutinins described above, which cause agglutination of red cells or platelets only in the presence of the appropriate drug, this agglutinin causes agglutination of white cells even if no drug is added to the suspension of cells in the patient's serum. It disappears as the patient recovers. It is of no value in diagnosis because such leucoagglutinins are also found in some cases of agranulocytosis which are not due to drugs. However, an indication that the drug may be responsible is occasionally provided by the observation that the activity of the agglutinin can be increased by the addition of the drug to the suspension of white cells in the patient's serum.

Leucocyte agglutination dependent on the presence of the drug

Although platelet agglutination and red cell agglutination by the causative drug in the sera of sensitized patients have been reported fairly frequently, leucocyte agglutination dependent on the presence of a drug in the serum of a patient who has recovered from agranulocytosis due to that drug has been reported only very rarely. As in Moeschlin's (1954, 1958) case, described above, the antibody may be demonstrable for only a very short time after recovery. In order to detect this type of antibody it is therefore essential to attempt to demonstrate leucocyte agglutination by the drug in the patient's serum as early as possible.

The Significance of Positive Serological Findings in Patients with Haematological Disorders Suspected of being due to Drug Hypersensitivity

Positive results with any of the serological tests described above may be of the greatest help in diagnosis and, taken together with an accurate history and careful physical examination, may obviate the necessity for administering a test dose of the drug; a procedure which can never be entirely free from danger. None the less, a positive result of a serological test designed to show that a drug has caused a haematological disorder does not necessarily prove that the drug has caused that disorder. Thus, Dausset & Bergerot-Blondel (1961) have described a patient in whose serum *para*-aminosalicylic acid caused agglutination of red cells, white cells and platelets, although the patient had never had clinical haemolytic anaemia or agranulocytosis, and it seemed improbable that the thrombocytopenic purpura from which he subsequently suffered was due to this drug. Other workers have also encountered reactions of this type. Reference has already been made to the agglutination by certain sera of red cells which have been exposed to penicillin. Only a minute proportion of the patients from whom these sera were obtained had suffered from haemolytic anaemia due to penicillin. Of six patients whose sera agglutinated red cells which had been exposed to chloramphenicol, five had no history of ever having been treated with this drug (Watson & Joubert 1960). It is clear, therefore, that *in vitro* tests for haematological disorders suspected of being due to drug hypersensitivity may be misleading unless they are interpreted in the light of the clinical and pathological findings.

Test Dosing

If skin tests and *in vitro* tests, when these are applicable, give negative results, the administration of a test dose of the drug, after recovery, is the only means of establishing that the drug has caused the patient's symptoms. In a highly sensitized patient, this procedure may be dangerous, and test dosing should be performed only when it is essential to establish the identity of the causative agent. The potential danger of the procedure is indicated by the fact that, as mentioned above, Rose (1953) has reported a fatal reaction to the intradermal injection of penicillin given as a skin test to a sensitive patient. Drug hypersensitivity reactions causing a reduction in the number of the formed elements in the blood have not been reported as having caused death when such extremely small doses have been given. However, a fatal attack of thrombocytopenic purpura with death from intracranial haemorrhage has resulted from an oral test dose of one grain (65 mg) of quinine (Ackroyd 1958).

In highly sensitized patients, the reaction may develop within a few minutes or a few hours of taking a small dose of the causative drug. In less sensitive patients

there may be no detectable response to a single small dose, and the administration of the drug may have to be continued for several days or even for 2 or 3 weeks, and large doses may have to be given before the drug causes a reaction.

The dose required to produce a positive response may change markedly in the same patient. This is shown very strikingly by one patient who was sensitive to antazoline (Ackroyd 1964). Shortly after she had recovered from an attack of thrombocytopenic purpura due to this drug, she developed severe thrombocytopenia in response to an oral dose of 5.0 mg of the drug. As she vomited 5 min after taking the drug, the amount absorbed must have been considerably less. Eighteen months later she was given a test dose of 0.5 mg of the drug. This produced no reaction and the dose was increased daily until, on the 12th day, she received 300 mg; still with no untoward result. On the following day she was given 100 mg and developed a very mild attack of thrombocytopenic purpura from which she rapidly recovered. The total quantity of antazoline given during the 13 days was 817.5 mg. Nine days later, by which time it was thought that she might be highly sensitive to the drug, she was given 1.0 mg by mouth. There was no reaction and the dose was slowly increased until, on the 5th day, she was given 50 mg, making a total of 71 mg in 5 days. About 3 hr after this last dose had been given, she was found to have developed thrombocytopenia, and there were a few scattered pin-point petechial haemorrhages in her skin. She again recovered rapidly.

The Technique of Test Dosing

It will be clear from what has been written above that much of what passes for test dosing in current clinical practice employs doses so large that they might well kill a highly sensitive patient and yet, when given as a single dose, might not reveal the sensitivity of a less sensitive patient. According to a standard textbook of X-ray diagnosis, the usual method of detecting sensitivity to intravenous iodine-containing contrast media is to inject '3 c.c of the solution very slowly into the vein. If the patient shows a marked systemic reaction, further injection is stopped'. A well-known textbook of therapeutics recommends that 'a test dose of 0.2 ml of serum should be injected subcutaneously and the patient observed for a period of half an hour' before a therapeutic dose of antiserum is given to a patient not known to be sensitive. Such procedures could be guaranteed to kill almost any highly sensitive patient, and could better be described as treating the coroner rather than the patient.

It has already been emphasized that although quite large doses of a drug may be required to produce a reaction, extremely small doses have sometimes caused severe and even fatal reactions. Bierlein (1956) has reported what is probably the smallest recorded dose which has produced a severe reaction. This was 3×10^{-6} of a unit of procaine penicillin. It is clearly quite impracticable to make a routine of starting courses of test doses with quantities as small as this, because

the time which would be required slowly to increase the dose to a therapeutic level would be prohibitive. Moreover, in cases where the patient is likely to be sensitive to such minute doses, the chance of increasing the dose too rapidly, and accidentally giving a fatal dose, would be too great. Test dosing should therefore never be performed in conditions in which a fatal reaction is known to be a possibility unless treatment is essential and no alternative immunochemically unrelated drug can be given. Also, test dosing should never be attempted with drugs which form depots; the danger of precipitating a reaction which cannot be controlled because the drug is being continually released is too great. Test dosing should be undertaken only if the patient has recovered completely from the reaction thought to have been caused by the drug; if there is no immunochemically unrelated drug which can be used in place of this drug; if the patient is likely to suffer if he does not receive the drug; and if it is unlikely that test dosing will cause a fatal reaction. Certainly, if the patient has recovered from a severe reaction associated with a state of shock, or from aplastic anaemia, test dosing should never be attempted.

As already stated, before giving a test dose, it is essential to ensure that the patient has recovered completely from the condition thought to have been due to the drug and in the case of haemolytic anaemia, agranulocytosis or thrombocytopenic purpura a blood count should be shown to be completely normal. In the latter condition it should also be shown that the capillary fragility is normal.

If it is decided to undertake test dosing, it will clearly be essential to start with a very small dose, and it is recommended (Ackroyd 1964) that the initial oral dose should not be more than a microgram. If the patient has recovered from haemolytic anaemia, agranulocytosis, or thrombocytopenic purpura, a blood count should be performed immediately before, and again about 3 and about 6 hours after the test dose is given, and this routine should be repeated with each succeeding dose. If no change in the blood count or other signs of hypersensitivity are noted, the dose may be increased daily until the patient develops a reaction, or until a therapeutic dose is reached. The dose should be increased as follows: 10 μg is given on the 2nd day and then 0.1 mg; 1.0 mg; 10 mg; 50 mg; 100 mg; and so on, on successive days. If a therapeutic dose has been reached and no signs of hypersensitivity have developed, the dose should be maintained at that level for at least 14 days before it is concluded that the patient is not sensitive to the drug (Ackroyd 1964).

If the drug has to be given parenterally, it is recommended that the initial dose should not be more than 1 nanogram (1.0 $n = 10^{-9}$g) and that, if necessary 10.0 n should be given on the 2nd day; 0.1 μg on the 3rd; and 1.0 μg on the 4th. Thereafter, the dose may be increased as in the scheme for drugs given orally.

There is an important warning which must be given at this stage. If a previous attempt to demonstrate hypersensitivity by the administration of a test dose has been made, even though this produced no detectable evidence of hypersensitivity

it may have caused a rise in antibody titre so that the patient may be found to be highly sensitive when next tested. For this reason, the initial dose given should be much smaller than the one previously tolerated.

TREATMENT

The detailed treatment of the actual systemic or cutaneous reaction is well dealt with in textbooks of therapeutics, and needs no further mention here. Some points of value in prophylaxis and in the technique of desensitization may, however, not be out of place.

PROPHYLAXIS

The first point to emphasize is that drugs with a high potentiality for causing serious reactions should be given only when treatment is essential and there is no alternative drug. It might be thought that this is so obvious as not to be worth stating, but the annual consumption of drugs such as chloramphenicol makes it clear that this principle is not sufficiently widely observed. Chloramphenicol may be life-saving in typhoid fever and other infections due to organisms which are not highly sensitive to any other antibiotic. Owing to its well-known capacity for causing aplastic anaemia, it should be used only for such infections; yet as Garrod (1958) has pointed out '. . . many unfortunate victims (of aplastic anaemia) have received the treatment (chloramphenicol) for quite trivial conditions'.*

The second point of importance in prophylaxis is that if treatment with a drug which is known to cause serious reactions is contemplated, the patient should always be asked if previous administration of the drug has caused symptoms. Furthermore, the patient's statement that he is sensitive to a drug must never be ignored. Failure to observe these rules has led to a disturbingly large number of severe reactions, and some deaths. In this connection, it should be remembered that drugs, the sale of which is not in any way restricted, may occasionally cause severe and even fatal reactions. As an example of this, aspirin administered by a doctor who was unaware that the patient was highly sensitive to it, has caused the death of the recipient (Halpern 1958a). If the patient has previously developed symptoms suggestive of serious drug hypersensitivity, a chemically unrelated drug should be substituted. In cases where no other drug can be substituted, the patient's sensitivity should be investigated by the methods described above. If the sensitivity is confirmed, this must usually be taken as an absolute contra-indication to the use of the drug although, occasionally, a course of desensitization (see below) may be tried.

A third point is to inquire whether the patient has previously suffered from

* Net sales of chloramphenicol in 1958 were over $62,000,000, and in 1959 over $70,000,000 (Saidi, Wallerstein & Aggeler 1961).

asthma, hay fever, or infantile eczema, because as stated above, there is some evidence—although this is admittedly inconclusive—that such patients may be more liable than normal individuals to develop certain types of drug reaction.

One further point of prophylactic value has already been mentioned in considering the diagnosis of hypersensitivity reactions: unexplained fever may be the first sign that the patient is developing such a reaction, and a careful watch should be kept for even a small spike of fever which may herald the reaction. This is particularly important in the more severe reactions, such as those involving the formed elements of the blood, because if treatment is stopped at this stage a fatal result may possibly be avoided.

Desensitization

It has been shown above that the patient's sensitivity to a drug may persist for years or may apparently disappear within a few days. However, if the drug is given again to a patient whose hypersensitivity seems to have disappeared, symptoms of hypersensitivity may reappear after a few days to a week or two of continuous treatment. This makes it difficult to evaluate the efficacy of attempts at desensitization. The procedure is potentially dangerous and should be attempted only when the patient's health urgently demands the administration of a drug for which no effective substitute is available. In practice, desensitization is rarely likely to be required except in certain cases of hypersensitivity to streptomycin or other antituberculous drugs. The technique involves the administration of extremely small but slowly increasing doses, and is described by Smith & Zirk (1961), Crofton (1953) and Alexander (1953). Some authors claim a high proportion of successes particularly with *para*-aminosalicylic acid (cf. Chapter. 24). It is not known why this procedure sometimes enables the patient to tolerate the drug.

REFERENCES

Ackroyd J.F. (1949) A simple method of estimating clot retraction with a survey of normal values and the changes that occur with menstruation. *Clin. Sci.* **7**, 231–47.

Ackroyd J.F. (1953) Allergic purpura, including purpura due to foods, drugs and infections. *Am. J. Med.* **14**, 605–32.

Ackroyd J.F. (1954) The role of sedormid in the immunological reaction that results in platelet lysis in sedormid purpura. *Clin. Sci.* **13**, 409–23.

Ackroyd J.F. (1958) Thrombocytopenic purpura due to drug hypersensitivity, In *Sensitivity Reactions to Drugs*, pp. 28–62, ed. Rosenheim M.L. & Moulton R. Oxford: Blackwell Scientific Publications.

Ackroyd J.F. (1960) The pathogenesis of purpura. In *Lectures on Haematology*, pp. 217–47, ed. Hayhoe F.G.J. Cambridge University Press.

Ackroyd J.F. (1964) The diagnosis of disorders of the blood due to drug hypersensitivity caused by an immune mechanism. In *Immunological Methods*, pp. 453–509, ed. Ackroyd J.F. Oxford: Blackwell Scientific Publications.

ALARCÓN-SEGOVIA D., WORTHINGTON J.W., WARD L.E. & WAKIM K.G. (1965) Lupus diathesis and the hydralazine syndrome. *New Engl. J. Med.* **272,** 462–6.

ALEXANDER H.L. (1955) *Reactions with Drug Therapy.* Philadelphia and London: Saunders.

ALEXANDER L.J. (1953) Desensitization of the penicillin-sensitive patient. *A.M.A. Archs Derm. Syph.* **68,** 323–7.

AMOS H. (1964) Effect of actinomycin D and chloramphenicol on protein synthesis in chick fibroblasts. *Biochim. biophys. Acta* **80,** 269–78.

ANGERVALL L., LEHMANN L. & BENGTSSON U. (1964) The renal concentrating capacity in albino rats after long-term consumption of phenacetin, N,A.P.A. (N-acetyl-p-aminophenol) and acetylsalcylic acid. *Acta med. scand.* **175,** 155–60.

ASTER R.H., COOPER H.E. & SINGER D.L. (1964) Simplified complement fixation test for the detection of platelet antibodies in human serum. *J. Lab. clin. Med.* **63,** 161–72.

ATKINS E. (1960) Pathogenesis of fever. *Physiol. Rev.* **40,** 580–646.

ATKINS E. & SNELL E.S. (1965) Fever. In *The Inflammatory Process,* pp. 495–534, ed. ZWEIFACH B.W., GRANT L. & MCCLUSKEY R.T. New York: Academic Press.

BAER R.L. (1954) Cross-sensitization phenomena. In *Modern Trends in Dermatology* (2nd series), pp. 232–58, ed. MACKENNA R.M.B. London: Butterworth.

BAER R.L. & SCHWARZSCHILD L. (1955) Selected allergic skin diseases in older persons. *Geriatrics* **10,** 265–73.

BARKHAM P. & TOCANTINS L.M. (1954) Observations on the thrombocytopenia due to hypersensitivity to quinidine. *Blood,* **9,** 134–43.

BATCHELOR F.R., DEWDNEY J.M., FEINBERG J.G. & WESTON R.D. (1967) A penicilloylated protein impurity as a source of allergy to benzylpenicillin and 6-aminopenicillanic acid. *Lancet* **i,** 1175–7.

BEARE, M. (1962) Toxic epidermal necrolysis. *Archs. Derm.* **86,** 638–53.

BERGSTRAND A., BERGSTRAND C.G., ENGSTRÖM N. & HERRLIN K.M. (1962) Renal histology during treatment with oxazolidine-diones (trimethadione, ethadione and paramethadione). *Pediatrics, Springfield* **30,** 601–7.

BERKOWITZ M., GLASER J. & JOHNSTONE D.E. (1953) The incidence of allergy to drugs in pediatric practice. *Ann. Allergy* **11,** 561–6.

BEUTLER E. (1959) The hemolytic effect of primaquine and related compounds. *Blood* **14,** 103–9.

BIERLEIN K.J. (1956) Repeated anaphylactic reactions in a patient highly sensitized to penicillin. *Ann. Allergy* **14,** 35–40.

BLANTON W.B. & BLANTON F.M. (1953) Unusual penicillin hypersensitiveness. *J. Allergy* **24,** 405–6.

BLIZZARD R.M., HAMWI G.J., SKILLMAN T.G. & WHEELER W.E. (1959) Thyroglobulin antibodies in multiple thyroid diseases. *New Engl. J. Med.* **260,** 112–16.

BOUMA H.G.D. (1966) Trombocytopenie na het gebruik van acetosal. Doctoral thesis. Holland: University of Groningen.

BRAUN-FALCO, O. and GEISSLER, H. (1962) Zwei Epidermolysis acuta combustiformis. *Med. Welt.* **34,** 1737–42.

BREWER G.J., TARLOV A.R. & ALVING A.S. (1960) Methaemoglobin reduction test. A new, simple, *in vitro* test for identifying primaquine-sensitivity. *Bull. Wld Hlth Org.* **22,** 633–40.

BROWN B.C., PRICE E.V. & MOORE M.B. (1964) Pencilloyl-polylysine as an intradermal test of penicillin sensitivity. *J. Am. med. Ass.* **189,** 599–604.

BROWN E.A. (1955) Problems of drug allergy. *J. Am. med. Ass.* **157,** 814–19.

BROWN E.A. (1959) Reactions to drugs. In *International Textbook of Allergy,* pp. 609–23, ed. JAMAR J.M. Oxford: Blackwell Scientific Publications.

BUDD M.A., PARKER C.W. & NORDEN C.W. (1964) Evaluation of intradermal skin tests in penicillin hypersensitivity. *J. Am. med. Ass.* **190**, 203–5.

CAHAL D.A. (1966) Methyldopa and haemolytic anaemia. *Lancet* **i**, 201.

CARSTAIRS K.C., BRECKENRIDGE A., DOLLERY C.T. & WORLLEDGE S. (1966) Incidence of a positive direct Coombs test in patients on α-methyldopa. *Lancet* **ii**, 133–5.

CHARGIN L. & LEIFER W. (1940) Fixed eruptions due to the arsphenamines. *J. invest. Derm.* **3**, 443–63.

CHASE M.W. (1958) Antibodies to drugs. In *Sensitivity Reactions to Drugs*, pp. 125–34, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

COMENS P. (1956) Experimental hydralazine disease and its similarity to disseminated lupus erythematosus. *J. Lab. clin. Med.* **47**, 444–54.

COOKE R.A. (1919) Allergy in drug idiosyncracy. *J. Am. med. Ass.* **73**, 759–60.

COOMBS R.R.A. (1964) In *Immunological Methods*, p. 510, ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

CORR D.J. & WELLMAN W.E. (1956) Reactions to penicillin. *Minn. Med.* **39**, 599–610.

CREGER W.P. & HOUSEWORTH J.H. (1954) Erythrophagocytosis and thrombocytopathy occurring during the course of a Henoch-Schönlein syndrome due to quinine. *Am. J. Med.* **17**, 423–31.

CROFTON J. (1953) Desensitization to streptomycin and P.A.S. *Brit. med. J.* **2**, 1014–17.

CRONIN A.E. (1965) The immunology of allergic drug reactions. Ph.D. thesis. University of Cambridge.

CRUICKSHANK B. (1966) The basic pattern of tissue damage and pathology of systemic lupus erythematosus. In *Lupus Erythematosus: A Review of the Current Status of Discoid and Systemic Lupus Erythematosus and Their Variants*, pp. 10–53, ed. DUBOIS E.L. New York: McGraw-Hill Book Company.

DACIE J.V. (1962) *The Haemolytic Anaemias, Congenital and Acquired Part II, The Autoimmune Haemolytic Anaemias*, 2nd ed. London: J. & A. Churchill.

DALLDORF G. (1933) A sensitive test for subclinical scurvy in man. *Am. J. Dis. Child.* **46**, 794–802.

DAMESHEK W. (1965) *I.T.P.* In *Concepts of Autoimmunity and Their Application in Haematology*, Series Haematologica **9**, 61–75, ed. BJÖRKMAN S.E.

DAUSSET J. (1958) The role of 'peri-platelet atmosphere' in the mechanism of thrombocytopenic purpura due to sedormid. In *Sensitivity Reactions to Drugs*, pp. 63–74, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

DAUSSET J. & BERGEROT-BLONDEL Y. (1961) Étude d'un anticorps allergique actif en présence de para-amino-salicylate de soude (PAS) contre les hématies, les leucocytes et les plaquettes humaines. *Vox. Sang. (Basel)*, **6**, 91–109.

DAUSSET J., CONTU L. & MAGIS C. (1963) Anémies hémolytiques médicamenteuses de type allergique. *Proc. 9th Congress europ Soc. Haemat. Lisbon*, pp. 1062–9. Basel and New York: Karger.

DAVIES G.E. (1958) Chemical structure and pharmacodynamic action in relation to drug sensitivity. In *Sensitivity Reactions to Drugs*, pp. 149–72, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

DEVADATTA S., GANGADHARAM P.R., ANDREWS R.H., FOX W., RAMAKRISHNAN C.V., SELKON J.B. & VELU S. (1960) Peripheral neuritis due to isoniazid. *Bull. Wld Hlth Org.* **23**, 587–98.

DE WECK A.L. (1964) Penicillin allergy: its detection by an improved haemagglutination technique. *Nature (Lond.)* **202**, 975–7.

DONALDSON G.M. & SCARBOROUGH H. (1945) Toxic thrombocytopenic purpura following local sulphathiazole therapy. *Archs Dis. Childh.* **20**, 69–72.

DOXIADIS S.A., FESSAS P., VALAES T. & MASTROKALOS N. (1961) Glucose-6-phosphate dehydrogenase deficiency. *Lancet* **i,** 297–301.

DUBOIS E.L. (1966) The clinical picture of systemic lupus erythematosus. In *Lupus Erythematosus,* pp. 129–276, ed. DUBOIS E.L. New York and London: McGraw-Hill Book Company.

DUBOIS E.L., KATZ Y.L., FREEMAN V. & GARBAK F. (1957) Chronic toxicity studies of hydralazine (apresoline) in dogs with particular reference to the production of the 'hydralazine syndrome'. *J. Lab. clin. Med.* **50,** 119–26.

DUSTAN H.P., TAYLOR R.D., CORCORAN A.C. & PAGE I.H. (1954) Rheumatic and febrile syndrome during prolonged hydralazine treatment. *J. Am. med. Ass.* **154,** 23–9.

ELVES M.W., ROATH S. & ISRAËLS M.C.G. (1963) The response of lymphocytes to antigen challenge *in vitro*. *Lancet* **i,** 806–7.

EPSTEIN W.L. & MAYOCK R.L. (1957) Induction of allergic contact dermatitis in patients with sarcoidosis. *Proc. Soc. exp. Biol. Med.* **96,** 786–7.

EVANS D.A.P. & CLARKE C.A. (1961) Pharmacogenetics. *Brit. med. Bull.* **17,** 234–40.

EVANS D.A.P. & WHITE T.A. (1964) Human acetylation polymorphism. *J. Lab. clin. Med.* **63,** 394–403.

FEINBERG S.M. & FEINBERG A.R. (1956) Allergy to penicillin. *J. Am. med. Ass.* **160,** 778–9.

FISHER A.A. (1966) Systemic eczematous 'contact-type' dermatitis medicamentosa. *Ann. Allergy* **24,** 406–20.

FREEDMAN A.L., BARR P.S. & BRODY E.A. (1956) Hemolytic anemia due to quinidine: observations on its mechanism. *Am. J. Med.* **20,** 806–16.

FRIIS T., FOGH J. & NISSEN N.I. (1960) Erythrocyte survival time in persons abusing phenacetin, with or without renal insufficiency. *Acta med. scand.* **167,** 253–66.

GARDNER D.L. (1957) The response of the dog to oral L-hydrazinophthalazine (hydralazine). *Brit. J. exp. Path.* **38,** 227–35.

GARROD L.P. (1958) The production of bone marrow aplasia by chloramphenicol. In *Sensitivity Reactions to Drugs,* pp. 92–9, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

GONCALVES A.P. (1956) Friction skin test in fixed erythema. *Anais bras. Derm. Sif.* **31,** 115–24.

GOOD R.A., KELLY W.D., RÖTSTEIN J. & VARCO R.L. (1962) Immunological deficiency diseases. *Prog. Allergy* **6,** 187–319.

GOODMAN M.H. & ARTHUR R.D. (1941) Fixed eruptions. Report on an unusual condition due to sulfanilamide. *Archs Derm. Syph.* **43,** 692–7.

GRAHAM J.R. (1964) Methysergide for prevention of headache: experience in five hundred patients over three years. *New Engl. J. Med.* **270,** 67–72

GRAHAM J.R., SUBY H.I., LE COMPTE P.R. & SADOWSKY N.L. (1966) Fibrotic disorders associated with methysergide therapy for headache. *New Engl. J. Med.* **274,** 359–68.

GROTH O. (1961) Lichenoid dermatitis resulting from treatment with the phenothiazine derivatives metopromazine and laevomepromazine *Acta derm.-vener., Stockh.* **41,** 168–77.

GUTMAN A.B. (1957) Drug reactions characterized by cholestasis associated with intrahepatic biliary tract obstruction. *Am. J. Med.* **23,** 841–5.

HALPERN B.N. (1958a) The immunological character of humoral antibodies in drug allergy. In *Sensitivity Reactions to Drugs,* pp. 135–46, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

HALPERN B.N. (1958b) In *Discussion on Halpern* (1958a).

HALPERN B., KY N.T., AMACHE N., LAGRUE G. & HAZARD J. (1967) Diagnostic de l'allergie médicamenteuse *in vitro* par l'utilisation du test de transformation lymphoblastique (T.T.L.) *Presse méd.* **75,** 461–5.

HARBER L.C., TROMOVITCH T.A. & BAER R.L. (1961) Studies on photosensitivity due to dimethylchlortetracycline. *J. invest. Derm.* **37,** 189–93.

HARTL W. (1965) Drug allergic agranulocytosis (Schultz's disease). *Semin. Hemat.* **2,** 313–37.

HARTMANN H.A., MILLER E.C. & MILLER J.A. (1959) Periarteritis in rats given a single injection of 4-fluoro-10-methyl-1,2-benzanthracene. *Proc. Soc. exp. Biol. Med.* **101,** 626–9.

HARVEY A.M., SHULMAN L.E., TUMULTY P.A., CONLEY C.L. & SCHOENRICH E.H. (1954) Systemic lupus erythematosus; review of the literature and clinical analysis of 138 cases. *Medicine (Baltimore)* **33,** 291–437.

HASERICK J.R. (1954) Blood factor in lupus erythematosus. In *Modern Trends in Dermotology* (2nd series), pp. 76–86, ed. MACKENNA R.M.B. London: Butterworth.

HAZLETT B.E. & YENDT E.R. (1958) Thrombocytopenia following gold therapy with successful treatment. *Can. med. Ass. J.* **79,** 31–3.

HESS A.F. (1916) The blood and the blood vessels in hemophilia and other hemorrhagic diseases. *Archs intern. Med.* **18,** 203–20.

HEYMANN W., HACKEL D.B. & HUNTER J.L.P. (1960) Trimethadione (tridione) nephrosis in rats. *Pediatrics, Springfield* **25,** 112–18.

HILDRETH E.A., BIRO C.E. & MCCREARY T.A. (1960) Persistence of the 'hydralazine syndrome'. A follow-up study of eleven cases. *J. Am. med. Ass.* **173,** 657–60.

HIMSWORTH H.P. (1950) *Lectures on the Liver and its Diseases,* 2nd edn., p. 164. Oxford: Blackwell Scientific Publications.

HOFFMAN G.C., HEWLETT J.S. & GARZÓN F.L. (1963) A drug-specific leuco-agglutinin in a fatal case of agranulocytosis due to chlorpromazine. *J. clin. Path.* **16,** 232–4.

HOIGNÉ R. (1958) Nephelometric studies on the sera of patients sensitive to drugs. In *Sensitivity Reactions to Drugs,* pp. 118–23, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

HOLDSWORTH C.D., ATKINSON M. & GOLDIE W. (1961) Hepatitis caused by the newer amine-oxidase-inhibiting drugs. *Lancet* **ii,** 621–3.

HOLLAND P. & MAUER A.M. (1964) Drug-induced in-vitro stimulation of peripheral lymphocytes. *Lancet* **i,** 1368–9.

HOLLEY H.L. (1961) Drug therapy and the etiology of systemic lupus erythematosus. *Ann. intern. Med.* **55,** 1036–9.

HOLLISTER L.E. (1957) Allergy to chlorpromazine manifested by jaundice. *Am. J. Med.* **23,** 870–9.

HOROWITZ H.I. & NACHMAN R.L. (1965) Drug purpura. *Semin. Hemat.* **2,** 287–312.

HOROWITZ H.I., RAPPAPORT H.I., YOUNG R.C. & FUJIMOTO M.M. (1965) Change in platelet factor 3 as a means of demonstrating immune reactions involving platelets: its use as a test for quinidine-induced thrombocytopenia. *Transfusion* **5,** 336–43.

HOROWITZ H.I., YOUNG R. & NACHMAN R.L. (1964) Thrombocytopenia due to digitoxin: demonstration of antibody and mechanism of action. *J. clin. Invest.* **43,** 1236.

HUGULEY C.M., ERSLEV A.J. & BERGSAGEL D.E. (1961) Drug-related blood dyscrasias. *J. Am. med. Ass.* **177,** 23–6.

JACOBS J.C. (1963) Systemic lupus erythematosus in childhood. Report of thirty five cases, with discussion of seven apparently induced by anticonvulsant medication, and of prognosis and treatment. *Pediatrics, Springfield* **32,** 257–64.

JANEWAY C.A. (1962) Hypogammaglobulinemia and immunologic responses. In *Allergology. Proceedings of the Fourth International Congress of Allergology, New York City, October 15–20, 1961,* pp. 241–51, ed. BROWN E.A. Oxford: Pergamon Press.

JEFFERSON J. (1967) Lyell's toxic epidermal necrolysis: a staphylococcal aetiology? *Br. med. J.* **2,** 802–4

JILLSON O.F. & CURWEN W.L. (1959) Phototoxicity, photoallergy and photoskin tests. *A.M.A. Archs Derm. Syph.* **80,** 678–86.

JOHNSON F.D. & KORST D.R. (1961) Pancytopenia associated with sulfamethoxypyridazine administration. *J. Am. med. Ass.* **175,** 967–70.

KAPLAN J.M., WACHTEL H.L., CZARNECKI S.W. & SAMPSON J.J. (1965) Lupus-like illness precipitated by procainamide hydrochloride. *J. Am. med. Ass.* **192,** 444–7.

KATZ H.I., GILL K.A., BAXTER D.L. & MOSCHELLA S.L. (1964) Indirect basophil degranulation test in penicillin allergy. *J. Am. med. Ass.* **188,** 351–4.

KIRSHBAUM M.D. & BEERMAN H. (1964) Photosensitization due to drugs. A review of some of the recent literature. *Am. J. med. Sci.* **248,** 445–68.

KNOWLES F.C., DECKER H.B. & KANDLER R.P. (1936) Phenolphthalein dermatitis. An experimental study including reproduction of the eruption in skin transplants. *Archs Derm. Syph.* **33,** 227–37.

KNOX, J.M. (1961) Clinical aspects and types of drug-induced photosensitivity. *Ann. Allergy* **19,** 749–54.

KNUDSEN E.T., ROBINSON O.P.W., CROYDON E.A.P. & TEES E.C. (1967) Cutaneous sensitivity to purified benzylpenicillin. *Lancet* **i,** 1184–8.

KRAKOFF I.H., KARNOFSKY D.A. & BURCHENAL J.H. (1955) Effects of large doses of chloramphenicol on human subjects. *New Engl. J. Med.* **253,** 7–10.

LADD A.T. (1962) Procainamide-induced lupus erythematosus. *New Engl. J. Med.* **267,** 1357–8.

LANCET ANNOTATION (1903) Salicylic acid in strawberries. *Lancet* **i,** 1187.

LANDSTEINER K. & JACOBS J. (1936) Studies on the sensitization of animals with simple chemical compounds, II. *J. exp. Med.* **64,** 625–39.

LEPPER M.H., DOWLING H.F., ROBINSON J.A., STONE T.E., BRICKHOUSE R.L., CALDWELL E. R. & WHELTON R.L. (1949) Studies on hypersensitivity to penicillin. *J. clin. Invest.* **28,** 826–31.

LEVINE B.B. (1964) Immunochemical mechanisms involved in penicillin hypersensitivity in experimental animals and in human beings. In *Therapy with the New Penicillins,* ed. BRUMFITT W. & WILLIAMS J.D., *Post-grad. med. J. Suppl.* to vol. **40,** 146–52.

LEVINE B.B. (1965) Induction of immune response and the role of antibody specificity in drug hypersensitivity-type hemolytic anemias and thrombocytopenias. *Semin. Hemat.* **2,** 338–46.

LEY A.B., HARRIS J.P., BRINKLEY M., LILES B., JACK J.A. & CAHAN A. (1958) Circulating antibody directed against penicillin. *Science* **127,** 1118–19.

LICHTENSTEIN M.R. & CANNEMEYER W. (1953) Severe para-amino-salicylic acid hypersensitivity simulating mononucleosis or hepatitis. *J. Am. med. Ass.* **152,** 606–7.

LOVEMAN A.B. (1934) Experimental aspect of fixed eruption due to Alurate, a compound of Allonal. *J. Am. med. Ass.* **102,** 97–101.

MACGIBBON B.H., LOUGHRIDGE L.W., HOURIHANE D.O'B. & BOYD D.W. (1960) Autoimmune haemolytic anaemia with acute renal failure due to phenacetin and p-aminosalicylic acid. *Lancet* **i,** 7–10.

MAGIS C., THIERFELDER S., SAINT-PAUL M. & DAUSSET J. (1962) Étude sérologique d'une agranulocytose allergique à l'aminopyrine. *Nouv. Revue fr. Hémat.* **2,** 602–8.

MAYER P.S., MOSKO M.M., SCHUTZ P.J., OSTERMAN F.A., STEEN L.H. & BAKER L.A. (1953) Penicillin anaphylaxis. *J. Am. med. Ass.* **151,** 351–3.

MAYER R.L. (1950) Compounds of quinone structure as allergens and cancerogenic agents. *Experientia (Basel)* **6,** 241–50.

MELTZER L. & BAER R.L. (1949) Sensitization to monoglycerol para-aminobenzoate: case report. *J. invest. Derm.* **12,** 31–9.

MENDES E., RODRIGUES A., FERNANDES A., ULHÔA CINTRA A. & LACAZ C.S. (1959) Allergic study of seven cases of sudden death ascribed to penicillin. *Acta allerg.* **14,** 445–56.

MEYLER L. (1952) *Side Effects of Drugs.* Amsterdam: Elsevir.

MEYLER L. (1955 onwards) Side effects of drugs. Periodical reviews. *Excerpta med.*

MIESCHER P. & MIESCHER A. (1952) Die Sedormid-Anaphylaxie. *Schweiz. med. Wschr.* **82,** 1279–82.

MIESCHER P. & PLETSCHER A. (1958) Zur Pathogenese der Anämie bei Patienten mit Abusus phenacetinhaltiger Analgetica. *Schweiz. med. Wschr.* **88,** 1056–60.

MOESCHLIN S. (1954) Weitere Beobachtungen über Immunoleukopenien und-agranulocytosen. *Schweiz. med. Wschr.* **84,** 1100–7.

MOESCHLIN S. (1958) Agranulocytosis due to sensitivity to drugs. In *Sensitivity Reactions to Drugs,* pp. 77–88, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

MOLLIN D.L. Sideroblasts and sideroblastic anaemia. *Brit. J. Haemat.* **11,** 41–8.

MORROW J.D., SCHROEDER H.A. & PERRY H.M. (1953) Studies on the control of hypertension by Hyphex. II. Toxic reactions and side effects. *Circulation,* **8,** 829–39.

MOSER R.H. (1956) Diseases of medical progress. *New Engl. J. Med.* **255,** 606–14.

MUELLING R.J., BEVEN T., SAMSON R.F., JENEVEIN E.P. & GUILLORY J.D. (1958) Studies in human sensitivity to antibiotics. I. Testing for precipitins after reaction to antibiotics. *Am. J. clin. Path.* **29,** 503–5.

MUIRHEAD E.E., HALDEN E.R. & GROVES M.T. (1958) Drug-dependent Coombs (antiglobulin) test and anemia: observations on quinine and acetophenetidin (phenacetin). *Archs. intern. Med.* **101,** 87–96.

MUNOZ J. (1964) Effect of bacteria and bacterial products on antibody response. *Adv. Immun.* **4,** 396–440.

NACHMAN R.L. & ENGLE R.L. (1965) Amino acid generation following platelet-antibody interaction. *Vox Sang.* **10,** 416–28.

NAEGELI O., DE QUERVAIN F. & STALDER W. (1930) Nachweis des zellulären Sitzes der Allergie beim fixen Antipyrinexanthem (Autotransplantationen, Versuch *in vitro*). *Klin. Wschr.* **9,** 924–8.

NOAH J.W. (1964) Anaphylactic histamine release and the Schultz-Dale technique. In *Immunological Methods,* ed. ACKROYD J.F. Oxford: Blackwell Scientific Publications.

NOAH J.W. & BRAND A. (1954) Release of histamine in the blood of ragweed-sensitive individuals. *J. Allergy* **25,** 210–14.

NOAH J.W. & BRAND A. (1955) Correlation of blood histamine release and skin test response to multiple antigens. *J. Allergy* **26,** 385–93.

PARKER C.W. (1964) The immunochemical basis for penicillin allergy. In *Therapy with the New Penicillins,* ed. BRUMFITT W. & WILLIAMS J.D., *Post-grad. med. J. Suppl.* to vol. **40,** pp. 141–5.

PARKER C.W., SHAPIRO J., KERN M. & EISEN H.N. (1962) Hypersensitivity to penicillenic acid derivatives in human beings with penicillin allergy. *J. exp. Med.* **115,** 821–38.

PERLMAN F. (1965) Value of skin testing for penicillin allergy. *J. Am. med. Ass.* **192,** 64–5.

PERRY H.M. & SCHROEDER H.A. (1954) Syndrome simulating collagen disease caused by hydralazine (apresoline). *J. Am. med. Ass.* **154,** 670–3.

PETERS G.A., HENDERSON L.L. & PRICKMAN L.E. (1955) Anaphylactic penicillin reactions: three nonfatal cases of reaction to oral penicillin with positive skin tests and one fatal case following intramuscular penicillin. *Proc. Staff Meet. Mayo Clin.* **30,** 634–40.

PETZ L.D. & FUDENBERG H.H. (1966) Coombs-positive hemolytic anemia caused by penicillin administration. *New Engl. J. Med.* **274,** 171–8.

PISCIOTTA A.V. (1965) Studies on agranulocytosis. VII. Limited proliferative potential of C.P.Z. sensitive patients. *J. Lab. clin. Med.* **65,** 240–7.

PISCIOTTA A.V., EBBE S., LENNON E.J., METZGER G.O. & MADISON F.W. (1958) Agranulocytosis following administration of phenothiazine derivatives. *Am. J. Med.* **25,** 210–23.

PRESCOTT L.F. (1965) Effects of acetyl salicylic acid, phenacetin, paracetamol, and caffeine on renal tubular epithelium. *Lancet* **ii,** 91–6.

PRICKMAN L.E. & BUCKSTEIN H.F. (1937) Hypersensitivity to acetylsalicylic acid. *J. Am. med. Ass.* **108,** 445–51.

RICH A.R. & GREGORY J.E. (1943) The experimental demonstration that periarteritis nodosa is a manifestation of hypersensitivity. *Bull. Johns Hopkins Hosp.* **72,** 65–82.

RITZ N.D. & FISHER M.J. (1960) Agranulocytosis due to administration of salicylazosulfapyridine (azulfidine). *J. Am. med. Ass.* **172,** 237–40.

ROBINSON D.S., MACDONALD M.G. & HOBIN F.P. (1965) Sodium diphenylhydantoin reaction with evidence of circulating antibodies. *J. Am. med. Ass.* **192,** 171–2.

ROSE B. (1953) Allergic reactions to penicillin: a panel discussion. In BOGER W.P., SHERMAN W.B., SCHILLER I.W., SIEGAL S. & ROSE B. *J. Allergy* **24,** 383–404.

ROSE G.A. & SPENCER H. (1957) Polyarteritis nodosa. *Q. Jl Med.* **26,** 43–81.

ROSNER F., LAI M. & RITZ N.D. (1966) Hemolytic anemia due to penicillin hypersensitivity. *New Engl. J. Med.* **274,** 973.

RUBIN D., WEISBERGER A.S., BOTTI R.E. & STORAASLI J.P. (1958) Changes in iron metabolism in early chloramphenicol toxicity. *J. clin. Invest.* **37,** 1286–92.

RYTEL M.W., KLION F.M., ARLANDER T.R. & MILLER L.F. (1963) Detection of penicillin hypersensitivity with penicilloyl-polylysine. *J. Am. med. Ass.* **186,** 894–8.

SAIDI P., WALLERSTEIN R.O. & AGGELER P.M. (1961) Effect of chloramphenicol on erythropoiesis. *J. Lab. clin. Med.* **57,** 247–56.

SALTZSTEIN S.L. & ACKERMAN L.V. (1959) Lymphadenopathy induced by anticonvulsant drugs and mimicking clinically and pathologically malignant lymphomas. *Cancer* **12,** 164–82.

SAMTER M. (1959) Pathophysiology of bronchial asthma. In *International Textbook of Allergy,* pp. 224–39, ed. JAMAR J.M. Oxford: Blackwell Scientific Publications.

SCHROEDER H.A. (1959) The pharmacology of hydralazine. In *Hypertension—The First Hahnemann Symposium on Hypertensive Disease,* pp. 332–44, ed. MOYER J.H. Philadelphia: Saunders.

SEHON A.H. (1959) The detection and nature of non-precipitating antibodies in allergic sera. In *Mechanisms of Hypersensitivity,* pp. 61–83, ed. SHAFFER J.H., LO GRIPPO G.A., & CHASE M.W. London: J. & A. Churchill.

SHELLEY W.B. (1962) New serological test for allergy in man. *Nature (Lond.)* **195,** 1181–3.

SHELLEY W.B. (1963) Indirect basophil degranulation test for allergy to penicillin and other drugs. *J. Am. med. Ass.* **184,** 171–8.

SHELLEY W.B. & COMAISH J.S. (1965) New test for penicillin allergy. *J. Am. med. Ass.* **192,** 36–40.

SHELLEY W.B. & JUHLIN L. (1961) A new test for detecting anaphylactic sensitivity: the basophil reaction. *Nature (Lond.)* **191,** 1056–8.

SHELLEY W.B. & JUHLIN L. (1962) Functional cytology of the human basophil in allergic and physiologic reactions: technic and atlas. *Blood* **19,** 208–16.

SHERLOCK S. (1963) *Diseases of the Liver and Biliary System,* 3rd edn., pp. 293–313. Oxford: Blackwell Scientific Publications.

SHERMAN W.B. (1949) Drug allergy. *J. Am. med. Ass.* **140,** 447–50.

SHULMAN L.E. (1963) Inducing agents and relationship to other diseases. *Arthritis Rheum.* **6,** 558–71.

SHULMAN N.R. (1963a) Mechanism of blood cell damage by adsorption of antigen-antibody

complexes. In *Immunopathology. Third International Symposium*, pp. 338–52, ed. GRABAR P. & MIESCHER P. Basel: Schwabe.

SHULMAN N.R. (1963b) Mechanism of blood cell destruction in individuals sensitized to foreign antigens. *Trans. Ass. Am. Physns* **76**, 72–83.

SHULMAN N.R. (1964) A mechanism of cell destruction in individuals sensitized to foreign antigens and its implications in auto-immunity. *Ann. intern. Med.* **60**, 506–21.

SIDI E., HINCKY M. & LONGUEVILLE R. (1958) Cross-sensitization between neomycin and streptomycin. *J. invest. Derm.* **30**, 225–7.

SIEGEL B.B. (1962) Studies in penicillin hypersensitivity. V. Further studies on the antigenic properties of altered penicillin. *J. Allergy* **33**, 349–55.

SIMPSON D.G. & WALKER J.H. (1960) Hypersensitivity to para-aminosalicylic acid. *Am. J. Med.* **29**, 297–306.

SLEEPER J.C., TYOR M.P. & SMITH A.G. (1960) Hepatitis due to p-aminosalicylic acid hypersensitivity. A clinical-pathologic correlation in two nonfatal cases. *Gastroenterology* **39**, 208–14.

SMITH J.M. & ZIRK M.H. (1961) Toxic and allergic drug reactions during the treatment of tuberculosis. *Tubercle, Lond.* **42**, 287–96.

SNELL E.S. (1961) An examination of the blood of febrile subjects for pyrogenic properties. *Clin. Sci.* **21.**, 115–24.

STAVITSKY A.B. & ARQUILLA E.R. (1955) Micromethods for the study of proteins and antibodies. III. Procedure and applications of haemagglutination and haemagglutination-inhibition reactions with bisdiazotized benzidine and protein-conjugated red blood cells. *J. Immun.* **74**, 306–12.

STEIN J.H., HAMILTON H.E. & SHEETS R.F. (1964) Agranulocytosis caused by chlorpropamide A case report with confirmation by leukoagglutination studies. *Archs intern. Med.* **113**, 186–90.

STEWART G.T. (1967) Allergenic residues in penicillins. *Lancet* **i**, 1177–83.

SWANSON M.A., CHANMOUGAN D. & SCHWARTZ R.S. (1966) Immunohemolytic anemia due to antipenicillin antibodies: report of a case. *New Engl. J. Med.* **274**, 178–81.

SWIFT S. (1954) Hypersensitivity to penicillin. *Lancet* **ii**, 602–4.

SYMMERS W.ST.C. (1958) The occurrence of so-called collagen diseases, and of diseases systemically affecting the connective tissues, as a manifestation of sensitivity to drugs. In *Sensitivity Reactions to Drugs*, pp. 209–22, ed. ROSENHEIM M.L. & MOULTON R. Oxford: Blackwell Scientific Publications.

THIEL J.A., MITCHELL S. & PARKER C.W. (1964) The specificity of hemagglutination reactions in human and experimental penicillin hypersensitivity. *J. Allergy* **35**, 399–424.

THIERFELDER S., MAGIS C., SAINT-PAUL M. & DAUSSET J. (1964) Die Pyramidon-Agranulozytose: eine immunhämatologische studie. *Dt. med. Wschr.* **89**, 506–12.

TORII T. & KOHORIUCHI Y. (1961) Antigenicity of penicillin and its relation to albumin binding. *Nature (Lond.)*, **192**, 429–31.

TULLIS J.L. (1961) The role of leukocyte and platelet antibody tests in management of diverse clinical disorders. *Ann. intern. Med.* **54**, 1165–80.

URBACH E. & GOTTLIEB P.M. (1946) *Allergy*, 2nd edn., p. 318. London: Heinemann.

URBACH E. & SIDARAVIČIUS B. (1930) Zur Kritik der Methoden der passiven Übertragung der Überempfindlichkeit. *Klin. Wschr.* **9**, 2095–9, 2304.

VAN ARSDEL P.P. & GILLILAND B.C. (1965) Anemia secondary to penicillin treatment: studies on two patients with 'non-allergic' serum hemagglutinins. *J. Lab. clin. Med.* **65**, 277–85.

VAN ARSDEL P. P., O'ROURKE T.K., HORAN J.D. & KUMASAKA Y. (1963) Serum hemagglutinating antibodies in penicillin allergy. *J. Am. med. Ass.* **185**, 584–7.

VERWILGHEN R., REYBROUCK G., CALLENS L. & COSEMANS J. (1965) Antituberculous drugs and sideroblastic anaemia. *Brit. J. Haemat.* **11,** 92–8.

WATKINSON G. & HILLIS B.R. (1947) Photosensitivity to sunlight from use of prophylactic sulphonamides: its relation to vaccination reactions. *Brit. med. J.* **ii,** 609–11.

WATSON K.C. & JOUBERT S.N. (1960) Haemagglutination of cells treated with antibiotics. *Nature (Lond.)* **188,** 505–6.

WATSON K.C., JOUBERT S.M. & BENNETT M.A.E. (1959) Penicillin as an antigen. *Nature (Lond.)* **183,** 468–9.

WATSON K.C., JOUBERT S.M. & BENNETT M.A.E. (1960) The occurrence of haemagglutinating antibody to penicillin. *Immunology* **3,** 1–10.

WAYNE E.J. (1958) Problems of toxicity in clinical medicine. In *A Symposium on the Evaluation of Drug Toxicity*, pp. 1–8, ed. WALPOLE A.L. & SPINKS A. London: J. & A. Churchill.

WERTHER J.L. & KORELITZ B.I. (1957) Chlorpromazine jaundice: analysis of twenty-two cases. *Am. J. Med.* **22,** 351–66.

WIJNJA L., SNIJDER J.A.M. & NIEWEG H.O. (1966) Acetylsalicylic acid as a cause of pancytopenia from bone-marrow damage. *Lancet* **ii,** 768–70.

WINTROBE M.M. (1961) *Clinical Hematology*, 5th edn., p. 611. London: Kimpton.

WISE F. & SULZBERGER M.B. (1933) Drug eruptions: fixed phenolphthalein eruptions. *Archs Derm. Syph.* **27,** 549–67.

WORLLEDGE S., CARSTAIRS K.C. & DACIE J.V. (1966) Autoimmune haemolytic anaemia associated with α-methyldopa therapy. *Lancet* **ii,** 135–9.

ZINKHAM W.H., LENHARD R.E. & CHILDS B. (1958) A deficiency of glucose-6-phosphate dehydrogenase activity in erythrocytes from patients with favism. *Bull. Johns Hopkins Hosp.* **102,** 169–75.

CHAPTER 27

ALLERGIC CONTACT DERMATITIS

CHARLES D. CALNAN

INTRODUCTION

DERMATITIS AND ECZEMA

For many decades there has been argument amongst dermatologists as to the correct use of the words dermatitis and eczema. The individual physician may be clear in his own mind as to what he means when he uses each of these terms, but there is no national or international agreement as to their usage. For practical purposes, in this chapter dermatitis and eczema will be treated as synonymous, and will denote a pathological change in the skin with particular histological features (Plate 27.1*), not all of which are necessarily present in any one instance. These features are both dermal and epidermal. In the dermis the small vessels and lymphatics are dilated, with accompanying oedema and exudation of inflammatory cells, mostly small and large lymphocytes, with occasional polymorphonuclear and eosinophil leucocytes, which migrate

* Plates in this chapter follow p. 768

towards and invade the epidermis. The epidermis shows focal oedema of the prickle cell layer, producing 'spongiosis', and intracellular oedema resulting in rupture and breakdown of epidermal cells to produce characteristic vesicles. Immigrating inflammatory cells are present in the spongiotic epidermis and in the vesicles, which may burst and exude serum. The normal process of keratinization is disturbed and is manifest by a loose hyperkeratosis or by parakeratosis (nucleated stratum corneum cells).

In the majority of cases this histological change can be readily diagnosed by the clinician, the signs being redness, swelling, papules, vesicles or pustules, scaling, and sero-cellular exudation and crusting (Plates 27.1, 27.2, 27.3, and 27.4). Again, not all these signs will be present in any one instance. The only symptom of note is itching.

Contact Dermatitis

Patients with eczema are classified on a basis of clinical pattern and aetiology, although an appreciable proportion (20% or more) defy classification. Until the end of the nineteenth century all eczema was believed to be of endogenous origin, but in 1895 Jadassohn showed that he could produce the reaction at will by the simple application of iodoform to the skin of a surgical patient who had shown an adverse reaction to it. In 1904 Nestler and Cranston Low independently showed that they could produce eczema with the juice from *Primula obconica* leaves. The extensive human and animal studies of Bloch and others which followed resulted in the segregation of cases of exogenous or contact dermatitis from all the other types, which are grouped as endogenous. Evidence was at the same time being provided, largely by physicians in industrial medicine, that dermatitis could be caused by chemical injury or irritation of the skin, irrespective of allergic sensitization.

The current conception of contact dermatitis which has evolved is as follows. A dermatitis can be produced by contact between any part of the skin surface and a noxious substance. There are two distinct mechanisms possible—one involves purely chemical damage (the so-called irritant, primary irritant or toxic dermatitis), while the other is dependent on a specific immunological reaction (called allergic contact dermatitis). The two mechanisms may be combined in one patient, and a single substance may be capable of acting via either mechanism separately. Allergic contact dermatitis is the only type of dermatitis for which the causative agent and its effects have been subjected to extensive experimental analysis; partly because skin testing can identify the agent and partly because it can be reproduced and studied in animals. No satisfactory test procedure can be used to prove cases of irritant or toxic contact dermatitis; and the diagnosis is normally made on the basis of the history and clinical features.

The causative agents in allergic contact dermatitis vary enormously. The most frequent in men are substances encountered in the course of their work,

especially chromates, formalin, synthetic resins, turpentine, and other chemicals; in women, nickel from nickel-plated stocking suspenders and other clips and fasteners on clothing is by far the most common, in addition to cosmetics, rubber, articles of clothing, and various cleaning agents. In both sexes topically applied medicaments form a big group of contact sensitizers.

SKIN TESTING

Allergic contact dermatitis is a form of the delayed hypersensitivity response to an allergen, although it may have immediate type sensitivity associated with it. Contact dermatitis is sometimes spoken of as epidermal sensitivity, in contrast to dermal sensitivity manifest by urticaria. This distinction is inaccurate and a misnomer. Both the dermis and epidermis are concerned in the contact dermatitis reaction, although the proportional involvement of each may vary to extremes. Hence, the terms dermal and epidermal sensitivity should be regarded as anachronisms.

Methods

While immediate type reactions may be demonstrated clinically in the skin by the prick, scratch, or intradermal tests, contact dermatitis sensitivity is shown by the patch or intradermal tests. The usual technique of the closed patch test, which is substantially the same as the method devised by Bloch, is as follows. The test material is applied directly to the skin surface on a piece of lint or linen of about 1 cm in size (Plate 27.4). The latter is covered by a slightly larger piece of cellophane, which is then kept in position with a piece of adhesive tape (Plate 27.4). For the open test drops of test solution are placed on the skin and allowed to dry. In general, this method has few advantages but considerable limitations, and hence is not frequently used. The same is true of applying the test substance by electrophoresis. Special non-spill 'adhesive chambers', which can be held in position on the skin of the forearm, have been devised for exposing a test area to the vapour only of a noxious liquid.

Some investigators advocate preparing the skin test sites by cleaning with fat solvents, or by scarification or abrasion, but such procedures are not normally necessary or even desirable since they may be conducive to false positive reactions. Kligman (1966) has recently advocated the use of a 'provocative patch test', which increases the sensitivity of a patch test to detect weak allergic reactions. Magnusson (1966) has usefully discussed the pros and cons of several different patch testing techniques.

Since the hypersensitivity is generalized over the whole skin surface the test patches may be applied anywhere. The most convenient are the back, the arms and the thighs. The writer's method is to use the back and apply the patches in rows of four, numbering them from left to right and from above downwards,

so that they can be listed in the patient's case record. A control patch of lint, cellophane and tape is always included. The patches are removed and the results read after 48 hr (24 hr is adequate time for application, although the reaction may not be seen until 48 hr later). It is not necessary to apply any material for longer than 48 hr. A second reading is made 96 hr after application, since about 10% of positive reactions do not appear until this time. A few reactions are delayed even longer, up to 7 or 10 days. If, however, a positive reaction appears some 10–14 days or more after application, it is almost certainly due to primary sensitization by the test patch. This can usually be confirmed by re-applying the test material, when a reaction will usually appear at 48 hr.

Positive Reactions

Positive reactions (Plate 27.5) are recorded in a standard way: + for redness only; ++ for redness, swelling and papules; +++ for intense redness, swelling and numerous papules and vesicles; ++++ for large confluent blisters. In practice, it is not always easy to quantitate patch test results in this way, and the degree of reaction is not necessarily reproducible. For quantitation the preferred method is to use a known amount of the chemical dissolved in a solvent such as acetone, chloroform, or methyl ethyl ketone and apply it evenly over a known area of skin inside a short cylinder of metal or plastic material. Ten-fold increases in dilution may be applied together and the patient's sensitivity 'titrated'. It is, however, more important for the clinician to know whether a positive reaction, of any degree, indicates specific allergic hypersensitivity to the substance or not. This will only be true if: 1. the test substance can never produce an irritant or toxic reaction in any concentration, 2. the patient is not in 'status eczematicus', and 3. the other causes of false positive reactions (mentioned later) are not present.

As with many biological test procedures, the patch test is simple to carry out but can prove extremely difficult to interpret correctly. The principal problem is to distinguish the toxic and false positive reactions. A typical toxic reaction will show redness, swelling and pustules, necrosis or bullae. One form is the so-called 'soap reaction' which shows a characteristic wrinkling and glistening of the horny layer (Plate 27.6). False positive reactions, however, may exactly mimic all the stages of genuine positive reactions described above.

Evaluation of Reactions

Experience is frequently the best guide in separating true from false reactions, but the following points may be a guide to the investigator, bearing in mind the fact that all the evidence in a case should be assessed together:

1. The nature of the test substance and its chemical compositions should be noted. Materials which are frequently applied to the skin such as cosmetics,

clothing and topical medicaments are unlikely to be irritant. Alkalis, detergents, soaps, shampoos and the common organic solvents are frequently irritant but virtually never sensitizers. Reference can be made to published lists of test materials.

2. A specific allergic reaction is usually constant. Hence, if the test is repeated one or more times at intervals and gives the same result, it is more likely to be a true positive. Toxic reactions tend to be more variable.

TABLE 27.1

Effects of dilution on patch test results with irritants and sensitizers

Concentration (%)	Substance A	Substance B	Substance C
100	++++	++++	++++
50	+++	−	+
25	++	−	−
10	+	−	++
5	±	−	−
Conclusion:	Sensitizer	Irritant	Irritant

TABLE 27.2

Tests on control subjects with an irritant substance showing variation in time and individual response

Subjects	1	2	3	4	5	6	7	8	9	10
Substance A	−	−	+	+	−	+	−	−	+	−
Repeated one month later	−	+	−	+	−	+	−	−	−	+

3. Testing with graded dilutions of a sensitizer will produce graded results, whereas in the case of an irritant it is likely to give irregular results as shown in Table 27.1.

4. Control subjects may be used. If the test material produces a positive reaction on a person not previously exposed to it, the substance is likely to be an irritant. The greater problem is when it does not. A group of about ten or twenty subjects should be used, since toxic reactions show considerable individual variation, as shown in Table 27.2.

5. A focal flare-up of the patient's dermatitis coincidentally with a positive patch test is often taken to be evidence that the test substance is the specific cause of the dermatitis. It would appear to be analogous to the flare-up of a pulmonary lesion after a strongly positive tuberculin test. While this is frequently a specific association, it is not always so and should not be regarded as proof. Strongly irritant patch test reactions may also cause such a focal flare.
6. It might be supposed that histological examination of the test site would be of value. An allergic lesion will show the changes of dermatitis with immigration of mononuclear cells into the epidermis. A typically toxic lesion shows cellular damage to the outer layers of the epidermis, and the immigration into it of polymorphonuclear leucocytes. The two lesions may sometimes be separated by a differential cell count in the blister fluid. These histological and cellular changes are, however, only present when grossly irritant substances are used; they provide no additional evidence when purely clinical examination of the test result requires it. However, use of the skin window technique has shown that basophil leucocytes immigrate early into the area of an allergic reaction.

False Positive Reactions

The greatest difficulties in interpretation occur with the false reaction that mimics a genuine positive result. The principal causes of the false positives are the following:
1. The patient is in so-called 'status eczematicus'. It is not possible to define accurately the limitations of this state. In general, it indicates the presence of

Table 27.3
Comparative test results with turpentine in eczematous and normal subjects
(Geiger 1929)

Concentration of turpentine (%)	Eczema patients positive	Controls positive
60	most	6%
30	20%	none

active acute eczema of wide extent over the skin surface. The 'state' may persist for some weeks even after the eczema has subsided. It denotes a non-specific hyper-irritability of the skin surface, and may be illustrated by Geiger's (1929) results with turpentine shown in Table 27.3. In these circumstances the patch test should be repeated after the eczema has subsided for a few months.

Repeated patch testing of a patient will also be likely to induce this state and give false positive results (Calnan 1964).

2. Local hyper-irritability of the surrounding skin may be induced by a strongly positive reaction, to such a degree as to produce a reaction in an adjacent test site. This is not common; it may be checked by repeating the doubtful test at a more distant site after the very strongly reacting site has subsided.

3. Severe reactions to the adhesive plaster used may induce a false reaction to the test material. The test should be repeated with a more suitable adhesive*.

4. Contamination by the applicant's finger of the lint used for subsequent patches may mislead. It usually only occurs adjacent to strongly positive reactions, but is particularly prone to happen when testing with plants.

5. Solid materials will indent the test site and may cause non-specific pressure damage which can simulate a positive reaction. Solid materials should be dissolved in a suitable solvent or grated to a fine powder before application.

It must be remembered that these factors are non-specific. Such is the biological variability of man that they do not occur in every case, do not affect every patch applied to a patient, and are not always reproducible under what appear to be the same circumstances.

False Negative Reactions

False negative reactions are equally important, although they are much less frequently encountered. Some of the possible causes are the following:

1. Sometimes too small an amount of the test material is applied. This may stem from fear of a violent reaction in a clinically very hypersensitive patient. Either the area of lint used or the amount of test substance applied may be inadequate. Pieces of leather or fabric cut out from patient's materials may be too small.

2. The concentration of the substance may be too low. It is, of course, best to start with a low concentration of a substance for which there is no known standard, and then it can be increased in stages.

3. The allergen may not penetrate the stratum corneum of the epidermis in sufficient amount to produce a reaction. This has been shown to occur with eosin in lipsticks, with neomycin and lanolin, and may apply to other substances. It can be overcome by raising the concentration of the test allergen considerably, or by applying it to an area of 'stripped skin'—(skin from which the stratum corneum has been stripped with Scotch tape). Pretreatment of the test area with sodium lauryl sulphate (Kligman 1966) will also obviate this difficulty. The use of some types of adhesive tape (e.g. Micropore) may produce

* A special hypoallergenic type of patch test plasters, which contain no rubber or resin, is made by Laboratoires Vigier-Huerre, 9 Rue de la Gare, Levallois-Perret, Seine, France (Sidi & Hinck 1957). The use of a plastic-based adhesive tape also greatly reduces the reaction as compared with colophony resin-based tapes.

insufficient occlusion and maceration of the horny layer, resulting in inadequate penetration and a false negative response.

4. The use of a piece of solid material (such as wood, rubber or plastic) may bring an inadequate amount of the test material into contact with the skin, while the pressure of the solid may actually suppress the eczematous reaction. The material should be applied as a powder. Hence solid materials can produce false positive and false negative reactions. Fernstrom (1954) has recommended additional pressure with a piece of plastic sponge, and states that it will produce positive responses when the routine method gives a negative result. The writer has not been able to confirm this.

5. An allergen may not always be leached out of, or separated from, the material containing it when applied. One example is the leather of a hat band; another is chromate from cement.

6. The patient may be in a refractory phase. This is a rare, but well authenticated, cause (Kligman 1958). It occurs especially during or following a very severe contact dermatitis; when the patient is re-tested after an interval of several weeks or months a positive reaction can be obtained.

7. Local sensitivity (hypersensitivity confined to a local area of the skin) does not occur in the strict immunological sense, although it may be apparent clinically. Patients with lipstick dermatitis may react to a lipstick on the lips only or to medicaments only on an area of dermatitis. Patch tests should not, if possible, be applied to sites of previous dermatitis.

8. Epstein (1958) has put forward the concept of 'dermal contact dermatitis'. He describes this as a popular type of dermatitis produced by specific allergic sensitization to externally applied substances. Patch tests are negative but intradermal tests positive. He gives examples of causative agents as neomycin, gentian violet, nickel, chromate, penicillin and rivanol. Allergens do sometimes give a positive intradermal and a negative patch test, but it is rare and does not necessarily indicate a basically different immunological response.

Diluents

The correct concentration of the test substance and a satisfactory diluent are important. The diluent must be entirely non-irritant itself, nor should it facilitate toxic reactions from test substances. Water, olive oil, castor oil or liquid paraffin, and soft paraffin are suitable. Propylene glycol is not completely satisfactory, but methyl ethyl ketone (MEK) is suitable. Butyric acid amyl ester has also been recommended. Materials containing an organic solvent should not be applied to the skin until the solvent has been allowed to evaporate.

Concentration of Test Substances

The usual list of standard concentrations for test substances is that of Sulzberger, Rostenberg & Goodman (1939). Their concentrations are maximal,

and lower ones are often indicated. It has, unfortunately, not been revised or brought up to date, and its practical value is limited. When a substance is never an irritant it may be applied neat (as it is), and any reaction produced is likely to be an allergic one. However, many substances give irritant reactions in high concentrations, and an appropriate dilution is necessary to detect allergic sensitivity. Other compounds are irritants in high concentrations, and never act as allergens. When dilution is necessary, an appropriate concentration to start with is 1%. If the result is positive in the patient, it should be tested on about ten or twenty control subjects. If any of them react, the procedure should be repeated with a 0.1% solution. If there is no reaction in the patient at 1%, the concentration should be raised stepwise to 5, 10, 20, 50 and 100%, using controls at each level.

CLINICAL CONSIDERATIONS

Cross-sensitivity

Although allergy is by definition a specific reaction, Cranston Low showed that the specificity was not absolute in the case of flowers, and that it could be part of a group reaction. His own skin was very sensitive to *Primula obconica*, and less so to *P. sinensis*, the common wild primrose and the coloured hybrid primrose. He gave no reaction to *P. polyanthus*, *P. auricula*, *P. veris*, and *P. malacoides*. He had seen a patient sensitive to jonquil who also reacted to daffodil, and knew of the cross-reactions between poison ivy, poison oak and the Japanese lacquer tree *R. vernicefera*. Bloch (1924) reported his experience of cross-reactions to several chemical compounds such as iodoform, quinine, resorcin and formalin. Cross-sensitization is the term used to denote the phenomenon by which allergic sensitivity to one substance extends to other chemically related substances. It is becoming more important in dermatology and immunology with the rapidly increasing number and complexity of synthetic chemicals produced for medical, industrial and domestic usage. Numerous examples are known (Baer 1954), but the most widely studied is cross-sensitization to substances with a primary amino group in the para position on a benzene ring. Study of this 'para group' sensitivity began with the work of Meyer (1928) who showed that patients sensitive to paraphenylene-diamine may also react to aniline, aminophenol, diaminophenol, methylaniline, amino-azobenzene and many other related compounds (Fig. 27.1). Such patients, however, do not react to every substance containing a para-amino group, and some patients do not appear to cross-react to any other related compound. In fact, the pattern of cross-sensitization varies very considerably from patient to patient, and appears to be determined partly by the primary sensitizer and partly by the individual himself. For example, 100% of patients sensitized to

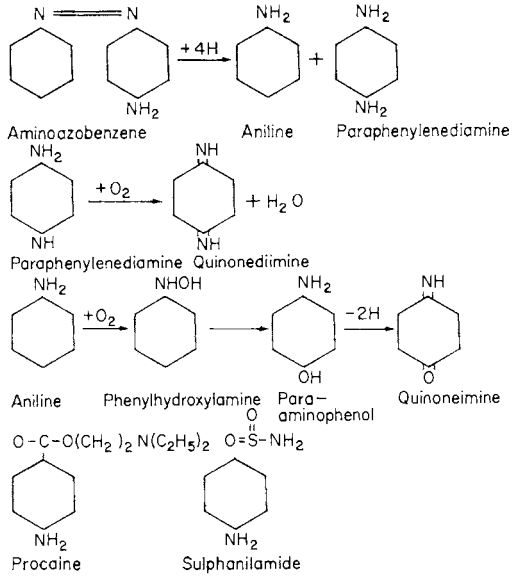


FIG. 27.1.

sulphonamide react to paraphenylenediamine and to local anaesthetics, whereas only 20% of those primarily sensitized to paraphenylenediamine react to local anaesthetics. In general, the primary allergen gives a much stronger reaction than any of the secondary allergens, although aniline has been reported as an exception to this rule. Mayer believes that the mechanism of cross-sensitization

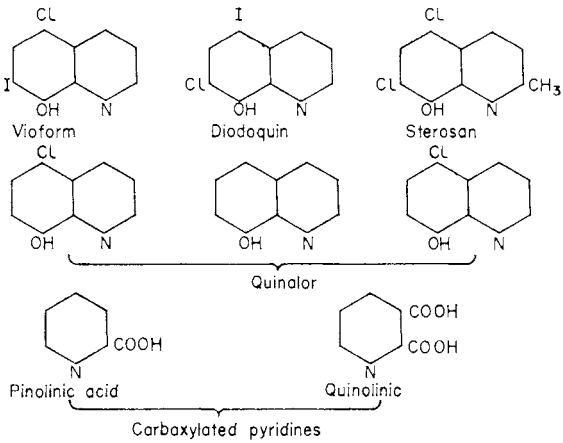


FIG. 27.2.

depends on the conversion of the applied allergen to a common haptenic substance, via oxidation, reduction, or other chemical reactions in the skin. In the case of para-amino compounds they could all be converted to quinone-imines and quinonediamines or their derivatives (Fig. 27.1). In the case of halogenated hydroxyquinolines it has been suggested that all of four therapeutic substances, Vioform, Diodoquin, Steroxan and Quinolor (which may all cross-react), could be converted into carboxylated pyridines as the actual antigenic determinants (Fig. 27.2). Again, not all patients sensitized to any one of these compounds will cross-react to any or all the others. To explain such anomalies the protagonists of the common antigenic determinant thesis suggest that some patients have a defect in their skin which renders them unable to make the necessary conversion. Unfortunately, it has not been shown whether human skin can or does carry out these chemical reactions on the compounds already mentioned. The view has also been expressed that the allergenic potential of paraphenylenediamine is partly due to the para position of the amino group, and partly due to its chemical reactivity and lability. A nitro group in the ortho position considerably reduces the chemical reactivity of paraphenylenediamine, and with it the biological reactivity. Orthonitroparaphenylenediamine is about fifty times less allergenic than paraphenylenediamine, with equally reduced effectiveness as a dye for hair or fur.

Another example illustrating the common antigen principle is shown by the recent studies of Levine (1960) on penicillin. He has shown that certain physiologically possible degradation products of penicillin, particularly D-benzylpenicillenic acid, D-penicillamine and D-α-benzylpenicilloic acid, are potent inducers of delayed contact sensitivity in the guinea-pig, and that they cross-react with penicillin G. The antigenic determinant is probably a benzylpenicilloyl-lysine group.

Polysensitivity

Polysensitivity is more of a clinical term than an immunological concept. It may take three forms: first, the cross-sensitization between a number of substances which are closely related chemically, as discussed above; secondly multiple acquired specific sensitivities to chemically unrelated substances; and thirdly, multiple non-specific or false positive reactions. If a patient's skin is in a very reactive state as a result of a severe or extensive dermatitis, they may react to numerous patches in a test series, most or all of which do not appear to be of clinical significance; and which, when re-tested separately after allowing sufficient time for the skin to return to normal, will give negative results.

Photo-contact Sensitivity

Photo-contact dermatitis is another clinical concept with a questionable and poorly understood immunological basis. Some patients develop an apparently

allergic contact dermatitis from a specific substance only on areas of skin exposed to light. It may only be possible to obtain a positive patch test by exposing the test site to a suberythema dose of ultraviolet light 24–48 hr after application of a patch or intradermal injection of the test substance. Examples of such photo-contact sensitizers are sulphonamides, phenothiazine derivatives and halogenated salicylanilides (Harber *et al* 1966). The mechanisms involved are obscure, but it is usually suggested that photo-decomposition of either the hapten or a hapten-protein conjugate results in the formation of the actual antigen to which the antibody is directed. (pl. 27.7)

Desensitization

Specific desensitization of hyposensitization to low molecular weight allergens can be achieved, but only with great difficulty. It frequently requires several months and involves the risk of quite severe reactions. It has been successful with penicillin, streptomycin and poison ivy. It is usually only a temporary state and should not normally be advised or attempted.

Duration of Sensitivity

Allergic contact sensitivity tends to persist indefinitely—certainly for many years. There is a tendency for it to diminish in old age, perhaps as part of a general decrease in immunological reactivity. Photo-contact sensitivity is far less persistent and hence it may have a somewhat different pathogenesis.

IMMUNOLOGICAL MECHANISMS

Hapten Theory

Antigens have always been considered to be proteins or polysaccharides and immunological theory was based on this assumption. Specific and apparently allergic reactions to simple chemical compounds such as iodoform and para-phenylenediamine must therefore involve a different mechanism or else undergo some modification. ('Low molecular weight' is a more acceptable term than 'simple' to describe these chemicals, since many of them are extremely complex.) Landsteiner (1945) bridged the gap with his hapten theory. The low molecular weight chemical is called the hapten, which conjugates with a protein to form a complete antigen. This complete antigen is then capable of initiating antibody production and sensitization of either the immediate or delayed type; the antibody, however, is normally specific to the hapten but the carrier protein may sometimes impart specificity as well. The hapten has acquired antigenic properties by the protein linkage. Although it has not been entirely proved that such a protein linkage is the mechanism involved *in vivo*, there is a great deal

of circumstantial and experimental evidence to support it; and no alternative theory has a greater claim.

Animal Sensitization

Few animal species are capable of developing contact type sensitization, but it can be induced in the guinea-pig, the rabbit and the primates. The guinea-pig is the most suitable, although its behaviour in this regard differs from man in a number of ways. First, its epidermis is too thin to show the histological changes of eczema, except on the skin of the nipple, but the dermal changes by producing redness and swelling are adequate for diagnostic purposes. If required, the epidermis can be thickened by pre-treatment with acanthotic agents. Secondly, the degree of sensitization it develops is comparatively low. Man can readily be sensitized to react to dinitrochlorbenzene at one part per million, whereas with the guinea-pig the maximal sensitivity is one in forty thousand. Thirdly, neither focal responses nor secondary dermatitis lesions are at all frequently encountered in the guinea-pig except under special conditions (de Weck & Frey 1966). Without the guinea-pig, however, our knowledge of this subject would be scanty indeed. The factors involved in the pathogenesis of allergic contact dermatitis are now moderately well understood, as a result of studies of various parameters, principally the contact agent or allergen, the mode of exposure, the skin, and the individual.

The Allergen

The low molecular weight substances capable of causing contact sensitization vary enormously—from single ions such as mercury, iodine, nickel, cobalt, zirconium and beryllium, to simple compounds such as formaldehyde, iodoform, paraphenylenediamine, halogenated nitrobenzenes and mercaptans, up to more complex substances such as epoxy resins, higher alcohols, and sterols. It is not easy to see what chemical property, if any, all the known contact sensitizers have in common. A strong affinity for protein is thought to be the most important, but some appear to possess none at all. At least, chemical lability or a high reaction constant is necessary. If the hapten theory is correct, the formation of a stable non-dissociable covalent type of bond is essential. Halogenated nitrobenzenes react with the terminal amino group of lysine in this way. Similarly, some sulphur-containing nitrobenzenes will react with the cystine and cysteine of epidermal protein, and the reaction may be dependent on pH. Mercaptans are especially reactive chemically, and are potent contact sensitizers. Any form of dissociable chemical bond would not be effective. When a chemical cannot itself readily conjugate with protein, one of its degradation products may do so; for example, the D-benzyl-penicillenic acid or penicilloic acid formed from penicillin (Levine 1960). In addition to its chemical character, the concentration of the contact agent is of paramount

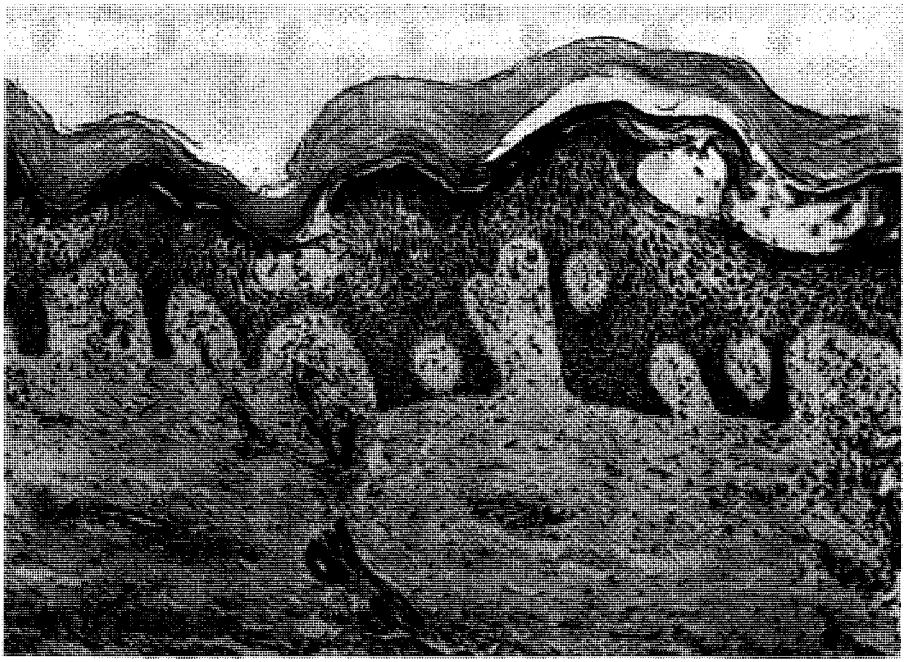


PLATE 27.1. Histology of dermatitis. Note patchy 'spongiosis' and vesicle formation with immigration into the epidermis of inflammatory cells from the perivascular infiltrate.

facing p. 768

PLATE 27.2a. Contact dermatitis on the front of the chest from nickel-plated brassière ring.

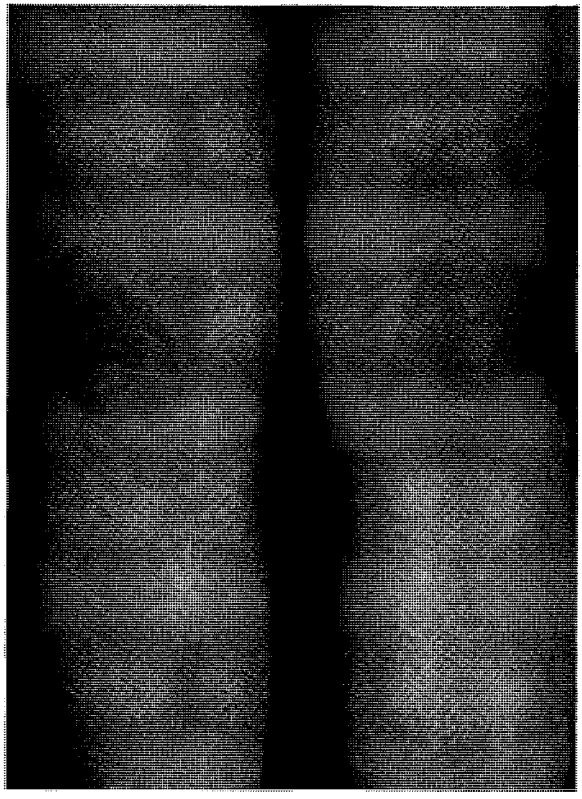


PLATE 27.2b. Contact dermatitis of the popliteal spaces from azo dye in nylon stockings.

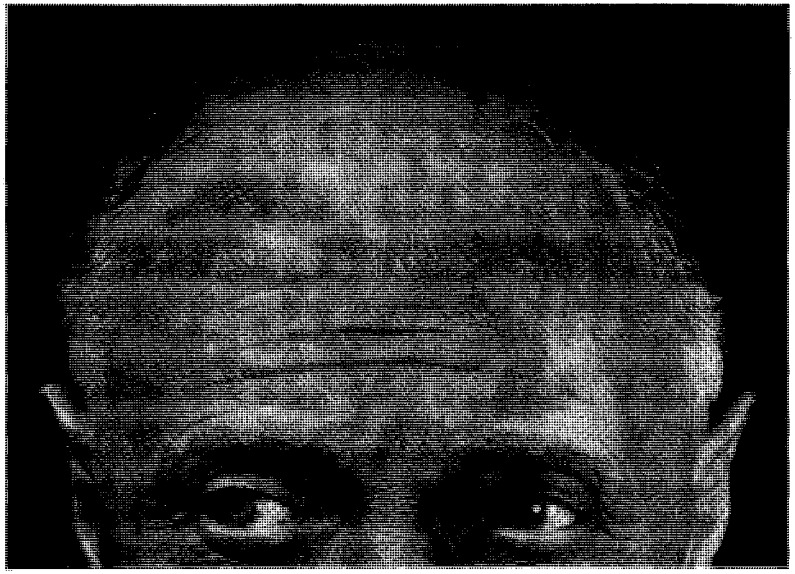


PLATE 27.3a. Contact dermatitis of the forehead from a leather cap band.

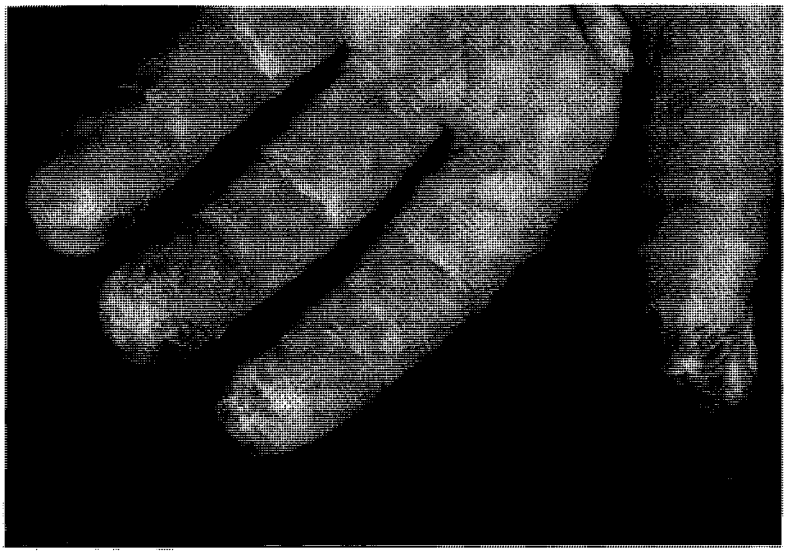


PLATE 27.3b. Contact dermatitis of finger-tips from garlic.

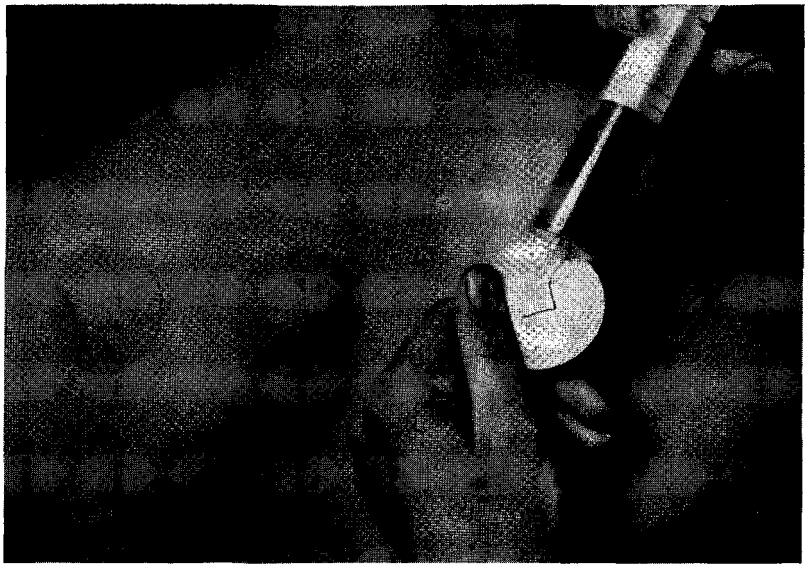


PLATE 27.4. Method of patch testing.

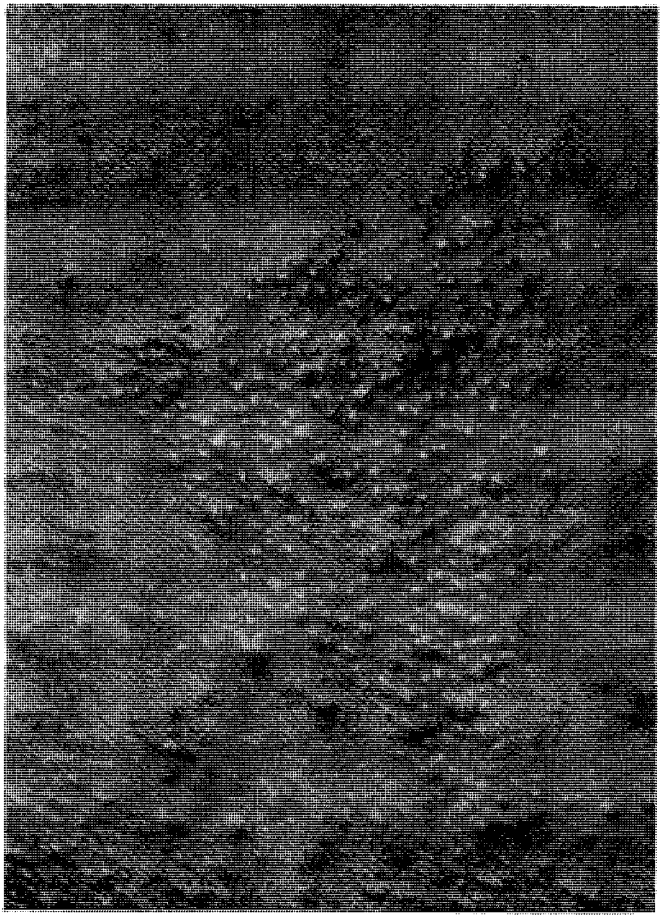


PLATE 27.5. Positive patch test reaction. Note group of closely set papules and vesicles.

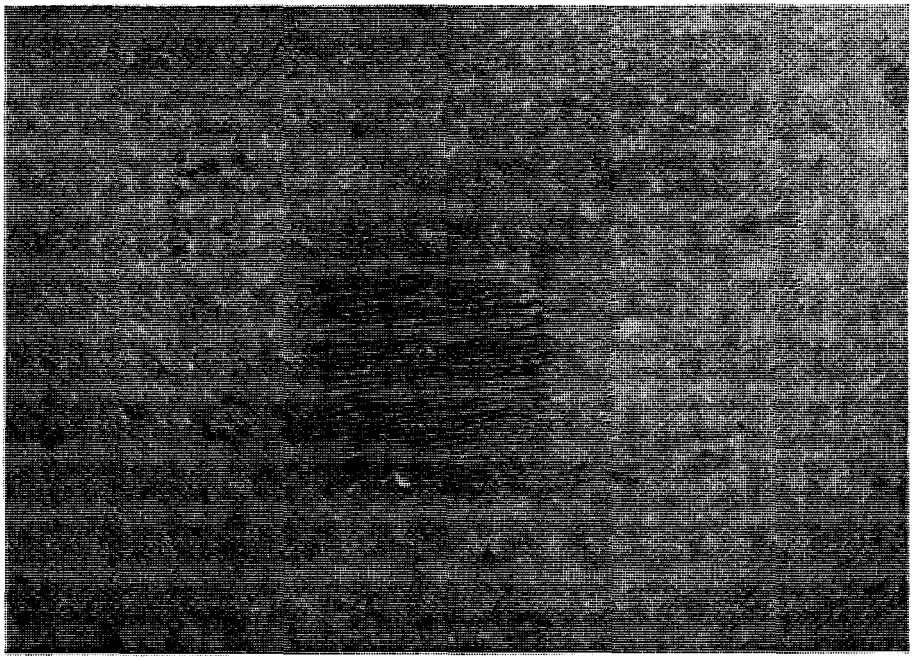


PLATE 27.6. False positive patch test result (from a soap). Note the wrinkled appearance of the horny layer and absence of grouped vesicles.

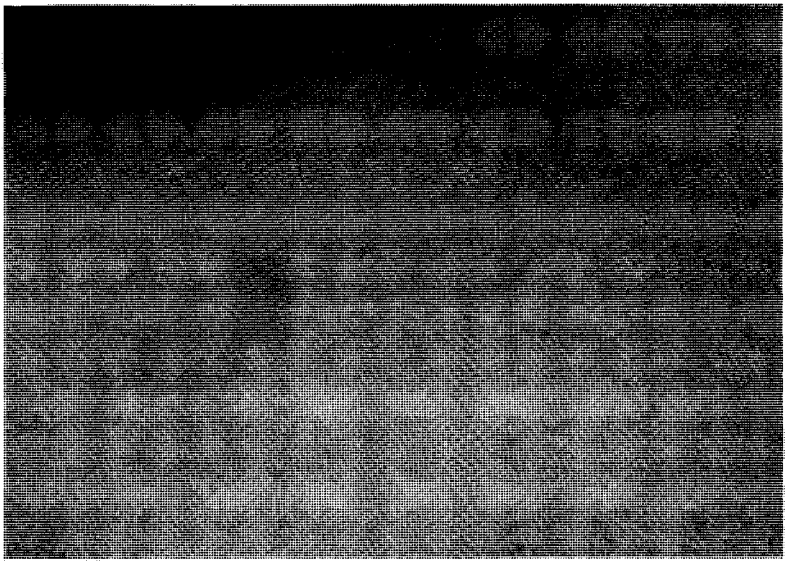


PLATE 27.7. Positive photo-patch test—results at 96 hours, after application of phenergan. The patch on the left was irradiated 48 hours after application with a sub-erythema dose of ultra-violet light. The patch on the right (which was not irradiated) remains negative.

importance. Sensitizing power is directly proportional to concentration, and not dependent on the quantity of chemical or the area of skin to which it is applied. The whole body may be immersed in an extremely dilute solution of dinitrochlorbenzene without inducing any reaction, whereas one drop of the pure substance will sensitize.

There is some evidence that the sensitization index of a chemical does not necessarily parallel its sensitizing potency. Some substances may induce a very high degree of allergic sensitivity, but only sensitize few of the individuals exposed. Other substances may sensitize a high proportion of persons exposed but not produce a very high degree of sensitivity in any of them.

The Mode of Exposure

The special feature of contact dermatitis type sensitivity in man is that it is induced by application of the sensitizer on to the skin surface. Intradermal, subcutaneous, intravenous or intramuscular routes are generally ineffective. It is normally mandatory in man, but not in experimental animals, to apply the agent in this way, apart from three exceptional circumstances. One is with overwhelmingly strong sensitizers such as dinitrochlorobenzene which, applied by almost any route in high concentration, will induce contact type sensitivity. The second is by the use of tubercle bacilli, and Freund's adjuvant, injected with the hapten, or by previous conjugation with a protein. The third is by injection directly into lymph nodes. But for almost all examples in clinical practice, contact type sensitization only results if the allergen has been applied to the skin surface and never from a purely parenteral route. Beryllium is an exception to this rule. Cases of pulmonary berylliosis and sarcoidal nodules in the dermis are usually associated with a positive eczematous patch test. However, in animals sensitization has been obtained by injecting hapten which has been conjugated with either serum protein, skin protein or red cell stromata.

The precise role that the epidermis plays is obscure. The usual explanation is that it supplies the protein moiety or pro-antigen with which the hapten conjugates. It has been shown that such conjugation (in the case of dinitrochlorbenzene) does take place *in vivo*, but it has not been demonstrated that the resultant conjugate plays any further part in the sensitization process although hapten-protein complexes prepared *in vitro* can induce sensitization. Such a conjugate cannot readily enter the immunologically competent cells with the ease of the hapten alone. Mayer (1957) has put forward the thesis that delayed hypersensitivity results when a hapten conjugates with a fibrous type protein (such as keratin or collagen); whereas conjugation with a globular protein (such as the serum proteins) elicit classical antibody formation with immediate type sensitivity. It is true that many low molecular weight chemicals can produce both types of antibody, but there is no experimental evidence to support the view that it is the type of protein which is determinant.

The Skin

Many substances merely require to be applied to the normal epidermal surface on one or more occasions to induce contact sensitivity in man. There is considerable evidence, however, that certain alterations of the epidermis will facilitate sensitization; chemical irritation, burns, freezing, epidermal 'stripping', sandpapering and scarification will have this effect. Many clinicians believe that the presence of dermatitis also predisposes the contact site, but there is little experimental evidence to support the view. Kligman & Epstein (1957) found that maximal sensitization occurred when the allergen was applied at weekly intervals on three occasions to a skin site prepared by freezing or light sandpapering. The three sites should all be drained by the same group of regional lymph nodes. The particular solvent in which the allergen is diluted may sometimes facilitate sensitization, possibly by some action on the epidermis. A solvent consisting of ethyl alcohol, methyl cellosolve and Tween 80 has a remarkable influence on penicillin contact sensitization.

Climate and season also appear to be relevant factors and can considerably alter the incidence of sensitization, although whether they act by altering the skin surface or in some other way is not known.

A notable feature of contact type sensitivity as encountered in clinical practice is the refractory period. The skin may be repeatedly exposed to a contact allergen for months or years before the immunological processes for sensitization (which only require a latent period of 6–10 days) are initiated. With the very potent antigens used for experimentation, there is virtually no refractory period; a single exposure is enough. It is not known what specific or non-specific changes, if any, take place in the skin or elsewhere, but it is possible that the individual has been 'altered' in some way by such repeated exposures before the 10-day latent period begins.

In the guinea-pig it has been shown that oral pre-feeding of the antigen, before application of a sensitizing dose to the skin, will effectively prolong the refractory period and induce one form of immunological tolerance. Such an effect has not been shown to occur in man.

The Individual

Genetic predisposition is of inverse importance to the potency of the sensitizer. The skin of all persons can be made to show the pathological changes of dermatitis by the application of very potent sensitizers, but with progressively weaker allergens fewer can be sensitized. Animal studies, particularly, and some human studies, show that this susceptibility or 'preparedness' (Chase 1957) is genetic. Guinea-pigs can be bred to produce strains which are highly susceptible or highly resistant to individual allergens. They can also be made resistant to sensitization by prior oral feeding of small doses of the allergen. It is not known

whether these findings are true for man, but they are likely to be so. Of racial differences it is agreed that the Negro is rather less sensitizable than the Caucasian or Mongolian.

Systemic disturbances may influence the contact sensitization rate in man. Disorders of the reticulo-endothelial system such as leukaemia, Hodgkin's disease, reticuloses and sarcoidosis tend to diminish the sensitizing capacity but do not abolish it. Age alone does not affect it. Some clinicians believe that

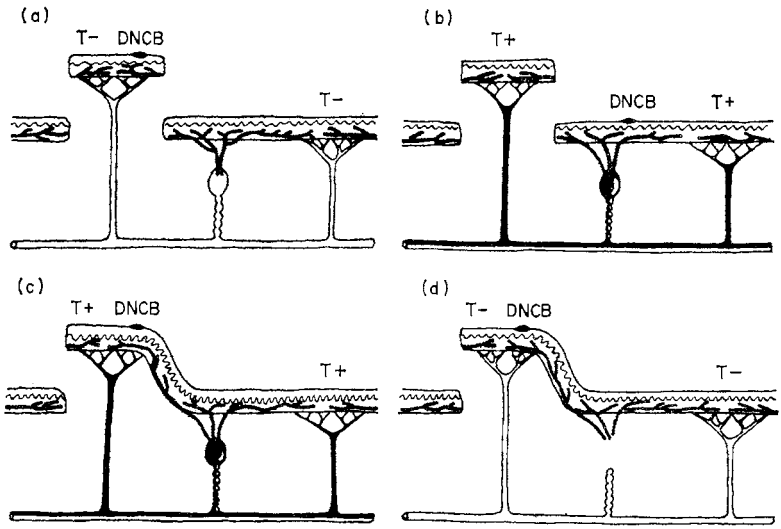


FIG. 27.3. Schematic representation of contact sensitization with dinitrochlorobenzene and challenge results on skin explant and on normal skin (Frey & Wenk 1956).

(*a*) Primary contact on explant island (lymphatics cut)—test on explant and normal skin positive.
(*b*) Primary contact on normal skin—test on explant island and normal positive.
(*c*) Primary contact on explant with isthmus (lymphatics intact)—test on explant and normal skin positive.
(*d*) Primary contact on explant with isthmus, but regional lymph nodes excised—test on explant and normal skin negative.

psychic trauma predisposes but no satisfactory evidence is available. Persons with agammaglobulinaemia behave no differently from normals.

ROLE OF LYMPHATIC SYSTEM

For a long time it was thought that contact dermatitis allergy was entirely confined to the skin, and that when an allergen was applied to the skin sensitization became generalized via, and because of, the continuity of the epidermis

over the whole surface. This erroneous idea was fostered by certain faulty experiments: Strauss and Coca cut a 'ditch' around an isolated island of skin in a monkey and concluded that a chemical applied to the island was unable to sensitize the rest of the skin of the animal; Bloch transferred a Thiersch graft from a patient sensitive to iodoform and said that the graft remained sensitive. These statements are now known to be wrong, as shown by the work of Landsteiner, Chase, Kligman, Frey and Wenk, and others. Frey & Wenk (1956) devised a most ingenious experiment. They found that they could isolate and explant a reasonably large area of skin on the flank of the guinea-pig so that it would remain viable when connected to the body only by a neurovascular pedicle. They prepared some animals in which the island was complete, and others in which the island was attached to the skin of the groin by a narrow isthmus of skin (so that the regional lymphatic drainage was intact). They then proceeded to sensitize the animals with dinitrochlorobenzene, either on the explanted island or on the opposite flank, and to test each site for the development of sensitization 2 weeks later. The experiment and the results are illustrated in Fig. 27.3.

The conclusions which they were able to make are:

1. Intact lymphatic drainage from the site of application and regional lymph nodes are necessary.
2. Contact sensitivity, once developed, is transmitted to the whole skin via the blood stream.
3. The minimal time of contact for the sensitizing substance is 32 hr. (A figure of 6–8 hr is more correct for man.)
4. The incubation time is 6–9 days.
5. The integrity of the nerve supply to the skin is not essential.

It was previously thought that the vital processes of sensitization took place only in the lymph nodes. It is now accepted that sensitization can also be peripheral to the nodes. Immunologically competent cells are found in the peripheral blood, and sensitization can be induced in these percolating lymphocytes at the skin site before going to the regional nodes. It is possible that all sensitization is a combined process, both peripheral and nodal (Medawar 1965).

The cellular changes in the regional lymph nodes during contact sensitization have been studied by various techniques (Turk & Stone 1963; Oort & Turk 1965; Diengdoh & Turk 1965). The major activity is in the paracortical region of the cortex of the gland adjacent to the medulla, whereas in classical antibody production of immediate type sensitivity the cellular activity is in the medulla. Contact sensitization induces an initial increase in large pyroninophilic cells in the paracortical area, followed by a progressive decrease and the appearance of large numbers of small lymphocytes. These cells show evidence of lysosomal activation in response to the allergenic stimulus.

Transfer of Sensitivity

The important role played by lymphocytes or other mononuclear cells has been demonstrated in many ways since Landsteiner and Chase successfully transferred sensitivity to picryl chloride by peritoneal exudate cells. Transfer has since been achieved in guinea-pigs with cells from the thymus, lymph nodes, spleen and direct from the thoracic duct. Lymphocytes and not polymorphonuclear leucocytes are necessary. Large quantities must be used, usually requiring the pooled cells from six to eight animals. A similar transfer of sensitivity has been claimed in man by Epstein & Kligman (1967) using poison ivy, dinitrochlorbenzene and paranitrosodimethylaniline. For success they found that 1. the donor must be very highly sensitized, 2. the recipient must be genetically susceptible, 3. the allergen must be selective, and 4. the quantity of cells must be large—at least 200 million lymphocytes (which is in marked contrast to the small number of cells required to transfer tuberculin sensitivity). Dead or ruptured cells are not effective (again contrasting with tuberculin sensitivity). Two unexpected findings were: first, that sensitivity to dinitrochlorbenzene (which is a much more potent antigen than poison ivy) was only transferred with difficulty, and secondly, that the acquired sensitivity in some patients persisted for many months—long after the transferred lymphocytes must have been destroyed by the homograft reaction. However, serious objections have been raised to such claims (Harber & Baer 1961).

Sensitivity is not transferred across the placenta. The Prausnitz-Küstner passive transfer test with serum is negative in contact dermatitis, as have been most attempts at transfer with cell-free fluids. Successful transfer of contact sensitivity to picryl chloride with cell-free extracts of lymphocytes has been claimed by Jeter *et al* (1954) and by Turk (1961), and it might appear to be but a matter of time before the technical problems are solved and the 'transfer factor' or 'contact type antibody' is separated from cells and identified. However, Turk has since shown that the transfer was not passive but active sensitization by antigen.

Tolerance

The conception of immunological tolerance or immunotolerance originated in relation to tissue antigens. There are five patterns of 'unresponsiveness' or absence of apparent response to an antigenic stimulus (Medawar 1960):

1. Immunological tolerance—as a result of antigen contact before or shortly after birth.
2. Radiation induced tolerance—following whole body X-irradiation.
3. Pre-feeding tolerance—induced by previous oral feeding or intravenous injection of chemical allergens.
4. Immunological paralysis—a centrally induced failure of the immunologic system.

5. Tolerance from protein overloading—induced by massive quantities of protein antigens.

In relation to contact type sensitivity immunotolerance has been studied in detail by de Weck & Frey (1966) using a single very large intravenous injection of allergen in guinea-pigs as their tolerogenic agent. The tolerogen is not necessarily identical with the antigen; and tolerance to immediate and delayed type responses can be dissociated. It is likely that the general principles of tolerance apply to contact sensitivity, but they have not yet reached a stage of application to man. The same may be said of immunosuppressive drugs, although experimental evidence suggests that complete inhibition of sensitization may not be achieved for some time to come.

THE FUTURE

A number of gaps in our knowledge remain to be filled by evidence in order to answer the following questions. It is established that the chemical, whether conjugated or not, must come into contact with immunologically competent cells, but does this occur in the skin or in the lymph nodes? Does the chemical conjugate with skin protein or with an intra-lymphocytic protein to form the effective allergen? Eisen *et al* (1957) have shown that dinitrochlorbenzene readily permeates the cell wall of lymphocytes and becomes concentrated at least twenty times inside these cells. Does any reaction take place *in vitro* between the haptenic chemical or its conjugate and the sensitized lymphocytes, similar to the lympholysis described with tuberculin? Can lymphocytes be sensitized *in vitro* by exposure to the conjugated or unconjugated chemical, as can be done with bacterial antigens? How is the sensitivity perpetuated?

Although this branch of immunology is at present being studied extensively there is still a great deal that has to be learnt before we can fully understand all of the precise mechanisms involved in contact type sensitization.

REFERENCES

Baer R.L. (1954) Cross-sensitization phenomena. In *Modern Trends in Dermatology*, 2nd series, p. 232, ed. Mackenna R.M.B. London: Butterworth.

Bloch B. (1924) Pathogenese des Ekzems. *Arch. derm. syph.* **145,** 34.

Bonnevie P. (1939) *Aetiologie und Pathogenese der Ekzemkrankheiten.* Leipzig: J.H. Barth.

Calnan C.D. (1964) The climate of contact dermatitis. *Acta Derm. Venereol.* **44,** 33.

Chase M.W. (1954) *Int. Arch. Allergy* **5,** 163.

Chase M.W. (1957) The mechanism of sensitization. *J. Allergy* **28,** 30–8.

Diengdoh J.V. & Turk J.L. (1965) *Nature (Lond.)* **207,** 1405.

Eisen H.N., Orris L. & Belman S. (1952) *J. exp. Med.* **95,** 473.

Epstein S. (1958) Dermal contact dermatitis. *Dermatologica (Basel)* **117,** 287.

Epstein W.L. & Kligman A.M. (1957) Transfer of allergic contact-type delayed sensitivity in man. *J. invest. Derm.* **28,** 291.

FERNSTROM A.I.B. (1954) Patch test studies. I. A new patch test technique. *Acta Derm. Venereol. (Stockh.)* **34,** 203.

FREY J.R. & WENK P. (1956) Experimentall Untersuchungen zur Pathogenese des Kontakekzems. *Dermatologica (Basel)* **112,** 265.

GEIGER R. (1929) *Arch. f. Dermat.* **158,** 76

HARBER L.C. & BAER R.L. (1961) *J. invest Derm.* **36,** 55.

HARBER L.C., HARRIS H. & BAER R.L. (1966) *Arch. Derm.* **94,** 255.

JETER W.S., TREMAINE M. & SEEBOHM P.M. (1954) Passive transfer of delayed hypersensitivity to 2,4-dinitrochlorbenzene in guinea pigs with leucocytic extracts. *Proc. Soc. exp. Biol. Med.* **86,** 251.

KLIGMAN A.M. (1958) Poison ivy dermatitis: an experimental study. *Arch. Derm.* **77,** 149.

KLIGMAN A.M. & EPSTEIN W.L. (1959) Some factors affecting contact sensitization in man. In *Mechanisms of Hypersensitivity*, p. 713. A Henry Ford Hospital Symposium, March 1958. London: J. & A. Churchill.

KLIGMAN A.M. (1966) *J. invest. Derm.* **46,** 573.

LANDSTEINER K. (1945) *The Specificity of Serological Reactions.* Cambridge, Mass.: Harvard University Press.

LEVINE B.B. (1960) Studies on the mechanism of the formation of the penicillin antigen. Part I. Delayed allergic cross-reaction among penicillin G and its degradation products. *J. exp. Med.* **112,** 1131.

MAGNUSSEN B. (1966) Europ. Congr. Allergy *Acta Derm. Venereol.* 38.

MARCUSSEN P.V. (1962) Variations in incidence of contact sensitivities. *Trans. St. John's Hosp. Derm. Soc.* **48,** 40.

MAYER R.L. (1954) *Progr. Allergy*, **4,** 79.

MEDAWAR P.B. (1960) In *Cellular Aspects of Immunity.* Ciba Foundation Symposium, p. 134. London: J. & A. Churchill.

MEDAWAR P.B. (1965) *Brit. Med. Bull.* **21,** 97.

OORT J. & TURK J.L. (1965) *Brit. J. Exper. Path.* **46,** 147.

ROSTENBERG A. JR. & SULZBERGER M.B. (1937) Some results of patch tests. *Arch. derm. syph.* **35,** 433.

SIDI E. & HINCKY M. (1957) Allergic sensitization to adhesive tape. *J. invest. Derm.* **29,** 81.

TZANCK A. & SIDI E. (1950) *Les dermatoses allergiques: la pathologie cutanée reactionnelle.* Paris: Masson.

TURK J.L. (1961) Passive transfer of contact sensitivity to picryl chloride in guinea pigs, with subcellular material. *Nature (Lond.)*, **191,** 915.

TURK J.L. & STONE S.H. (1963) In *Cell-bound Antibodies*, ed. AMOS S. & KAPROWSKI H., p. 51. Philadelphia: Wistar Institute Press.

URBACH E. & GOTTLIEB P.M. (1946) *Allergy.* London: Heinemann.

DE WECK A.L. & FREY J.R. (1966) *Immunotolerance to Simple Chemicals*, New York: American Elsevier Publishing Co. Inc.

CHAPTER 28

HAEMOLYTIC DISEASE OF THE NEWBORN AND OTHER CONDITIONS FOLLOWING ISO-IMMUNIZATION

W. WEINER

INTRODUCTION

Not many readers of this book will remember the time when 'erythroblastosis foetalis' was dealt with in textbooks as a condition bearing the label 'ill-understood'. The understanding of the disease came about through the genius of Levine who with his co-workers (Levine, Katzin & Burnham 1941) gave for the first time an explanation which co-ordinated all the known (and some of the then unknown) facts. Since then, knowledge has increased rapidly and though we cannot claim today to understand fully everything about the disease —and the understanding of a disease should lead to its prevention—we know a good deal more about it than even a few years ago. As with all great ideas, the theory proposed in this first paper was fundamentally simple. It has since been proved to be correct by innumerable papers and has been shown to apply not only to the rhesus system for which it was proposed but to all the other blood-group systems where 'immunization' of the mother can occur. Levine *et al*

(1941) suggested that foetal cells carrying antigens inherited from the father entered at some stage the maternal circulation. If the mother's cells lacked any of these antigens (if she was 'negative' for any of them) they could, under certain circumstances, lead to antibody in the mother. This antibody (or antibodies) would be passed through the placenta and when entering the foetal circulation cause damage to the foetal cells eventually resulting in 'erythroblastosis foetalis'.

BLOOD GROUP SYSTEMS INVOLVED

Rhesus System

Levine's *et al* (1941) first paper described iso-immunization within this system—and this system still provides by far the greatest number of cases. At first haemolytic disease of the newborn (HDN) due to the D antigen only was recognized (the antigen went by different names to start with but it is proposed to use the CDE terminology as it is certainly the most widely used and best understood). However, soon other antigens E, c and C, were recognized and proved to be capable of immunizing 'negative' mothers. Anti-E can be produced by either rhesus-negative or rhesus-positive E-negative mothers (e.g. CDe/CDe) whereas the production of anti-C is rare in D-positive people. Anti-c can, of course, be produced by C-homozygous mothers only (e.g. CDe/CDe). Anti-e is produced by mothers homozygous for the E antigen (for instance cDE/cDE). Rare antigens also lead occasionally to immunization, e.g. the C^w, and a most intriguing case of this sort was published by Van Loghem & Bakx (1953) where a mother negative for the c and C^w antigens (CDe/CDe) produced children with HDN due to anti-c and C^w consecutively. HDN due to the very rare antigen C^x has been described by Stratton & Renton (1954). Jakobowicz & Simmons (1959) described HDN due to the newly discovered G antigen and there is no doubt that if more antigens are discovered within the rhesus system, they will be responsible for HDN. It may even be that these rare (or frequent) antigens will be discovered through illness in babies.

The occurrence of anti-D in D+ve mothers is somewhat puzzling but at least nineteen cases of this type are known at present (Rosenfield, Haber & Gibbel 1958; Geiger & Wiener 1958). Two of our own cases (Weiner & Battey) had in their sera an antibody indistinguishable from a typical anti-D. The D of the first case was a D^u (genotype CD^ue/cde) whereas the second case was a strong D, probable genotype cDe/cde. All three babies (one mother had twins) were severely affected. The explanation of this phenomenon is not easy. It is possible that Wiener's mosaic theory (Wiener, Geiger & Gordon 1957) is right and it is conceivable that the D antigen of these people lacks one or other determinant group. When challenged with a 'complete' D, they might

produce an antibody against this 'determinant' group and as the latter is present in almost all 'normal' D antigens this antibody will behave like any other respectable anti-D. On the other hand, it is possible that what appears to be an anti-D, has in fact anti-G specificity. Some of the cases published by different authors have been re-investigated by Tippett & Sanger (1962) and these workers have indeed come to the conclusion that some antibodies mimic the anti-D pattern but could in fact be much more easily explained by the assumption that they were some sort of anti-G. One of our own cases apparently also falls into this category whereas the antibody of the other must still be regarded as an anti-D.

Other Blood Group Systems

The Kell and its allelomorphic antigen, Cellano, have been responsible for HDN and the Kell antigen is probably as efficient an antigen as the D. Fewer cases, however, must be expected as although approximately 90% of all mothers are Kell-negative, only about 10% of all fathers are positive. The cases of HDN due to Fy^a-anti-Fy^a are rare and occasional cases due to anti-Jk^a and anti-Jk^b have been described. In the MNSs system, anti-S has on several occasions been shown to be able to produce HDN (though it may occur as a 'natural antibody' and be quite harmless) and several cases of HDN due to anti-s are known. A_s to the M, there are several reports in the literature where this antigen wa reported as the villain of the piece but there is no doubt about its antigenic role in the case reported by Stone & Marsh (1959). The antigen Mi^a put in an appearance for the first time through HDN (Levine, Stock, Kuhmichael & Bronikovsky 1951). Anti-N has so far not caused any trouble.

In all the other systems (Lutheran, Lewis, Wright, etc.) occasional well-documented cases do occur but seem to be rather mild. In different ethnic groups still other systems might play an important part and in Mongolian people the Di^a is certainly worth watching. In short, no antigen, be it frequent or rare, seems to be unable to produce HDN. However, there is still the ABO system.

ABO System

Here the position is, to say the least, confused. A number of authors claim that disease due to incompatibility within the ABO system is as frequent as the one caused by incompatibility within the rhesus system or more frequent still. Others hardly ever recognize it. The truth might be somewhere in the middle. The controversy no doubt arises from the fact that while the diagnosis of HDN in most systems is really simple, viz. by a positive direct Coombs test on the infant's cells, this is not so in the ABO system. Here the diagnosis must be made on clinical grounds and is only scantily supported by a confusing and confused serology.

PATHOGENESIS

As pointed out, immunization is due to the entry of red cells carrying a 'foreign' antigen (or antigens) into the maternal circulation. This can be most easily achieved by injecting them intravenously, intramuscularly or subcutaneously. In HDN, immunization is produced by foetal red cells entering the maternal circulation at some stage. The question is when? It was noted that in a first pregnancy, HDN is very rare (unless the mother had received an antigenic stimulus beforehand). Subsequent children were affected and it was assumed that the antibody was produced by leakage of cells in the second or subsequent pregnancy. It was a further assumption that a first sensitizing dose must have occurred during a previous pregnancy and that the affected baby produced with his cells a 'secondary' response. We could show that in some patients, antibody was present some 6 months to a year after delivery of the first rhesus-positive baby (Weiner & Hallum 1950). Further, we found in our material fifty-five mothers (up to the end of 1966) whose sera during their second pregnancy contained typical anti-rhesus antibodies. None had ever received a transfusion or blood injection and the first children had all been rhesus positive with a negative direct Coombs test (DCT). Surprisingly the second babies were cde/cde and had, of course, a negative DCT. These could not have provided a stimulus for the maternal antibody production and the stimulus must therefore have been provided by the first baby. Most workers now assume that this first stimulus occurs during delivery, particularly before the placenta is fully delivered, and it could be shown that some babies give quite large 'transfusions' to their mothers at this stage (Wiener 1948). Particularly relevant here were the findings of Kleihauer, Braun & Betke (1957) who, with an elegant elution and staining technique, could show that in very many cases foetal cells, characterized by the different properties of the foetal haemoglobin, appeared in the maternal circulation. The volume of foetal blood thus transfused into the mother can be estimated and if the mother is rhesus negative and the foetus rhesus positive, a good correlation can be shown to exist between the volume of blood 'transfused' and the later development of antibodies (Woodrow *et al* 1965). However, there is good evidence that this cannot be the only time when foetal cells enter the maternal circulation. Amongst our material we have thirty-two primigravidae (up to the end of 1966) who during their first pregnancies developed antibodies against the rhesus factor and gave birth to affected babies (affected by the criteria enumerated below). None of these mothers had received a transfusion or blood injections and as far as humanly possible the fact that they were primigravidae was confirmed by interview with the mothers themselves (and sometimes their mothers). In no case did the clinical examination give ground to doubt their statements.

ABO Protection

The curious fact that women who bear babies with a compatible ABO group are more easily immunized against the rhesus factor than if the baby is incompatible was first noticed by Levine (1958a). Many further papers have since confirmed this on a large material. From our own material (Weiner & Battey 1962) we selected for investigation the primigravidae and the mothers immunized after the first baby (whose second baby was rhesus negative). Amongst the primigravidae, we could find only three exceptions (one mother Group O, child Group B; two mothers Group B, children Group AB) whereas there was only one exception amongst the twenty-nine mothers of the second category (a Group A baby born to a Group O mother). Nevanlinna & Vaino (1956), in a large-scale statistical investigation, arrived at the same conclusions. Only a few cases are known where a Group O mother married to a Group AB father became immunized. In their paper they mention two such matings. We ourselves have also encountered two such families. However, once immunization against the rhesus factor is established, the ABO group of the next baby is of no importance and though incompatible it will be affected. It should be noted, however, that Vos (1966) has lately shown that ABO incompatibility between child and mother often produces an antibody of lower titre (in later pregnancies) than if mother and child are ABO compatible. The 'protection' which incompatible ABO groups afford within the rhesus system extends also to the Kell system (Levine 1958b), and in all likelihood to other blood-group systems as well.

So far the facts: an explanation, however, is difficult. Several theories have been advanced and the most widely accepted at present seems to be the one suggested by Race & Sanger (1958). They assumed that incompatible cells would be destroyed rapidly and thus would not be able to provide an antigenic stimulus. One could think, however, that a different explanation (Weiner & Battey 1962) might fit better all the known facts and that the rapid sequestration of incompatible cells might play a different part. It is, of course, true that incompatible cells are sequestered rapidly but on the other hand it is also true that compatible cells will survive. Nevanlinna & Vaino (1956) point out that cells which are circulating and are not in contact with antibody-producing cells will not stimulate antibody production. This too must be true but we must not forget that these compatible cells consist of different populations with regard to their age. These cells are being sequestered over a much longer period (which may extend up to 3 months) and will thus provide a continuous stimulus. Thus the individual immunizing dose will be small but stimulation will go on for a very long time. This is analogous to the experience in experimental immunology when more potent antibodies are produced by repeated injections rather than by a single large dose. It also fits the known facts well: in an investi-

gation (unpublished) which was done some years ago, not a single rhesus-negative primigravida was found who had developed antibodies 10 days after delivery of her first baby but antibodies were found 4 months later in a few and the fifty-five mothers mentioned previously who in their second pregnancy gave birth to a rhesus-negative infant fall also into the same group. This theory would also explain the fact that on rare occasions primigravidae may produce an anti-rhesus antibody if one assumes that during the pregnancy foetal cells escape on frequent occasions into the maternal circulation. That this may happen is well known (Weiner *et al* 1958) and recent work using the Kleihauer *et al* (1957) technique gave evidence that this may happen quite frequently. If the assumption is accepted, it would also explain the occasional occurrence of immunization even if the foetus is incompatible (for instance the two incompatible matings quoted by Nevanlinna & Vaino (1956) and in our own material). Obviously even incompatible cells, if escaping frequently enough into the maternal circulation, will provide the same long-lasting stimulus though they are sequestered rapidly. It has already been mentioned above that the size of the foeto-maternal 'transfusion' is also important and Finn *et al* (1961) have shown that as little as 1 ml of foetal blood may act as an immunizing dose.

ABO Haemolytic Disease

The rule that a 'sensitizing' first pregnancy precedes immunization does not apply to HDN due to ABO incompatibility. Here it is the rule that the first incompatible child will show signs of HDN and that can easily be understood if one realizes how many people show what is regarded as an 'immune' antibody within the ABO system (an antibody which is mainly IgG) in their sera without known prior stimulation. These antibodies may be produced by injection of substances like horse serum (which has Group A specificity) but there are certainly a number of cases in our own material and elsewhere where such injections did not precede the pregnancy. It should, however, be pointed out, and this will be discussed at greater length later, that the diagnosis of ABO haemolytic disease is by no means as certain as it is in the other systems.

DIAGNOSIS

Ante-natal

It must be a unique occurrence that a probable and sometimes certain diagnosis is made in a patient who legally does not yet even exist. The more one learns of the disease, the more one realizes the importance of anticipation. This has become possible through the establishment of laboratories where appropriate tests can be done and thus it should not happen that an immunized mother slips through these nets. Prediction of the disease has become much more accurate in recent years. Before the introduction of the Coombs test and testing of sera

by means of enzyme-treated cells, a great many cases must have been missed. In an up-to-date laboratory this should be an exception now. How many cases are diagnosed depends, therefore, on the test procedures in the various laboratories and that again is not always a question of available knowledge but of available funds. It is never possible to equate human life and happiness with £ s. d. and ideally all the tests which are necessary to predict HDN should be done in every single expectant mother. This clearly is a counsel of perfection and some compromise is necessary.

It is convenient to divide the blood specimens from expectant mothers into groups and to test the various resulting groups at different levels. The first step is to establish the ABO and rhesus group and simultaneously with this investigation, the first level of testing is performed. On all mothers, irrespective of ABO group and rhesus type, irrespective of obstetric or transfusion history, a simple screening test is performed, but even at this level a more extensive investigation is performed on all specimens which are found to be rhesus negative. The second level should apply to all those sera in which the screening tests produce some, even the slightest, abnormality. It comprises also those mothers who have a significant obstetric history and thirdly all mothers who have had, at any time during their lives, transfusions or injections of blood. In the third level, those mothers are followed up who in the second level have given definite evidence of an abnormality. It should be noted that primigravidae are included and from what has been said above, it must be clear that in a large series, a certain number of genuine primigravidae who are immunized will be found. However, even if the primigravidae are primigravidae for their husbands' peace of mind or if a transfusion or blood injections which they had received had been forgotten, their history will still be given as 'primigravidae'. If they are excluded from thorough testing, some cases will certainly be missed. The tests at the various levels must be chosen taking into consideration the conditions in the laboratory, the number of trained staff, etc. There is, however, a minimum which should be done and it can probably be agreed that in the first level, all that would be necessary would be to test the sera against saline-suspended cells at 37 and 22°C (the latter can be combined with the routine ABO grouping) and in addition, against enzyme-treated cells at 37°C. For ease of performance, we prefer in our laboratory papain-treated cells, and have found that a significant reduction in labour simultaneously with an improvement of the results can be achieved by using cells which have been 'papainized' in bulk and then frozen in glycerol. These frozen cells can easily be reconstituted (Weiner 1961) and we have found that their reactivity is very well maintained for a number of months, possibly years. (This naturally also obviates the frequent bleeding of laboratory workers or donors which in a busy laboratory can very soon have great nuisance value to all concerned.) This also makes for confidence in reading the results as the technicians will

very soon learn to distinguish the strength and type of the agglutination and be able to separate the 'sheep' from the 'goats'. We have lately added to the papainized cells one further cell preparation treated with bromelin and preliminary results have shown that bromelin-treated cells may also be stored frozen. At present, however, we are still using fresh cells which are bromelin-treated every day. The choice of the cells has regard to the antibody or antibodies most frequently encountered. Thus it is advisable to use a CDe/cDE cell or perhaps even better, a mixture of CDe/cde and cDE/cde cells. Sera from rhesus-negative mothers are investigated more thoroughly. They are put up with a suspension of one rhesus-positive cell in saline, three different rhesus-positive cells in 25% albumin, one bromelin and one papain-treated rhesus-positive cell and two rhesus-negative cells in saline suspension.

At the second level, the nature of the antibody is further investigated. We use five cells in saline suspension which are: Cde/cde, cdE/cde, CDe/cde cDE/cde and cde/cde, five cells in albumin suspension (CDe/cde, cDE/cde CDe/cDE, two cde/cde) and again bromelin and papain-treated rhesus-positive cells. In addition to this, indirect Coombs tests (ICT) are now done with two different cells and the choice of these cells for the tests is as follows: one cell is of the constitution CDe/cDE, Kell negative and S negative. This cell may be either Duffy positive or Duffy negative. It should also be Le(a+). The second cell is rhesus negative and must be Kell positive, Fy(a+) and S positive. It should be L(a−). From the result obtained, one can already at this stage have a fairly shrewd guess at the nature of the antibody (e.g. if the first cell is positive, the second negative, possibly anti-rhesus; if the first cell is negative, the second cell positive, possibly anti-Kell or anti-S, etc.). At this level, the nature of an antibody will also be clarified and its specificity defined. If available, cells from a specimen taken from the father of the child should be included as test cells and used in the way other cells have given best reactions. If they are not available at this stage, some maternal serum may be kept back for later testing.

The third level of testing is adjusted for each case individually. From the second level the nature of the antibody is often obvious. If, for example, reactivity was best with enzyme-treated cells and ICT an antibody within the rhesus system is likely. If on the other hand, ICTs only have given positive results, the cells which reacted will give a hint how to test further. An appropriate cell panel is chosen and the cells used in the way in which best reactions have been obtained. (In saline, albumin, enzyme-treated, by ICTs and at 37, 22 and 4°C.) Enough different cells should be selected to give at least a probability of 1:200 of the nature of the antibody. As these tests are usually done 2–3 days after the screening tests have been performed the results may be much less convincing than on first testing if complement is required for the demonstration of the antibody. If this happens, the addition of complement may restore the positivity of the tests. Complement may be used by either mixing the patient's

serum with an appropriate amount of fresh AB serum and incubating the mixture together with the test cells (one-stage test) or alternatively one may incubate the test cells with the patient's serum first and replace this serum after a suitable length of incubation with fresh AB serum. This is then again incubated before the final wash (two-stage test) (Polley & Mollison 1961). Finally, after the recognition of its nature the titre of the antibody is determined. These tests will demonstrate almost all antibodies of clinical significance but this is only true if the presence of an antibody is suspected from the history or the tests done at the first level. Clearly it is impossible to test all sera at the third level and thus a very occasional case (e.g. first affected baby due to anti-Kell) might not be found ante-natally.

What has been said, however, does not apply fully to the ABO system. Here, the first indication that something might be wrong is the demonstration of a haemolysin in the maternal serum. As Rosenfield (1953) has shown, immunization within the ABO system occurs mainly in the setting: Group O mother, Group A child. This observation has been supported by a number of authors. It is also our experience as far as Caucasians are concerned. In Negroes, it appears that the B antigen is very often responsible for HDN and in a paper Wiener & Wexler (1948) quote two cases of HDN in Negroes, both due to the B antigen. If the correlation of a rhesus antibody and HDN (possibly expressed only as a positive DCT) in a rhesus-positive child is extremely great, the correlation of the presence of an α-haemolysin and an affected Group A child is nothing like as great. On the contrary, we have found on many occasions that the presence of an immune anti-A of great potency in the maternal serum had no visible, clinical or serological effect on a Group A baby. Furthermore, very similar findings in the maternal serum have been obtained when the baby is Group O. On the other hand, clinically obvious cases of ABO disease have escaped prediction in many cases and post-natal maternal specimens have given only scanty evidence of ABO immunization, so much so that we are at present very doubtful whether the searching for and reporting of these antibodies is worth while. There is work going on in many laboratories to find better means of predicting ABO disease and some of the tests are promising and may in future achieve a better correlation between clinic and serology than exists at present. Various new methods described lately seem, however, to work best in the hands of the authors. The antibody absorption test by Tovey, Lockyer, Blades & Flavell (1962) seems to give, in their hands, an excellent prediction. We have tried it in our laboratory but were not so successful. It must, however, be added that for theoretical reasons, it might not be easy to find a test of as great a significance in ABO disease as the ICT or enzyme test is in rhesus disease. Though it is true that a severe case of HDN due to ABO incompatibility is almost as easily recognizable clinically as a case due to rhesus incompatibility, milder cases might be mistaken for physiological jaundice and still milder

cases might be completely overlooked. The correlation with ante-natal serological findings thus becomes more and more difficult. In addition to all this, an anti-rhesus antibody almost always presupposes a prior experience of the antigen by the mother, which is not always obvious in the presence of an 'immune' antibody within the ABO system.

Cord Blood Findings

If there exists, in medicine, a single test which when positive indicates unequivocally a diagnosis, it is the positive Coombs test on cord blood cells, and apart from incompatibility within the ABO system, a positive DCT is a *conditio sine qua non* for a diagnosis of HDN. Occasionally one sees an apparent exception and that is a mother in whose serum a low-titred anti-rhesus antibody was discovered ante-natally giving birth to a baby whose cells gave, even when tested with very potent A.H.G. sera, negative results. The picture becomes much tidier and these cases 'fall into line' if one elutes the cord blood cells. A study made of just such cases (Weiner & Wingham 1966) showed that one has a good chance of demonstrating coating by demonstrating an antibody in the eluate in a very great percentage of these cases. Amongst ten cases thus investigated only two were found where this could not be demonstrated. It would appear, therefore, worthwhile to attempt an elution in such cases as a positive result confirms one's ante-natal findings. One might have expected that the strength of the Coombs test would correlate with the severity of the illness. This, however, is not entirely so. Walker (1958a and b) and other authors have commented on the lack of correlation. In our own experience, we would agree that a strongly positive Coombs test (that is a test where agglutinates form rapidly and attain a large size) may occur in very severe to comparatively light cases. A weakly positive test (late formation of small agglutinates), however, is almost always indicative of a mild disease (and the cases where antibody could be found in the eluate have without exception also been very mild indeed). It was tempting to try to assess the severity of HDN using similar criteria as are used in acquired haemolytic anaemia (AHA) of adults. There, antibody is formed constantly and it depends on the amount formed and the cell mass whether 'free' antibody will be found in the plasma. In our own experience, the reactivity, that is ability to absorb antibody in AHA, does not vary a lot from one patient to another and therefore these two parameters, that is the strength of the Coombs test and occurrence or absence of 'free' antibody in the serum, have some, very often good, correlation with the severity of the illness. In HDN, on the other hand, the amount of antibody is fixed once the child is born and no more is formed. If the child is born with a large red-cell mass, much antibody must have been passed through the placenta to give the red cells a heavy coating. Much less antibody is necessary to give the same coating if the cell mass is smaller. Again there is a difference

between AHA and HDN in as much as the reactivity of the cells in HDN differs. A D^u, for instance, will absorb less antibody (less coating of the cells but more antibody left 'free' in the serum) whereas a cDE/cde will absorb much more and little or no antibody will be found free. As Fudenburg, Barry & Dameshek (1958) have shown that cells coated heavily have a shorter survival than lightly coated cells, one can understand that the child with the heavier coated cells will show more severe signs of illness than a child whose cells are only lightly coated. As these two parameters do not, therefore, offer much help in assessing the severity of the illness, one has looked round for other criteria and these were found in the (*a*) haemoglobin (Hb.) and (*b*) bilirubin level. It is obvious that a baby's cells which had been exposed to an antibody *in utero*, must have suffered damage, and the products of cell destruction—bilirubin mainly—will be found to be present at a higher level than in normal infants. While *in utero* most of these products are being got rid of through the maternal circulation and a raised bilirubin level in the cord blood will thus indicate severe disease. As the cell destruction goes on after birth, the level of the bilirubin will go on increasing. The position as regards the Hb. level is not so simple. The initial level depends, as Mollison (1956a) has shown, to a great extent on the time at which the cord is clamped. If all the blood is allowed to enter the baby's circulation before the cord is clamped, the initial Hb. level will be higher than if the cord is clamped immediately after birth. In the cord blood, a normal level may, having regard to what has been said before, vary widely and is usually assumed to be between 14.8 g and 19 g%. The bilirubin level should not be higher than 2.5 mg% (but see discussion later).

ABO Haemolytic Disease

One might have hoped that the investigation of the cord blood would give diagnostic criteria suggestive for ABO HDN. In the same way, however, as the maternal ante-natal serology is not of the greatest help in predicting ABO disease of the newborn, investigation of the cord blood does not produce results in this disease which would be diagnostic with anything like the certainty one obtains in other diseases. Some authors (Rosenfield 1955) claim that if a special technique is used, ABO affected babies have a positive DCT. Others (Tovey, Gillespie, Guy, Valaes, Oppé & Lewis 1959) could not confirm these findings even with the same technique. Our own experience is that a certain number of Group A babies born to Group O mothers have a positive DCT though much weaker than in other systems and certainly not correlated, at least in our hands, with the antibody level in the maternal serum. The positivity of the Coombs test does not seem to depend on the type of Coombs serum used. The same sample may be positive with anti-IgG and anti-βic serum: others negative with both reagents. Tovey & Lockyer (1965) have lately described the direct bromelin test which

looks comparatively simple. The simplicity, however, is only apparent as much depends on the experience of the investigator. There is no doubt that, in the hands of the authors, this test has given good and reliable results which we in this laboratory, however, could not achieve.

Of other signs spherocytosis (and the increase in red-cell fragility) has often been regarded as an important sign. It is, however, certainly not diagnostic and though it is true that some of these babies show spherocytosis, it is not possible to correlate this sign with the severity of the illness. The whole problem hinges, as far as one can see at present, on a clear definition of ABO disease. In the rhesus and other systems, the serological diagnosis can easily be made and though not all serologically affected babies are clinically affected, all clinically affected babies are 'Coombs positive'. (The 'eluate positive' babies mentioned above are hardly ever clinically affected and do not even show a trace of jaundice.) There is, therefore, one certain parameter to support the clinical diagnosis. In ABO disease, on the other hand, one always looks to the clinician for the diagnosis and after more or less great efforts succeeds often to support it by serological means. Definite criteria have still, however, to be worked out. It will have to be shown why some children badly affected by ABO disease are often found not to have a positive Coombs test and on the other hand, children whose Coombs tests are positive are often not affected at all. It is likely that a test diagnostic for ABO disease might on occasion be 'positive', even if clinically the disease is absent. This would be on a par with a positive Coombs test (or positive eluate test) in the absence of clinical HDN in rhesus immunization but would certainly not invalidate a test as it has not invalidated the Coombs test. To start with, the test will have to be shown to be positive in all more severely affected babies and in those without any exception.

CLINICAL ASPECTS OF THE DISEASE

Clinical Diagnosis

The clinical diagnosis had for a long time been bedevilled by the fact that it was not realized that the various manifestations of the disease were all due to the same mechanism. So it came about that icterus gravis, haemolytic anaemia of the newborn or hydrops foetalis were all thought to be different diseases, though some observers noted their occurrence in the same family. There is no difficulty today to put all the different manifestations of the illness under one heading. The most prominent sign is jaundice. As has often been stressed every baby becoming jaundiced during the first 24–48 hr of life must be suspected of suffering from HDN. Pallor is often seen at birth and may develop later. Severe cases may show oedema or even anasarca. Bruising and petechiae are not uncommon. Spleen and liver may be enlarged. Severely affected babies

may be born with signs of heart failure—a raised venous pressure is the presenting sign.

Complications

Kernicterus (KI) must be regarded as the most serious complication. Much has been written about this condition but only comparatively lately has it been definitely realized that the cause of this disorder is a high level of bilirubin after birth which may occur in untreated severe cases. It is only the 'indirect' bilirubin which is toxic. In the adult, bilirubin is conjugated with glucuronic acid (with the help of the enzyme glucuronyltransferase) thus becoming 'direct' bilirubin and is excreted by the liver. In the newborn, the activity of glucuronyltransferase is low and conjugation does not take place to anything like the same extent as in the adult. There is, however, a second danger point. Normally both direct and indirect bilirubin are bound to the albumin fraction of the plasma (Odell 1959). This bilirubin-albumin complex does not enter the cells and if it stays in the circulation it does not do any damage. Only 'unbound' bilirubin is able to enter the cells of the CNS and interferes there with the metabolism of the cells, eventually leading to cell death. From this it is clear that if there is not enough albumin available to bind bilirubin the danger of KI increases. The albumin fraction in the newborn is lower than in adults and lower still in premature babies (hence their liability to contract KI or to increase the danger of KI in HDN). There is keen competition for the albumin-binding sites and substances other than bilirubin often win the race. Such substances are haematin, sulphonamides and salicylates and if the latter are given to the infants (and as they can pass through the placenta, to the mother) they may 'displace' bilirubin which is then free to enter the brain cells. Raising of the albumin level will, on the other hand, attract more bilirubin. The bilirubin level in the blood may then actually rise, but the danger to the child might decrease. Accordingly albumin transfusions have been tried in an attempt to prevent KI (Kitchen, Krieger & Smith 1960) and results up to now seem to have been favourable.

The next question to answer is: What is the dangerous level of bilirubin? This seems to be a simple and straightforward question which should be answerable after large enough numbers have been observed. Many methods for the determination of the bilirubin have been described but we are still without one which in different laboratories will always give the same answer. This might not apply to the spectrophotometric methods, which have three big advantages—they are physical methods rather than chemical ones, they enable bilirubin determinations in capillary blood samples and they do not give false high values in the presence of moderate amounts of Hb. They have, however, two disadvantages; one that they are rather slow and thus not very well suited for the investigation of numerous specimens, and secondly they are not suited

for the differentiation between 'direct' and 'indirect' bilirubin. In most laboratories chemical methods are used and thus results at present still vary from laboratory to laboratory so that different authors regard different levels as dangerous. The Newcastle group of workers (Walker 1958a and b; and Murray) regard a bilirubin of 25 mg% as just permissible (though one baby with a bilirubin of 23 mg% suffered KI). Mollison (1956b) has provided data which seem to show that a bilirubin level of 20 mg% should be regarded as dangerous and this is the value which is generally so regarded.

KI is unlikely to develop during the first few hours but may occur at any time during the first 5 days post-natally. Any rise of the bilirubin to near the danger level at any time must be regarded with the greatest caution and treatment should be instituted as soon as possible. Some workers have advocated frequent bilirubin determinations (this can easily be done using capillary blood and a spectrophotometric method) and they regard a steep rise of the bilirubin graph which reaches or passes the 15 mg% level and shows no sign of levelling off as an indication for active treatment.

For reasons mentioned above and to be discussed later, the ideal method should also distinguish between 'indirect' and 'direct' bilirubin. This at present is only possible if colorimetric methods are used.

The signs of KI are well known. The children become lethargic. There may be twitching movements of their limbs, opisthotonus, rolling movement of the eyes with coma and death supervening. Very characteristic is the high-pitched cry and the refusal of feeds. On the other hand, a very mildly affected baby, it stands to reason, need not show more than a very quickly passing state of lethargy or a faint twitching of an arm which may not have been observed if it happened at night in the nursery. Even such minor manifestations may lead to serious sequelae later on. These are spastic paraphegia and mental retardation. Almost always there is an impairment of hearing (Gerrard 1952) and it is sometimes difficult to decide whether the child is mentally backward or deaf as deafness will not be apparent even to the parents until a much later age of the child. The defect should, however, be diagnosed as soon as possible so that treatment and education can be adjusted. No child should remain backward through lack of hearing.

Another, less dangerous, but dramatic complication is the syndrome which has been given the name, probably wrongly, of 'inspissated bile syndrome' (Oppé and Valaes 1959). Experienced clinicians claim that they can diagnose the syndrome by the colour of the jaundice of their babies who appear much greener than usual. The importance of the recognition of this syndrome, which is apparently due to the massive extramedullary erythropoiesis in the liver obstructing the free flow of bile, lies in the fact that the bilirubin may reach what might appear to be a very dangerous level. This, at least in part, is conjugated bilirubin and as such is not toxic or dangerous to the CNS. The amount

of this bilirubin must be deducted from the total amount. In these cases, values over 20 mg% would not be dangerous provided the conjugated bilirubin forms a substantial part of the total and that the unconjugated bilirubin is well below the 20 mg% mark.

Prevention

For some time now it has been assumed that the first sensitizing stimulus to produce an anti-rhesus antibody in the maternal serum occurs during the delivery of a rhesus-positive baby. It thus makes sense to advise the obstetrician to achieve as untraumatic a delivery as possible with every baby in a rhesus-negative mother. Prolonged labour, manual removal of the placenta, Caesarian section, have all been incriminated and have no doubt often been instrumental in allowing foetal cells to escape into the maternal circulation. The fact that ABO-incompatible pregnancies lead to fewer cases of immunization than ABO-compatible ones provided the basis for Finn *et al* (1961) in this country to attempt prevention. Their idea was to imitate the 'natural' protection given by 'natural' antibody by sequestering the sensitizing cells by means of an anti-D. Interestingly enough the same procedure was followed by the American workers Freda, Gorman & Pollack (1965) who, however, started from a completely different idea. They assumed that in analogy to immunization in infectious disease, passively administered antibody would prevent the formation of the anti-rhesus antibody. The American team was first to use γ-globulin (rather than whole plasma) and both groups were able to show that by injecting male volunteers with IgG anti-D these could be protected against the development of anti-rhesus antibodies whereas other 'unprotected' volunteers developed this antibody very readily. It should, however, be pointed out that it is essential that the antibody injected should consist entirely of IgG as IgM had been shown by the Liverpool workers (Clarke 1966) to be enhancing rather than suppressing immunization. In addition, it seems to be important that IgG prepared by ethanol fractionation be used as this preparation has been shown (in other connections) not to be icterogenic and thus does not carry the risk of post-transfusional hepatitis. Work in many different places on this most important discovery is still going on and this work should settle primarily the practical problem of the dosage required to prevent primary sensitization. Apparently the dose given at first was rather higher than necessary and it is quite possible that much smaller doses will suffice. A further theoretical point (which may have practical implications) should be settled and that is how the treatment actually works. The Liverpool group assumes that its mode of action is primarily the sequestration of the antigen-carrying cells, whereas the American group assumes the preventative function to be the presence of the passively administered antibody. One could think of various experiments which could settle the problem but it would seem that the Liverpool workers might carry the

day as the American theory does not explain the undoubted deficit of immunized mothers with ABO-incompatible babies. If the American view is right, one could not explain this deficit and an additional theory would be necessary. The Liverpool hypothesis is thus simpler and seems to be right. It should not be very long now before definite results will be available and the problems which will present themselves then will be somewhat different from those presenting themselves now. One of the problems will be, for instance, how to produce enough IgG anti-D to make it available to all mothers needing protection. One will probably have to fall back on male volunteers artificially stimulated and the ethical problems connected with this will have to be considered. The question of the dosage mentioned above should be settled rather quickly. For this it will be of some importance to settle the question of how the treatment works as if sequestration is the modus operandi it will be easy to estimate the amount of IgG anti-D which is necessary to sequester all cells; if the American view should turn out to be correct, the determination of the dosage would necessitate a very lengthy controlled trial. It will also be necessary to decide whether protection should be given after every pregnancy or only after the first and/or second etc.

Until this, however, is decided, we have still to apply the usual precautions and it is, of course, still vital to prevent not only HDN but in particular KI. Thus, the drugs indicated above should not be given towards the end of the pregnancy and the use of albumin in the exchange transfusion treatment will have to be tried on a still larger material.

It is probably unnecessary to mention here that a rhesus-negative woman of child-bearing age should never be transfused with rhesus-positive blood. In the light of present-day knowledge, this must be regarded as a serious error, if not as negligence. With the growing availability of suitable blood for transfusion, this danger has receded but it is interesting that in our material of all mothers who were found to be immunized against antigens other than D, transfusion played a large part. Thus of twelve mothers (up to and including 1962) found to be immunized against the c antigen, no less than five had received a transfusion of c-positive blood at some stage. Again of mothers (most of them rhesus positive) who were found to be immunized against the E antigen (eleven) four had been transfused, three certainly with E-positive and one with rhesus-positive, probably E-positive, blood. Of two patients immunized against the c and E antigen, one was transfused and amongst the six mothers who developed a more or less pure anti-C (two of them rhesus positive) three had been transfused. The three mothers who had anti-e in their sera had all previously been transfused, certainly with e-positive blood. Of course, it would be impracticable to type all donors as far as the c, E, Kell, Duffy etc., and we have to accept this, admittedly small, risk of a rarely occurring immunization.

Prognosis

What is first of all the risk of immunization? The most frequent figure quoted is one affected infant amongst 250 liveborn babies. Normally this should be an acceptable risk to a couple who intend to marry and whose rhesus groups differ. Wiener (1946) suggested that the ability to become sensitized was under genetical control; this has been shown not to be the case (Gerrard & Waterhouse 1953). The risk of immunization seems to be somewhat bigger with homozygous fathers. This is understandable as all their children carry the immunizing antigen. But even within the Ds there seem to be marked differences. We would agree with Murray (1957) that children of the genotype cDE/cde are as a rule more severely affected than those whose genotype is CDe/cde. There are no doubt further differences amongst the various Ds; a child with a weak D(D^u) will no doubt be less affected than his brother who has inherited a 'proper' D. In this connection it does not seem to matter whether the 'D^u' is a genetically inherited one or a D whose potency has been weakened by a C in 'trans.' position (e.g. CDe/Cde). Mrs A.R.P. may be mentioned briefly. She was group A rhesus negative sub-group Cde/cde. Her first pregnancy in 1958 terminated with the birth of a healthy infant. In 1962 she had a 3-months miscarriage. In her third pregnancy, in 1963, a sample taken at 11 weeks showed no deviation from the norm. At 28 weeks, typical blocking anti-rhesus antibodies were found. These were at a low level. At 33 weeks, the titre had risen significantly and had passed the 'danger level'. The husband had been found to be Group O rhesus positive, phenotype CCDee (most likely genotype CDe/CDe). The baby was born at term: Group O rhesus positive, genotype CDe/cde, DCT very strongly positive: it required treatment. Next pregnancy started in January 1966. The first specimen was investigated at 16 weeks and an anti-rhesus antibody again found to be present was of a much lower titre than had been found in the last specimen from the previous pregnancy. Having regard to the apparent homozygosity of the husband, a rise in titre later in pregnancy was expected. This rise never materialized and the baby born at 38 weeks was found to be Group O and rhesus positive and the DCT was negative. (The eluate, however, yielded a typical anti-rhesus antibody.) When the cord blood cells were further investigated, the phenotype CCDee was determined which obviously corresponds to the genotype CDe/Cde. Thus the C in trans. position weakened the expressivity of the D which behaved as a D^u and obviously did not produce an efficient antigenic stimulus and also did not absorb as much antibody as it would have done had it not been hindered by the C in trans. position. It should be added that the baby never showed any signs of illness. Interestingly enough, the titre of the maternal antibody rose after delivery but even so did not reach the level found during the previous pregnancy.

As to the prognosis of the individual pregnancy, the question of the maternal

titre will be dealt with later. Suffice it here to say that in an approximately 10-year period, 4225 cord blood specimens of babies born to mothers whose sera contained anti-rhesus antibodies were investigated in our laboratory. Of these, 478 proved to be rhesus negative (11.3%) (in spite of the fact that some of the mothers had shown high and/or rising titres during this particular pregnancy). If the baby is born alive and is affected by HDN the most important factor seems to be whether an experienced team is available to assess, in the first instance, the severity of the illness and in the second instance to apply proper, rapid and effective treatment. If KI can be prevented, the prognosis for these babies seems to be perfectly good and apart from the occasional child which shows discoloured deciduous teeth, the babies should grow up into perfectly healthy and normal adults. The 'inspissated bile syndrome' has not been known for long enough for one to be certain what, if any, sequelae there might be. The few cases watched for some time did not seem to be any the worse for their experience as infants and in particular (Dunn p.c.) no case of cirrhosis of the liver had been reported as a consequence of this complication so far.

The prognosis for future pregnancies depends of course on the zygosity of the husband. If he is heterozygous the couple have a 50% chance of a healthy baby; if homozygous all babies will be affected. It was long thought that the inescapable sequence of events was that the illness went from bad to worse with each successive child. This, however, is not so. Though the above-mentioned pattern no doubt exists, families are well known where the severity of the babies' illness remains about the same in subsequent babies, and again in others, a very severely affected baby may be followed by a mildly affected one (Davies & Gerrard 1953). (It may well be that in these cases, the different antigenicity of the two Ds of the homozygous father may play some part, and see also above). It is, however, undoubtedly true that the prognosis for a pregnancy which follows a stillbirth is poor and it is in these cases that more active treatment is probably indicated.

TREATMENT

Whether the above-mentioned injections of IgG anti-D will always be an effective prophylaxis remains to be seen. For the time being, however, the disease does exist and treatment, early and efficient, is of paramount importance. Once the nature of the disease was recognized, it became clear that the children suffered and were killed by blood destruction. It was clearly essential (*a*) not to let them die from anaemia and (*b*) to avoid further damage from the products of blood destruction or further action of the circulating antibodies. As the greatest danger to these children is still KI the prevention of this was and still is imperative. Whereas previously it was assumed that some children were born with KI the consensus of opinion now is that this is never the case and that

it is a complication which arises after birth. The aim of an effective treatment is, therefore (*a*) to correct the anaemia, (*b*) to get rid of the antibodies and (*c*) most importantly to rid the child of the danger of KI by eliminating the bilirubin. Fortunately all three aims are achieved by an efficient exchange transfusion. This became a practical possibility when Diamond (1947) described the use of the plastic catheter making it unnecessary to resort to the mutilating operation of tying the radial artery. As the umbilical vein can be used repeatedly, repeat exchange transfusions are possible via this route. Many years were needed to find the proper indications for exchange transfusion. Forty per cent of all children born with HDN do not need treatment at all. They are not and do not become anaemic nor will their bilirubin levels rise to dangerous heights. It is, however, of paramount importance to decide as soon as possible whether an exchange transfusion will be necessary, as waiting in this condition is not 'masterly inactivity'. Walker & Murray (1954) and Mollison (1956c), besides a great number of other workers, have suggested the following cord blood values as indications for exchange transfusion: all children with a Hb. under 14.6 g% (100%) or with a bilirubin higher than 4 mg% should be exchange transfused. Those children whose Hb. is between 14.6 and 17 g% should be exchanged if the bilirubin is higher than 4 mg%. The aim of the transfusion is to replace 90% of the child's cells by compatible cells and Mollison (1956d) gives a useful nomogram to calculate the amount of donor blood necessary to achieve this result. In general, for a 6 lb baby, semipacked cells from two bottles of blood will suffice. The exchange transfusion removes a great amount of bilirubin and circulating antibody simultaneously. In severely affected babies, the bilirubin might rise again. This secondary rise is no doubt due to the influx of bilirubin from the tissues and possibly to a minor extent to further blood destruction of the child's own cells which are still being produced and still subjected to the action of the antibody which has possibly not been fully removed. Thus a second exchange transfusion is necessary if the bilirubin again rises to a dangerous level and from this it is obvious that the bilirubin must be watched carefully even after an exchange transfusion. If prematurity complicates the disease, and prematurity may be spontaneous or induced, it is the experience of most workers that three or possibly four exchange transfusions may be necessary. Even without prematurity, up to five exchange transfusions may exceptionally be necessary in very severe cases. Whether the use of albumin will improve matters only further experience can show.

All this applies to babies already born with the disease. The question of premature induction of labour will be dealt with below but it is obviously clear that if the baby dies *in utero* before viability is achieved, the foetal loss might be high in the most severely affected cases. As post-natal, so ante-natal, the greatest danger to the child is blood destruction and consequent anaemia (with

and without heart failure). It has long been known that blood delivered intra-peritoneally reaches the circulation fairly rapidly. It was Liley in 1963 who had the courage of his convictions and transfused for the first time a foetus *in utero*. He succeeded in saving the baby and since then a great number of intra-uterine transfusions have been performed. Though the technique is difficult, no doubt it is not too difficult to be learned by many. Equally the indications for this as yet heroic-appearing operation become more and more apparent and will be discussed below. There is, however, no doubt about it that already a number of children have been saved by intra-uterine transfusions who otherwise would have died *in utero* before reaching a viable age.

Selection of Blood

The red cells of the donor blood selected must not carry the antigen or antigens against which the mother has formed antibodies. Usually the rule is that the maternal type of blood may be used. It will certainly be compatible but there are cases where blood differing in some antigens from the maternal blood must be selected. A Group A rhesus-negative infant suffering from ABO disease must be transfused with Group O rhesus-negative blood even if the mother is Group O rhesus positive. A tricky case was a baby born to a mother of genotype CDe/Cde, who had produced anti-c. The baby girl was Cde/cde and the only blood suitable for her would have been the very rare Cde/Cde. Fortunately no transfusion treatment was needed. The ABO group should be the same in child and donor (this may again differ in the mother). Though it is true that agglutinins are usually 'borrowed' from the mother and are therefore identical, this is not always so and one would feel it quite wrong to transfuse a Group O baby, for instance, with Group A blood, the mother having been found to be of this type.

If by any chance the serology of the mother is not known, and one would have to supply suitable blood for the baby, the genotype of the baby is of no help whatsoever. Obviously a CDe/cde baby may be affected either by anti-D, anti-C or anti-c, not to speak of anti-Kell, etc., and to cross-match for the baby with cord blood serum is an unreliable method as most of the antibody may be absorbed on to the cells. The cord blood serum would then not indicate incompatibility. The way round this difficulty is either to obtain rapidly some blood from the mother and to cross-match from this or to produce, by any method which one masters, an eluate from the child's cells and use that for cross-matching. It is a sound practice to cross-match always with the maternal serum and cord blood serum simultaneously. This is particularly important in multiple immunization as, for example, the weaker antibody might be fully absorbed by the cord blood cells and an incompatibility thus overlooked.

It is important that whatever blood is chosen, this blood should be 'young'. It has been shown that blood older than 5 days does not survive so well in

newborn infants and though an efficient exchange transfusion had been performed, the baby might be found after the exchange transfusion with 'aged' blood to be anaemic a short time afterwards. Thus a further transfusion with its inherent dangers and unpleasantness might be necessary. It is recommended that to raise the Hb. of the baby sufficiently, the donor's cells should be partially packed and even fully packed cells have been used successfully. As in all procedures, it is quite wrong to proceed if there are some untoward reactions and very severely affected babies, particularly those in heart failure, do not tolerate a large exchange transfusion easily. It is essential, and this has been stressed by almost all people with experience, to have due regard to the venous pressure which can easily be measured. If this is found to be raised, it is essential to start the transfusion by withdrawing blood first, or rather let it squirt out after the catheter has been introduced. Only after withdrawing 20 or 30 ml of the child's blood should the transfusion be started. It should take a long time and if the baby shows signs of distress, one should stop for an hour or two and may leave the catheter *in situ*. Even in most experienced hands, a very occasional unexpected and sudden death may occur during an exchange transfusion and Anderson, Smith & Walker (1961) found a positive correlation between high plasma citrate concentration and illness or death in the babies exchanged. Their statement 'It is possible that citrate directly or indirectly is the cause of this crisis' invites further investigation. It is probably, therefore, wise to interrupt an exchange transfusion at the slightest sign of distress and to resume it when the baby has again regained its biochemical balance. Other workers in this field (Dunn p.c.) advocate injections of small amounts of calcium gluconate. If a baby has withstood a first exchange transfusion well, it does not automatically follow that a second or third will be equally well tolerated and the same precautions should be taken as with the first exchange transfusion. The results of treatment if conducted on the above lines are very good indeed. Walker and Murray surveying the results for the year 1960 in the Newcastle region had amongst 205 babies born alive only seven deaths of which at least three were not due to HDN: of the remaining four, one baby was admitted when 26 hr old and was then already suffering from KI. Similar results are obtained in other places, but to achieve them, two things are essential. First there must be a team of workers fully experienced in the clinical and laboratory aspects of the disease. In other words, an exchange transfusion must not be a dramatic, exciting event which happens once in a blue moon, it must be routine. This means that as the number of patients is small, only a few places should be selected where this treatment is applied. Secondly, all babies must be assessed and if necessary treated as early as possible—in other words all these babies should be born in designated hospitals to enable early treatment if necessary. The selection of blood for intra-uterine transfusions presents some special problems. The same principles apply here as in post-natal transfusion. Knowing the maternal serology,

it is easy to select blood with the 'missing' antigen but to select blood with a compatible ABO group is more difficult. One is certain only if both mother and father are of Group O as the baby must also be of this group, but in all other settings, the prediction of the baby's ABO group would be somewhat dicey and it is probably wise, therefore, to select for all cases, Group O blood from which the plasma has been removed as far as possible.

Induction of Labour

To induce or not to induce is a question which has been discussed for many years. It would appear logical to assume that a baby exposed for a long time to the action of the maternal antibody would be more severely affected than a baby exposed for a shorter period. To arrive at an unbiased answer, a 'blind' trial was held in Britain in 1952 (Mollison & Walker 1952) to find out whether babies of mothers induced earlier have a better chance of survival than those not so induced. The main aim of induction, however, should be to lower the rate of the stillbirth. It is obvious that all babies who die before or at the 32nd week of pregnancy would not be born alive at all as an induction before 32 weeks would produce such a degree of prematurity as to provide its own nearly insoluble problems. (This statement was true when the trial was held, that is before the introduction of intra-uterine transfusion.) A later date was therefore chosen and the results obtained when induction was performed at 36 weeks did not really improve the survival rates significantly. In the meantime, the Australian workers Kelsall, Vos & Kirk (1958) came forward with the idea that an induction is necessary if the antibody titre in the mother rises or surpasses a certain level. They titrate the maternal sera by means of ICTs and rely on these tests to indicate to them the severity of the illness to be expected. More or less the same line is taken by Tovey & Valaes (1959) in whose laboratory a Coombs titre of 1:40 or over is regarded as an indication for an early induction. If one, however, looks at the results of these workers, one is not completely convinced that these criteria can be applied in all cases. All workers depending on the results of titres stress that one of the indications for early induction is the homozygosity of the father of the child, thereby admitting that even with very high titres, a rhesus-negative and unaffected baby might be produced (from a heterozygous father). To improve on this, Vos (1958) described the 'partial absorption test' for the assessment of the severity of the illness of the baby but again the results, after having applied this test, do not seem to be entirely satisfactory. Even after a positive result of this test, rhesus-negative babies were apparently born. This would not have been so bad had they not been induced prematurely. *A priori* it seems astonishing that the Coombs titre has been chosen to assess the severity of the disease as this extremely valuable and sensitive test is notoriously tricky, depending on many variables and on the thoroughness and conscientiousness of the technicians. It appeared, therefore,

to us that a simpler test with the same or greater sensitivity might be more suitable and we therefore chose to determine the titres by means of enzyme-treated cells laid down in glycerol. Though, by and large, a high antibody titre was more likely to produce a severely affected baby than a low one, there were a few disturbing exceptions (Halley & Battey in press). A few severely affected babies were born to mothers whose antibody titres, carefully followed up, were never very high and on the other hand, there were a few cases where a rhesus-negative unaffected baby was born to a mother whose titre had been high throughout the pregnancy and in two showed a significant and definite rise towards the end. The danger of induction is the risk of prematurity of the baby. There might, however, be one indication for premature induction which seems to be agreed by all workers in this field and that is induction should be performed if the previous baby was either stillborn or very severely affected. One will not always achieve a surviving baby in this way but the early induction in these cases seems to offer the best prospect for a living infant. It is important in all these cases to be prepared for an exchange transfusion immediately the child is born and suitable blood should be available at the hospital before the induction or Caesarian section is performed. If necessary several bottles of different ABO group may be prepared, having regard to the ABO group found in the mother and father of the child and cross-matched with the maternal serum.

Since the first edition of this book, matters have improved considerably. One is now in a very much better position to assess the prognosis of a baby after the investigation of the amniotic fluid obtained by means of an amniotic tap has given a further lead. It was shown by Bevis (1956) that a high level of bilirubin in the amniotic fluid at certain stages of the pregnancy indicates a gravely affected foetus. Liley (1961) and Walker & Jennison (1962) gave further evidence of the usefulness of this parameter and there are now two criteria to assess the prognosis of a pregnancy. These two parameters, the antibody titre in the maternal serum and the bilirubin level in the amniotic fluid, are certainly more reliable than anything we could offer before and even the fact that the father of the child is heterozygous does not prevent a more accurate prognosis and thus appropriate action. It appears that the following procedure might give the best results and this is actually the procedure followed by a great number of obstetricians: a low-titred antibody found in the maternal serum (unless there are other indications that the baby might be badly affected e.g. previous stillbirth etc.) will not usually suggest an amniotic tap. If the titre of the antibody is high or rising, no matter whether the husband is homo- or heterozygous, an amniotic tap should be considered. In the hands of an experienced obstetrician this does not seem to carry a risk either to the mother or the baby and the information obtained is potentially so valuable that most people now advocate this diagnostic procedure. The determination is fairly simple (and will probably

be simplified in future still further) and in several cases has been performed repeatedly during the same pregnancy without any detriment to the patient.

The management of a severe case of rhesus immunization might thus be as follows: frequent determination of the antibody titre by a method tested and tried in the laboratory. If the titre rises up to the danger level (this again must be found out for each laboratory individually) an amniotic tap is indicated. If the bilirubin level is low, this should be repeated at suitable intervals. If the bilirubin level is high and at danger point, intra-uterine transfusion should be considered to prolong the intra-uterine life of the foetus up to a point when an induction will produce a viable infant. The later in pregnancy this can be achieved, the better the prognosis, and cases have become known where an intra-uterine transfusion has been done repeatedly with good results.

In spite of all these dramatic and exciting advances, there are still some cases where even the most expert management will not prevent the death of the foetus *in utero* and one is thus again driven to the conclusion that prevention would be better than cure.

CONGENITAL NEUTROPENIA AND THROMBOCYTOPENIA

For a long time occasional cases have been known to occur where a newborn infant showed a more or less severe degree of neutropenia or thrombocytopenia. When the pathogenesis of HDN was clarified, it was not too far-fetched to postulate a similar mechanism for the two conditions. The great difficulty which presented itself, however, to almost all investigators was the technical aspect of the problem. To isolate and handle white cells appropriately is at the best a laborious procedure and platelets behave, if anything, still more erratically —one of their biological properties and justification of existence is 'aggregation'. It would appear, however, that three techniques are now offering good prospects for further elucidation. The mixed antiglobulin technique of Chalmers, Coombs, Gurner & Dausset (1959) gives certainly convincing and repeatable results and in the hands of the expert is obviously an excellent method. The Antiglobulin Consumption Test of Steffen & Schindler (1955) and Steffen (1955) appears somewhat more laborious but again workers who have experience find this method useful. It is astonishing, however, that when both techniques are used together, the results are sometimes not identical. The Complement Consumption Test described by Chudomel, Ježková & Libánský (1959) seems simple but more experience is needed to evaluate this test for the conditions mentioned. None of the three tests is technically too difficult but they are all somewhat time-consuming and need a well-thought-out organization. From this it appears that none of them can at present be used on large numbers of samples but results obtained up to now indicate clearly that in some

cases at least, immunological forces are at work. If the suspicion arises that further investigation on these lines is essential or indicated, the maternal sera should be sent to laboratories specializing in these tests.

One must distinguish, in these conditions, two different settings. One setting appears to be analogous to the one found in HDN, that is the mother lacks an antigen on either the white cells or platelets which the child's cells carry and produces an antibody against this antigen during pregnancy. The other setting is quite different. The antibodies in the maternal serum are not due to a stimulus by external antigens but must be classed as autoantibodies against their own white cells or platelets. If these antibodies are IgG globulins, as they sometimes are, they may pass the placenta and affect the child's cells. This would be analogous to a mother suffering from AHA passing an antibody through the placenta which would affect the child's red cells. Naturally both of these conditions are self-limiting as both are due to an antibody passively transmitted to the child. Examples have been given by Rood, Leeüwen & Eernisse (1959), Hitzig & Gitzelmann (1959) for leucocyte (iso-) antibodies and Garrett, Giles, Coombs & Gurner (1960) for platelet and white cell (iso-) antibodies. The cases described by Sauer & Van Loghem (1955) and Gwynfor Jones, Goldsmith & Anderson (1961) seem to be examples of an autoantibody in idiopathic thrombocytopenia transmitted to the child. An isoantibody against platelets demonstrated by the mixed antiglobulin reaction and transmitted to the child, produced in the latter a thrombocytopenia which, in spite of treatment, proved to be fatal (Coombs & Garvie p.c.).

Cases of this sort are not too frequent and only few children are severely enough affected to need treatment. This, however, is still an unsolved problem, as white-cell transfusions are quite ineffective and, for that matter, platelet transfusions are not faring much better in the presence of anti-platelet antibodies.

REFERENCES

ANDERSON J., SMITH B.M. & WALKER W. (1961) Certain biochemical changes during exchange transfusion. *Vox. Sang. (Basel)* **6,** 525.

BEVIS D.C.A. (1956) Blood pigments in haemolytic disease of the newborn. *J. Obstet. Gynaec. Brit. Comm.* **63,** 68.

CHALMERS D.G., COOMBS R.R.A., GURNER B.W. & DAUSSET J. (1959) The mixed antiglobulin reaction in the detection of human iso-antibodies. *Brit. J. Haemat.* **5,** 225.

CHUDOMEL V., JEŽKOVÁ Z. & LIBÁNSKÝ J. (1959) Detection of leucocyte antibodies by the complement consumption test. *Rev. Czech. Med.* **1,** 8.

CLARKE C.A. (1966) Prevention of Rh haemolytic disease. A method based on the post-delivery injection of the mother with anti-D antibody. *Vox Sang.* **11,** 641.

COOMBS R.R.A. & GARVIE, J.M. Personal communication.

DAVIES BARBARA S. & GERRARD J. (1953) Haemolytic disease of the newborn with special reference to exchange transfusion 1950 to 1953. *Birmingham med. Rev.* **viii,** No. 2, 1.

DIAMOND L.K. (1947) Erythroblastosis foetalis or haemolytic disease of the newborn. *Proc. Roy. Soc. Med.* **40,** 546.

DUNN P.N. Personal communication.

FINN R., CLARKE C.A., DONOHOE W.T.A., MCCONNELL R.B., SHEPPARD P.M., LEHANE D. & KULKE W. (1961) Experimental studies on the prevention of Rh haemolytic disease. *Brit. med. J.* 27th May, 1486.

FREDA V.J., GORMAN J.G. & POLLACK, W. (1965) Prevention of rhesus haemolytic disease. *Lancet* **ii,** 690.

FUDENBERG H.H., BARRY ISABEL & DAMESHEK W. (1958) The erythrocyte-coating substance in autoimmune hemolytic disease. Its nature and significance. *Blood* **13,** 201.

GARRETT J.V., GILES H.McC., COOMBS R.R.A. & GURNER B.W. (1960) Neonatal purpura with platelet iso-antibody in maternal serum. *Lancet* **1,** 521.

GEIGER J. & WIENER A.S. (1958) An Rh_0 positive mother with serum containing potent Rh antibodies, apparently of specificity anti-Rh_0 causing erythroblastosis fetalis. *Proc. VI Cong. Int. Soc. Blood Trans.* p. 36.

GERRARD J. (1952) Kernicterus. *Brain* **75,** 526.

GERRARD J.W. & WATERHOUSE J.A.H. (1953) Incidence of haemolytic disease of the newborn in the offspring of sisters. *Brit. J. prev. soc. Med.* **7,** 24.

GWYNFOR JONES T., GOLDSMITH K.L.G. & ANDERSON I.M. (1961) Maternal and neonatal platelet antibodies in a case of congenital thrombocytopenia. *Lancet* **ii,** 1008.

HALLEY HELEN M. & BATTEY DIANA A. In preparation.

HITZIG W.H. & GITZELMANN R. (1959) Transplacental transfer of leucocyte agglutinins. *Vox Sang. (Basel)* **4,** 445.

JAKOBOWICZ RACHEL & SIMMONS R.T. (1959) Iso-immunization in a mother which demonstrates the 'New' Rh blood antigen G(rh^G) and anti-G(rh^G). *Med. J. Austr.* 12th Sept., 357.

KELSALL G.A., VOS G.H. & KIRK R.L. (1958) The case for the induction of labour in the treatment of haemolytic disease of the newborn. *Brit. med. J.* **2,** 468.

KITCHEN, W.H., KRIEGER VERA L. & SMITH MARGERY A. (1960) The use of human albumin during exchange transfusion. *Med. J. Austr.* **11,** 781.

KLEIHAUER E., BRAUN E., & BETKE J. (1957) Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutausstriches. *Klin. Wschr.* **35,** 637.

LEVINE P., KATZIN E.M. & BURNHAM L. (1941) Isoimmunization in pregnancy, its possible bearing on the etiology of erythroblastosis fetalis. *J. Amer. med. Ass.* **116,** 825.

LEVINE P., STOCK A.H., KUHMICHAEL A.B. & BRONIKOVSKY N. (1951) A new human blood factor of rare incidence in the general population. *Proc. Soc. exp. Biol. (New York)* 402.

LEVINE P. (1958a) The influence of the ABO system on Rh-haemolytic disease. *Hum. Biol.* **30,** 13.

LEVINE P. (1958b) quoted in *Blood Groups in Man,* 3rd ed., p. 135. RACE R.R. & SANGER RUTH. Oxford: Blackwell Scientific Publications.

LILEY A.W. (1961) Liquor amnii analysis in the management of the pregnancy complicated by rhesus sensitization. *Amer. J. Obstet. Gynaec.* **82,** 1359.

LILEY A.W. (1963) Intrauterine transfusion of foetus in haemolytic disease. *Brit. med. J.* **2,** 1107.

LOGHEM J.J. VAN JR. and BAKZ C.J.A. (1953) Haemolytic disease of the newborn due to iso-immunization by the antigens c and C^W *Vox Sang. (Basel)* **3,** 130.

MOLLISON P.L. (1956a) *Blood Transfusion in Clinical Medicine,* 2nd edn., p. 430. Oxford: Blackwell Scientific Publications.

MOLLISON P.L. (1956b) *Ibid.* p. 473.

MOLLISON P.L. (1956c) *Ibid.,* p. 489.

MOLLISON P.L. (1956d) *Ibid.,* p. 495.

MOLLISON P.L. & WALKER W. (1952) Controlled trials of the treatment of haemolytic disease of the newborn. *Lancet* **i,** 429.

MURRAY SHEILAGH. Personal communication.

MURRAY SHEILAGH (1957) The effect of Rh genotypes on severity in haemolytic disease of the newborn. *Brit. J. Haemat.* **3,** 143.

NEVANLINNA H.R. & VAINO T. (1956) The influence of mother-child ABO incompatibility on Rh immunization. *Vox Sang. (Basel)* **1,** 26.

ODELL G.B. (1959) The dissociation of bilirubin from albumin and its clinical implications. *J. Pediat.* **55,** 268.

OPPÉ T.E. & VALAES T. (1959) Obstructive jaundice and haemolytic disease of the newborn. *Lancet* **i,** 536.

POLLEY MARGARET J. & MOLLISON P.L. (1961) The role of complement in the detection blood group antibodies. Special reference to the antiglobulin test. *Transfusion (Philad.)* **1,** 9.

RACE R.R. & SANGER RUTH (1958) *Blood Groups in Man,* 3rd edn., p. 315. Oxford: Blackwell Scientific Publications.

ROOD J.J. VAN, LEEÜWEN A. VAN & EERNISSE J.G. (1959) Leucocyte antibodies in sera of pregnant women. *Vox Sang. (Basel)* **4,** 427.

ROSENFIELD R.E. (1953) AB hemolytic disease of the newborn. *Proc. Amer. Ass. of Blood Banks.*

ROSENFIELD R.E. (1955) AB hemolytic disease of the newborn. Analysis of 1480 cord blood specimens, with special reference to the direct antiglobulin test and to the role of the Group O mother. *Blood* **10,** 17.

ROSENFIELD R.E., HABER GLADYS & GIBBEL NATALIE (1958) A New Rh variant. *Proc. VI Cong. Int. Soc. Blood Trans.* p. 90.

SAUER A.J. & LOGHEM J.J. VAN (1955) Platelet autoantibodies and haemorrhagic disease in the newborn. *Vox Sang. (Basel)* **5,** 45.

STEFFEN C. (1955) Untersuchungen uber die Verwendung der Antihumanglobulin-ablenkungs-methode zum Nachweis von Thrombozytenantikörpern. *Wien Z. inn. Med.* **36,** 246.

STEFFEN C. & SCHINDLER H. (1955) Bericht uber die Verwendung des Antihumanglobulin-ablenkungsversuches für den Nachweis eines Antileukocyten-antikörpers bei Agranulozytosen. *Münch. med. Wschr.* **97,** 469.

STONE B. & MARSH W.L. (1959) Haemolytic disease of the newborn caused by anti-M. *Brit. J. Haemat.* **5,** 344.

STRATTON F. & RENTON P.H. (1954) Haemolytic disease of the newborn caused by a new Rh antibody, anti-C^x. *Brit. med. J.* **1,** 962.

TIPPETT PARTICIA A. & SANGER RUTH (1962) Observations and subdivisions of the Rh. antigen D. *Vox Sang.* **7,** 9.

TOVEY G.H., GILLESPIE, E.M., GUY JOAN, VALAES T., OPPÉ T.E. & LEWIS F.J.W. (1959) Cord blood findings in the ABO haemolytic disease. *Lancet* **i,** 860.

TOVEY G.H., LOCKYER J.W., BLADES A.N. & FLAVEL H.C.G. (1962) Ante-natal prediction of ABO haemolytic disease. *Brit. J. Haemat.* **8,** 251.

TOVEY G.H. & LOCKYER J.W. (1965) Cord blood test for ABO haemolytic disease. *Proc. 10th Congr. int. Soc. Blood Transf. Stockholm* **1964,** p. 875.

TOVEY G.H. & VALAES T. (1959) Prevention of stillbirth in Rh haemolytic disease. *Lancet ii,* 521.

VOS G.H. (1958) The partial absorption test: a new technique for titrating Rh antibodies of pathological significance. *Aust. Ann. Med.* **7,** 340.

Vos G.H. (1966). Antenatal immunization by the Rh factor and ABO blood group incompatibility between mother and child. *Vox Sang.* **11,** 460.

Walker A.H.C. & Jennison R.F. (1962) Antenatal prediction of haemolytic disease of the newborn Comparison of liquor amnii and serological studies. *Brit. med. J.* **2,** 1152.

Walker W. (1958a) The changing pattern of haemolytic disease of the newborn (1948–57). *Vox Sang. (Basel)* **3,** 225.

Walker W. (1958b) The changing pattern of haemolytic disease of the newborn (1948–57) *Vox Sang. (Basel)* **3,** 336.

Walker W. & Murray Sheilagh (1954) The management of haemolytic disease of the newborn. *Brit med. J.* **11,** 126.

Walker W. & Murray sheilagh. Personal communication.

Weiner W. (1961) Reconstitution of frozen red cells. *Lancet* **1,** 1264.

Weiner W. & Battey Diana A. (1962) Rhesus incompatibility enhanced by ABO compatibility. *Vox Sang.* **7,** 228.

Weiner W., Child Rosalie M., Garvie J.M. & Peek W.H. (1958) Foetal cells in the maternal circulation during pregnancy. *Brit. med. J.* **11,** 770.

Weiner W. & Hallum Jean L. (1950) Follow-up of rhesus negative primigravidae. Development of rhesus immunization. *Brit. med. J.* **11,** 868.

Weiner W. and Wingham June (1966) Rhesus-immunized mothers and direct Coombs test negative babies. *Lancet* **ii,** 85.

Wiener A.S. (1946) Pathogenesis of congenital hemolytic disease (erythroblastosis fetalis). 1. Theoretical considerations. *Amer. J. Dis. Child.* **71,** 14.

Wiener A.S. (1948) Diagnosis and treatment of anaemia of the newborn caused by occult placental haemorrhage. *J. Amer. Obstet. Gynaec.* **56,** 717.

Wiener A.S., Geiger J. & Gordon Eve B. (1957) Mosaic nature of the Rh_0 factor of human blood. *Exp. Med. Surg.* **15,** 75.

Wiener A.S. & Wexler I.B. (1948) Erythroblastosis fetalis in negroid infants. *Blood,* **3,** 414.

Woodrow J.C., Clarke C.A., Donohoe, W.T.A., Finn R., McConnell, R.B., Sheppard P.M., Lehane D., Russell Shona H., Kulke W. & Durkin Catherine M. (1965) Prevention of Rh-haemolytic disease: A third report. *Brit. med. J.* **1,** 279.

CHAPTER 29

AUTO-ALLERGIC BLOOD DISEASES

J.V.Dacie and Sheila M.Worlledge

INTRODUCTION

The recognition that certain blood disorders were brought about by an auto-antibody mechanism stems from observations made early in this century.

In 1904, Donath and Landsteiner showed that in paroxysmal cold haemoglobinuria the patient's serum contained an antibody, a haemolysin, which was adsorbed to red cells at a low temperature and which led to lysis by complement subsequently if the cell-serum suspension was warmed to body temperature. Donath and Landsteiner's observations provide a classic example of how laboratory tests can furnish an explanation for clinical phenomena.

A few years later Widal and his associates reported in France the first observations which suggested that acquired haemolytic icterus might have its origin in the development of autohaemagglutinins, and a little later Chauffard and Vincent emphasized the role of haemolysins in the causation of acute haemolytic anaemia associated with haemoglobinuria. The importance of these early observations was not, however, widely appreciated until the late 1930s and it was not until well after the introduction of the antiglobulin test (Coombs test) in 1946 that the role of auto-antibodies in the causation of acquired haemolytic anaemia was generally accepted and the terms 'auto-immune' or 'auto-allergic' (as used in this book) were commonly used.

The idea that 'idiopathic' thrombocytopenic purpura might have a similar pathogenesis and be caused by antiplatelet auto-antibodies is of more recent origin and is even now not much more than 15 years old. Still later have antileucocyte auto-antibodies been thought possibly to be responsible for certain leucopenias of obscure origin.

In addition to acquired haemolytic anaemia, thrombocytopenic purpura and certain granulocytopenias, all of unknown aetiology, certain drug-induced blood dyscrasias are now known to be brought about by an immune-body mechanism. After Ackroyd's pioneer observations on thrombocytopenic purpura following the administration of the hypnotic Sedormid, a similar mechanism has been found to operate in purpuras due to certain other drugs and occasionally in drug-induced haemolytic anaemias. It is possible, too, that drug-induced granulocytopenia can be brought about in this way. The antibodies formed are directed primarily against the drug and only secondarily involve the red cells, platelets and possibly leucocytes, either because the drug or a drug-fragment is firmly combined with the cell surface or because drug-anti-drug complexes are non-specifically adsorbed on to the cells. The haemolytic anaemia that may follow α-methyldopa administration, however, appears to be serologically indistinguishable from the 'idiopathic' auto-allergic disease and the antibodies formed do not appear to be directed against the drug. This type of haemolytic anaemia will, therefore, be discussed with the other auto-allergic anaemias of the warm-antibody type.

In the following pages brief descriptions will be given of the auto-allergic blood disorders. The serological aspects, aetiology and pathogenesis will be particularly emphasized.

AUTO-ALLERGIC SYNDROMES INVOLVING THE RED CELLS

The syndromes to be discussed in relation to the red cells comprise:

1. The auto-allergic haemolytic anaemias.
2. Paroxysmal cold haemoglobinuria.
3. Allergic drug-induced haemolytic anaemia.

The warm-antibody and cold-antibody types of auto-allergic haemolytic anaemia will be discussed separately.

As is well known, both primary or 'idiopathic' and secondary or symptomatic auto-allergic haemolytic anaemias exist, and more and more it is being recognized that cases apparently 'idiopathic' in origin are not infrequently in reality based upon an underlying disease. However, it cannot be doubted that 'idiopathic' cases occur and that they comprise probably the majority of cases met with in this country. Secondary cases most commonly occur in association with malignant disease of the lympho-reticular system, chronic lymphocytic leukaemia and reticulosarcoma in particular, and systemic lupus erythematosus. A particularly interesting haemolytic anaemia occasionally develops in patients being treated with α-methyldopa; the haemolysis usually lessens fairly rapidly after the drug is withdrawn. Possible relationships between the drug and the antibodies are discussed under 'Aetiology and Pathogenesis' p. 825. Other transient haemolytic anaemias may sometimes develop after pneumonia, due to mycoplasma or possibly viruses, and in association with glandular fever; the antibody in such cases is always of the cold type.

The auto-antibodies formed in secondary cases appear to be identical from the serological and immunochemical points of view with those found in 'idiopathic' cases. For this reason they will not be described separately.

Auto-allergic Haemolytic Anaemia (Warm-antibody Type)

Clinical Findings ('Idiopathic' Type)

Auto-allergic haemolytic anaemia affects all ages, from infancy upwards, and both sexes. The disease is of very variable severity, and it may be transient, lasting weeks or months, or be of long duration, persisting for years. Not infrequently the patient may suffer a succession of acute relapses interspersed by periods of months or even years of relatively good health. In some patients the disease starts acutely and then gradually lightens in severity. The outlook is serious and although complete recovery is possible, the mortality is considerable and has even exceeded 40% in selected series (Dacie 1962). Steroid hormones are valuable in controlling the disease and splenectomy, too, is often of benefit. Azathioprine (Imuran), 6-mercaptopurine, 6-thioguanine and nitrogen-mustard have all been tried by various workers and have sometimes

been found useful in reducing the amount of antibody produced. At present, however, there is little information as to their value. Azathioprine seems, at the moment, to be the most useful drug in reducing antibody production and it appears to have the least side-effects. Its use should be considered in any patient for whom prolonged high-dose corticosteroid therapy seems to be necessary and in patients who relapse after splenectomy. Thymectomy has been reported to have been beneficial in at least two infants with severe auto-allergic haemolytic anaemia uncontrolled by corticosteroid therapy and splenectomy (Wilmers & Russell 1963; Karaklis *et al* 1964).

Serology

Direct antiglobulin test

The diagnosis of auto-allergic haemolytic anaemia depends on the demonstration of auto-antibody either attached to the patient's red cells or free in his serum. The former is best done by the application of the direct antiglobulin test, but other methods such as auto-agglutination in serum-albumin or in polyvinylpyrrolidone (PVP) have been used and may occasionally prove to be more sensitive (Evans & Weiser 1957; Jandl & Castle 1956).

It is important not to equate the presence of a positive antiglobulin test with auto-allergic haemolytic anaemia. In the first place, it may not mean that auto-antibody protein is bound to the red cell surface; positive results may be due to adsorbed antigen-antibody complexes, antibodies against drugs bound to the red cell surface and possibly abnormalities of the red cell surface itself. In the second place, a positive antiglobulin test may occur without any past or present evidence of increased red cell destruction, as exemplified by the vast majority of patients with a positive antiglobulin test following α-methyldopa administration (Carstairs *et al* 1966). Nevertheless, a positive antiglobulin test, in a patient presenting signs of haemolytic anaemia, is strong presumptive evidence that the haemolytic anaemia is of the auto-antibody type.

The use of antiglobulin sera reacting with γ-globulins only or with non-γ-globulins only has provided interesting information on the type of proteins coating the red cells. Three patterns of reaction can be found when the red cells of patients suffering from 'warm' auto-allergic haemolytic anaemia are tested, but some γ-globulin is detected in over 80% (Worlledge 1965). Recent work has defined more precisely the type of antibody protein detected on the red cells and Leddy (1966) has published a study of the direct antiglobulin test using rabbit antisera against the heavy chains of γG (γ), γA (α) and γM (μ) and against human complement (C′) components. He obtained three patterns of reaction with this panel of antisera. Firstly, agglutination only with anti-γ sera; secondly, agglutination only with anti-C′ sera, and thirdly, agglutination with both anti-γ and anti-C′ sera. The anti-α, and anti-μ sera and a fifth serum absorbed with γG, γM and γA proteins and with human complement gave no

agglutination. In a larger series tested with anti-γG, anti-γM and anti-C′ sera, Engelfriet and co-workers (1966) obtained occasional agglutination with anti-γM sera but the majority of the reactions were one of Leddy's three types. These three types of reactions are the same as the three types obtained with anti-γ-globulin and anti-non-γ-globulin sera (Dacie 1962; Worlledge 1965) and these sera can be equated with anti-γG and anti-C′ respectively.

In Leddy's (1966) series 50% of the antiglobulin tests were of the 'C′ only' variety, but he has included all patients with a positive direct antiglobulin test both with and without evidence of haemolytic anaemia. Binding of complement without detectable immunoglobulins on the red cell surface and in the absence of cold agglutinins in the serum is an interesting phenomenon which is not uncommon. It can be the result of fixation of complement by an antigen-antibody complex which is then removed during washing or it may mean that the antiglobulin test, as ordinarily performed, is not a very sensitive method of detecting γM proteins. γA antibodies do not appear to bind complement and the lack of reaction with anti-α and the fact that γA-globulins have not yet been incriminated as auto-antibodies suggests that these immunoglobulins do not play a major role in auto-allergic haemolytic anaemia.

There is no direct correlation between the intensity of an antiglobulin reaction and the clinical evidence of haemolysis, but in an individual patient clinical improvement is usually reflected in a diminution in the strength of the direct antiglobulin test or in the amount of antibody that can be transferred to normal red cells *in vitro*.

Free antibody in serum

Auto-antibody can often but not invariably be demonstrated free in a patient's serum. In general, the worse a patient is affected, the greater are the chances of demonstrating free antibody. It depends, too, on how the antibody is sought. If enzyme-treated red cells are used, the percentage of positives will be found to be higher than if the indirect antiglobulin technique using normal cells is alone employed, e.g. 62% positive using enzyme-treated cells compared to 38% positive by the antiglobulin technique in our latest series of patients.

A positive indirect antiglobulin test can be found to be due to γG auto-antibodies, γG auto-antibodies and adsorbed complement components or adsorbed components only; the exact type parallels the direct test. The type of direct antiglobulin test also gives a clue to the reaction that may be expected when auto-antibody is tested for with enzyme-treated cells: thus, where the direct test is positive only with anti-γG sera, the free antibody agglutinates but does not usually lyse enzyme-treated cells and is likely to be a γG protein; where the direct test is positive only with anti-C′ sera, the free antibody agglutinates and lyses such cells and is likely to be a γM protein (Engelfriet *et al* 1966); where the rest is positive with both sera, the free anti-

body may lyse and/or agglutinate enzyme-treated cells and may be either a γG or γM protein.

Unusual antibodies

In most cases the auto-antibodies are of the incomplete non-agglutinating type. Occasionally, however, in-saline-acting auto-agglutinins are developed, in which case the patient is usually severely ill. Very rarely, autohaemolysins are formed, active at 37°C, and their presence, too, is usually associated with severe haemolysis, often accompanied by haemoglobinuria.

Specificity of the auto-antibodies

Studies of red cell specificity should be done with antibody eluted from the patient's cells, rather than with serum, in which the amount of free auto-antibody may be small and in which iso-antibodies, stimulated by previous transfusions, may be present. Satisfactory eluates, however, can only be made when there is γG antibody on the patient's cells. Several successful methods of antibody elution have been described (Jensen 1959). It is now well known that many of the antibodies show Rh specificity, the incidence depending on the genotype of the cells used for the tests: with cells of the common Rh genotypes Rh specificity can be demonstrated in about 30% and the most frequent antibody is anti-e; if –D–/–D– cells are added to the test panel about 45% can be shown to be Rh specific, and if Rh_{null} (– – –) cells are used, too, and extensive absorption studies carried out, the incidence rises to over 70% (Weiner & Vos 1962), suggesting that some of the antibodies are reacting with a 'basal' Rh antigen common to almost all human cells, as was suggested by Wiener, Gordon & Gallop (1953). The antigen against which a 'specific' antibody is directed is usually present on the patient's cells but occasionally this is not so: for example, anti-E has been eluted from E-negative cells (Hubinont *et al* 1959). This suggests that cross-reacting antibodies are sometimes formed. Auto-antibodies that lyse enzyme-treated cells seem to react indiscriminately with all cell samples. A summary of the results of antibody tests in these patients is given in Table 29.1 (p. 810).

Serum complement

Complement levels may be reduced in auto-allergic haemolytic anaemia. There seem to be two mechanisms producing this. When the rare warm haemolysins are present, complement may be utilized faster than it is regenerated; in such cases the absence of complement may well protect the patient from the worst effects of his disease. Sometimes, however, complement levels are low at a time when haemolysis is not intense; such cases are usually secondary auto-allergic haemolytic anaemias and the complement may be utilized

by antigen-antibody reactions other than those involving the red cells, as for instance in systemic lupus erythematosus.

TABLE 29.1

Characteristics of the auto-antibodies in auto-allergic haemolytic anaemia of the warm-antibody type

	Type of protein detected by the direct antiglobulin test		
	γG	γG and complement components	Only complement components
Incidence of this type of reaction*	50%	30%	20%
Specificity of eluted antibody	Usually rhesus	Only γG antibody eluted, occasionally rhesus, usually 'non-specific'	No antibody eluted
Immunoglobulin class of free auto-antibody	γG ?always	γG, sometimes γM also	γM†; γG not demonstrated
Type (complete or incomplete) of free auto-antibody	Incomplete, very rarely complete	Incomplete	Incomplete
Reaction of free auto-antibody with enzyme-treated cells	Agglutination	Sometimes agglutination, sometimes haemolysis and agglutination	Always haemolysis (and agglutination)
Specificity of auto-antibody in serum	Usually rhesus	Occasionally rhesus, usually 'non-specific'	Always 'non-specific'

* Worlledge (1965). † Engelfriet *et al* (1966).

α-METHYLDOPA HAEMOLYTIC ANAEMIA

A significant proportion of patients taking α-methyldopa (Aldomet) give a positive direct antiglobulin test. A positive test appears to take about 6–12 months to develop and the incidence is dose-dependent, being 11% in patients taking 1 g or less daily, 20% in patients taking 1–2 g and 36% in patients taking over 2 g (Carstairs *et al* 1966). The patient's cells are agglutinated only by anti-γG sera and free γG auto-antibody is often present in the plasma. Both this and the antibody eluted off the cells will react with normal red cells and this reaction cannot be enhanced or inhibited by addition of the drug. These antibodies frequently show some Rh specificity and the commonest single

antibody is anti-e. The vast majority of these patients have no evidence of increased red cell destruction.

Nevertheless, patients taking α-methyldopa do occasionally develop auto-allergic haemolytic anaemia. The incidence has been calculated as 0.02% of the patients at risk, and the occurrence is not obviously dose-dependent; most affected patients were receiving under 1 g of the drug per day (Worlledge *et al* 1966). The anaemia takes some months to develop and the earliest reliably

TABLE 29.2

Serological findings on thirteen patients with α-methyldopa-induced haemolytic anaemia, tested at time of diagnosis

Patient's number	Direct anti-globulin reaction*	Specificity of antibody in the eluate	Specificity of antibody in the serum	Patient's probable Rh genotype
1	4½	Anti-c	Anti-e	rr
3	4	Anti-e	Anti-e	R_1R_1
4	5	Not tested	'Non-specific'	R_2R_2
7	4	'Non-specific'	Weak-D-†	R_1R_2
8	4	Weak-D-†	Weak-D-†	R_1r
9	4½	Not tested	Weak-D-†	R_1r
10	4½	Anti-e	Anti-e	R_1r
20	5	'Non-specific'	Weak-D-†	R_1R_2
21	4½	Anti-e	Anti-e	R_1r
29	4½	Weak-D-†	Weak-D-†	R_1R_2
30	4	Anti-e	Anti-e	R_2r
31	4½	Anti-e	Anti-e	R_1r
32	4½	Anti-c	Anti-c	R_2r

* The direct antiglobulin reaction was graded in increasing strength from 0 to 5.

† – D – cells were agglutinated weakly; cells of all other Rh genotypes were agglutinated strongly and equally.

documented case occurred after 5 months' treatment. The clinical and serological findings are the same as those in 'idiopathic' auto-allergic haemolytic anaemia of the warm-antibody type and the diagnosis can only be made from the patient's history. These patients have antibodies which are qualitatively similar to those in the serum of a proportion of non-anaemic patients on this drug (see above); quantitatively, however, more antibody appears to be present. The serological findings on patients whose blood was examined at the time of diagnosis are shown in Table 29.2. The most satisfactory treatment of the anaemia is to stop the drug; the haemoglobin should then rapidly return to normal. In this respect the short course of the illness in these patients differs

from that of most patients who have the 'idiopathic' disease whose illness is usually long-continued. When withdrawal of the α-methyldopa is inadvisable or when the patient is very seriously ill, steroids will usually control the haemolysis but this will recur if the steroids are discontinued and treatment with α-methyldopa is continued. The direct antiglobulin test takes 7–18 months to become negative after cessation of therapy.

Auto-allergic Haemolytic Anaemia of the Cold-antibody Type

Clinical Features

Both 'idiopathic' and secondary cases occur in which the auto-antibodies are of the 'cold' variety. The patients present as a rule with two clinical features in addition to anaemia and jaundice; namely, Raynaud's phenomena and haemoglobinuria. The syndrome, which is conveniently referred to as the cold-haemagglutinin syndrome, differs in many ways from auto-allergic haemolytic anaemia of the warm-antibody type.

The 'idiopathic' type of the syndrome is a disorder of elderly people, both men and women; it is long-continued and does not appear ever to lessen in intensity. It may, however, not affect the patient very seriously.

Steroid treatment and splenectomy are relatively ineffective but treatment with chlorambucil may be useful when the disease is crippling. Leucopenia and thrombocytopenia must be guarded against and small doses of the drug (2 mg/day) are advisable, at least at the onset of treatment.

Secondary cases occur most commonly in association with reticulosarcoma and other malignant lymphomas and after atypical pneumonias. Occasionally, too, a patient, for long regarded as suffering from the 'idiopathic' type of disease, may present signs of a lymphoma (Schubothe, Baumgartner & Yoshimura 1961).

The type of disease that may follow pneumonia due to *Mycoplasma pneumoniae* (and possibly some viruses too), is characteristically of sudden onset and short duration. Except for minor and often subtle difference in behaviour *in vitro*, the auto-antibodies are essentially similar in both the 'idiopathic' and secondary cases. Exceptionally, an acute transient haemolytic anaemia may follow glandular fever; in this case the antibody often has a different specificity (see below).

Serology

Direct antiglobulin test

The antiglobulin test is typically positive even if strict precautions are taken to avoid cooling the patient's blood below 37°C after withdrawal, and despite washing the red cells in repeated changes of warm saline.

Tests with specific antisera show that positive results are due to complement

components on the red cell surface and not to adsorbed antibody. The antibody, which is a γM-globulin, is usually only active *in vitro* up to 30–32°C. The temperature in blood vessels in the exposed skin may well be within this range, and since this type of antibody is potentially lytic, it will bind complement

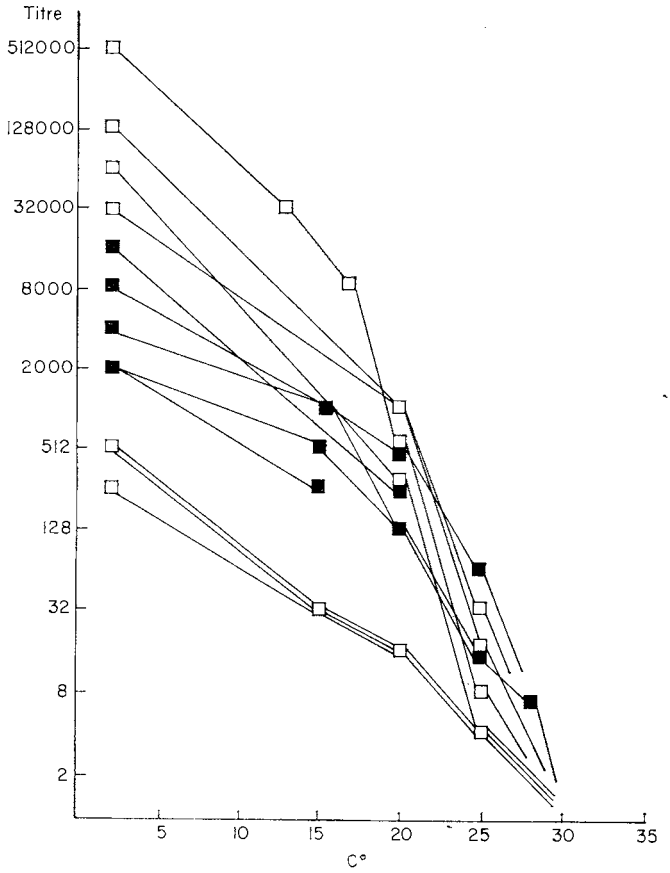


FIG. 29.1. Relationship between temperature and cold-agglutinin titre.
□ Cases of 'idiopathic' auto-allergic haemolytic anaemia of the cold-antibody type.
■ Cases of 'auto'-allergic haemolytic anaemia following a typical pneumonia.

components to the red cell surface as well as causing agglutination. After rewarming to 37°C, any agglutination will disappear and the antibody will elute off the cells, but the complement components remain and these cause the red cells to agglutinate with anti-C′ sera if they do not cause actual lysis.

Free antibody in serum

Cold auto-antibody is characteristically present in very large amounts in the patient's serum, so much so that it quite often forms a definite abnormal peak if the serum is submitted to electrophoresis.

Titrations of the cold antibody with normal red cells may give titres of or exceeding 64,000, and if the cells are enzyme-treated the titres may be higher still. The effect of temperature on the agglutination of normal red cells is shown in Fig. 29.1.

The antibodies are potentially lytic, but they are usually only weakly so for normal human red cells and then as a rule only if the pH of the cell-serum mixture is reduced to within the range pH 6.5–7.5. Enzyme-treated cells are,

TABLE 29.3

Characteristics of the auto-antibodies in auto-allergic haemolytic anaemia of the cold-antibody type

Type of protein detected by the direct antiglobulin test	Only complement components
Incidence of this type of reaction	100%
Specificity of eluted antibody	No antibody eluted
Immunoglobulin class of free auto-antibody	γM
Type (complete or incomplete) of free auto-antibody	Complete
Reaction of free auto-antibody with enzyme-treated cells	Agglutination is usually markedly enhanced, rarely inhibited. Usually some haemolysis
Specificity of auto-antibody in the serum	Usually anti-I; occasionally anti-i; rarely 'non-specific'

however, lysed to higher serum dilutions and the lysin titre seems to parallel the degree of anaemia more closely than the agglutinin titre. The characteristics of the auto-antibody are summarized in Table 29.3.

Specificity of cold antibodies

High-titre cold antibodies agglutinate human red cells irrespective of the known blood groups. However, the cells of newborn infants and certain rarely-met-with samples of adult blood have been found to be only very weakly agglutinable (Wiener *et al* 1956; Jenkins, Marsh *et al* 1960). As a consequence of these findings normal adult cells have been termed 'I' and the relatively inagglutinable cells 'i'. It is now known that I-positivity is a characteristic

which is normally developed after birth, full positivity being achieved in about 2 years (Marsh 1961). Sera causing strong agglutination of normal adult red cells and only weak agglutination of cord-blood cells and adult 'i' cells are termed anti-I. Sera causing the opposite pattern of agglutination are termed anti-i.

Most sera containing high-titre cold agglutinins react as anti-I sera, but occasionally some are seen in which the antibody is markedly inhibited by enzyme treatment of the cells; these seem to agglutinate all red cell samples indiscriminately and equally.

The transient haemolytic anaemia which occasionally follows glandular fever is often caused by antibodies with i-specificity (Jenkins *et al* 1965). The cold agglutinins which are common after *Mycoplasma pneumoniae* infections seem to have I-specificity and the auto-allergic haemolytic anaemia, which sometimes follows such infections, is caused by antibodies of similar specificity.

It is interesting to note that if the agglutinability of a patient's own red cells by a high-titre antibody is compared with that of normal adult red cells the patient's cells generally appear to be less agglutinable. In part, at least, this could be due to selection, with the most sensitive cells (i.e. those which are agglutinated at the highest temperature *in vivo*) being eliminated first. The presence of adsorbed complement, if it does not cause lysis, may paradoxically inhibit agglutination to some extent (Evans *et al* 1965). In practice, the relative insensitivity of the patient's cells can be life saving, as in the case of a patient whose serum agglutinated normal cells at 37°C, but her own cells only at temperatures less than 32°C.

Serum complement

Complement concentrations are often reduced in the serum of patients forming high-titre cold agglutinins. This is especially true of those in whom intravascular haemolysis is a major feature. In such patients, serum-complement activity may not even be demonstrable and this probably helps to protect the patient from the worst effects of his illness.

Paroxysmal Cold Haemoglobinuria

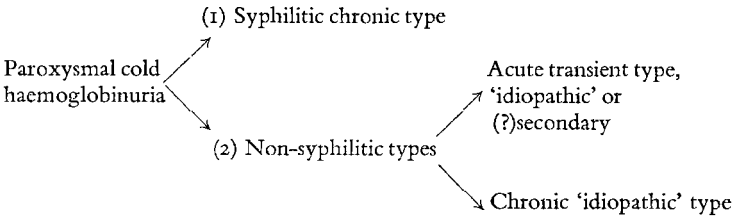
Classically associated with congenital syphilis, paroxysmal cold haemoglobinuria is now a rare disease. Antibodies of the Donath-Landsteiner type are, however, formed under several different circumstances and it is now widely recognized that paroxysmal cold haemoglobinuria is a syndrome and that 'idiopathic' and secondary cases occur (Table 29.4).

The duration and severity of the disorder vary widely: at one end of the scale a patient may suffer from but a single attack of haemoglobinuria; at the other, he or she may experience repeated attacks whenever the weather is cold and this may persist for many years. Because it has been thought that the presence

of the Donath-Landsteiner antibody can be the only stigma of congenital syphilis, it is not always easy to determine with certainty whether the disorder is related to syphilis. Positive reactions for syphilis, too, are not uncommon in the acute phase of the disease (where a non-syphilitic origin is probable). Such reactions are usually transient and should be checked by specific reactions for syphilis such as the Treponema immobilization test.

TABLE 29.4

A classification of the paroxysmal cold haemoglobinurias



(From *The Haemolytic Anaemias: Congenital and Acquired.* Vol. 2: *The Autoimmune Haemolytic Anaemias*, 2nd edn., 1962, by J. V. DACIE. London: J. & A. Churchill.)

SEROLOGY

The Donath-Landsteiner (D-L) *antibody*

The most remarkable characteristic of the D-L antibody is its ability to lyse normal human red cells, even when present at only modest concentrations. In this respect it is much more active at comparable titres than are the high-titre cold antibodies of the cold-haemagglutinin syndrome.

The D-L antibody is often referred to as a biphasic cold antibody because chilling at 0°C followed by warming to 30–37°C is necessary for the demonstration of lysis. This cold-warm procedure is the basis of the Donath-Landsteiner test. In fact, lysis will sometimes occur 'monophasically' if the suspension of red cells in serum is left at room temperature (15–25°C). The determining factor is the thermal range of the antibody which, however, in syphilitic cases at least seldom exceeds 15°C.

Donath-Landsteiner antibodies will generally be found to agglutinate red cells at approximately the same dilutions at which they cause lysis and to sensitize red cells to agglutination by antiglobulin serum at slightly higher dilutions. This antiglobulin reaction can always be shown to have a γG-globulin component if the test is done after washing the cells in cold saline at 4°C. The antibody is a γG antibody but rapidly elutes off the red cells as the temperature

TABLE 29.5

Comparison of the reactions *in vitro* of high-titre cold antibodies (anti-I) and the Donath–Landsteiner antibody

	High-titre cold antibody	Donath–Landsteiner antibody
Titre	High (→512,000)	Moderate (→64)
Thermal range	High (→30–32°C)	Moderate (→18–20°C)
Effect of pH	Usually lysis only in acidified serum (pH 6.5–7.5)	Lysis always in unacidified serum, optimum pH 7.5–8.5
Specificity	Anti-I	Anti-P
Physical-chemical nature	γM	γG

TABLE 29.6

Relationship of the Donath–Landsteiner antibody to iso-antibodies of the P blood group system

	P_1 cells	P_2 cells	pp cells	P^k cells
Anti-P_1 (made by P_2 persons)	+	−	−	+ (usually)
Anti-P (made by P^k persons)	+	+	− (or w)	−
Anti-P + P_1 + P^k (= anti-Tj^a, made by pp persons)	+	+	−	+
Donath–Landsteiner antibody	+	+	−	−

+ = reaction. − = no reaction.

(From WORLLEDGE S.M. (1965) Serological aspects of autoimmune haemolytic anaemia. In *Autoimmunity, a Symposium of the 5th Congress of the International Academy of Pathology*, p. 52, ed. BALDWIN R.W. & HUMPHREY J.H. Oxford: Blackwell Scientific Publications.)

is raised. The *in vitro* reactions of Donath-Landsteiner antibodies are compared with those of high-titre cold antibodies of the anti-I type in Table 29.5.

Role of pH

As contrasted with the high-titre cold antibodies of the cold-haemagglutinin syndrome, lysis by the D-L antibody always seems to take place in unacidified serum and is almost maximal at pH 8.0.

Specificity of the D-L *antibody*

In 1963 Levine and his colleagues showed that the Donath-Landsteiner antibody has a specificity within the P blood group system. Further studies with pp (Tj^a-negative) and P^k cells have shown that the haemolysin has the specificity of anti-P (Worlledge & Rousso 1965). The relationship of the Donath-Landsteiner antibody to iso-antibodies in the P blood group system is shown in Table 29.6. The P groups of six patients suffering from paroxysmal cold haemoglobinuria were found to be normal; four were P_1 and two were P_2 and all six cell samples were agglutinated by anti-P and anti-Tj^a (anti-$P+P_1+P^k$). Thus the Donath-Landsteiner antibody is an auto-antibody which shows well-defined blood group specificity.

Relationship between the D-L *antibody and the Wassermann or Kahn reacting material in sera from syphilitics*

All workers seem to agree that the D-L antibody is quite distinct from the material in the serum of syphilitics which gives rise to positive Wassermann and Kahn reactions.

Direct antiglobulin reactions

Patients' red cells withdrawn at the time of or soon after an attack of haemoglobinuria give positive antiglobulin reactions of the 'C′ only' type. The test, however, becomes negative between attacks. In this respect paroxysmal cold haemoglobinuria contrasts with the cold-haemagglutinin syndrome in which the direct antiglobulin test is continuously positive. This is a reflection of the lower thermal range of the D-L antibody which usually requires a major degree of chilling for its adsorption.

Allergic Drug-induced Haemolytic Anaemia

Confirmation that drug-induced haemolytic anaemia may have an allergic origin stems from the work of Harris (1956) in America, with the drug Fuadin (stibophen), an antimony-containing compound. Harris's observations were made on a patient suffering from schistosomiasis who was retreated with

Fuadin. Within a few days of the start of the second course of treatment the patient experienced acute haemolytic anaemia associated with haemoglobinuria. Harris was able to show

1. That the direct antiglobulin test was positive at the height of the patient's illness, and

2. That the patient's serum contained an antibody which would unite with normal red cells only in the presence of the drug Fuadin or a closely related compound.

Harris pointed out the similarity between his observations and those made by Ackroyd in thrombocytopenic purpura due to the hypnotic Sedormid.

Since Harris's publications, several other reports of haemolytic anaemia produced by the same mechanism have appeared. It is, however, clear that this type of drug-induced haemolytic anaemia is rarely met with; however, cases mediated by quinidine, quinine, phenacetin, PAS (sodium salt of *para*-aminosalicylic acid), antistin, salicyl-azosulphapyridine, insecticide preparations and penicillin have been reported. In each case the patient probably forms an antibody against the drug. This antibody then reacts *in vivo* with the drug and involves the red cell by one of three methods. Firstly, as Ackroyd suggested, the drug may be loosely bound to the red cell; secondly, as is the case with penicillin (de Weck 1964) the drug or a drug-fragment can become chemically combined with the red cell; and thirdly, as Schulman (1963) has suggested, the drug-anti-drug complex may be adsorbed by the red cells in a non-specific manner. Although the patient's own red cells are destroyed by the antibody, the reaction cannot be considered as truly auto-allergic as the antibody is not primarily directed against the red cells, and their involvement is only incidental to the reaction of the drug with the drug antibody. This type of drug reaction is discussed further in Chapter 26.

AUTO-ALLERGIC SYNDROMES INVOLVING PLATELETS

Many analogies can be drawn between platelet deficiency states and the anaemias and it is highly probable that some cases at least of thrombocytopenic purpura have an auto-allergic origin analogous to that of auto-allergic haemolytic anaemia. As with the haemolytic anaemias, thrombocytopenia can be 'idiopathic' or secondary. The discussion here, however, will be confined to 'idiopathic' cases (idiopathic thrombocytopenic purpura or ITP) and the drug-induced thrombocytopenias.

'IDIOPATHIC' THROMBOCYTOPENIC PURPURA (ITP)

The possible relationship between antibodies directed against platelets and

thrombocytopenic purpura was first demonstrated experimentally by Bedson who, administering an antiplatelet serum to guinea-pigs, produced thrombocytopenic purpura and also obtained evidence of damage to capillary endothelium. Much later, Evans and his associates (1951) pointed out that thrombocytopenia was a common accompaniment of auto-allergic haemolytic anaemia and suggested that the thrombocytopenia, too, could be due to an auto-agglutinin. They also pointed out that transitory thrombocytopenia was not infrequently found in infants born of mothers suffering from ITP, which suggested that a platelet-destroying factor might have passed through the placenta. Other evidence of the presence of a platelet-destroying mechanism, which might have been an antibody, was the observation of the unusually short survival of platelets after transfusion into patients suffering from ITP. In the same year, Harrington, Minnich & Moore (1951) showed that the transfusion into normal recipients of blood or plasma from patients with ITP was followed by thrombocytopenia in 60% of the cases studied. They also showed that the factor responsible for this thrombocytopenia was probably a serum globulin and relatively stable on storage. Recently, Shulman, Marder & Weinrach (1964) have extended these observations by showing that this factor is present in the 7S γ-globulin fraction, that it is adsorbed by normal human platelets, that it is species-specific and that it affects autologous as well as homologous platelets. All this evidence suggests that the ITP factor is an antibody but the direct demonstration of this by conventional immunological methods has proved surprisingly difficult.

Clinical Features

ITP can be divided into two clinical groups. Firstly, a relatively transitory acute form which occurs mainly in children of either sex and which is usually associated with a previous infectious illness. In this disease there is an abrupt onset of purpura and a very low platelet count but the prognosis is usually good and the platelet count will have returned to normal levels within 3 months in 90% of the patients. Secondly, there is the chronic form of ITP which occurs mainly in adults, particularly in women, and is not usually associated with previous infections. This disease, like auto-allergic haemolytic anaemia, is of very variable severity but most patients usually present with platelet counts below 40,000 per cu.mm and these low levels persist until treatment is given. Initial treatment is usually with corticosteroids and in Baldini's (1966) experience few patients fail to respond if the dose is high enough.

Splenectomy is usually reserved for those patients who after 6 months' treatment still need corticosteroids in high doses. Recently, azathioprine, 6-thioguanine and 6-mercaptopurine have been used but it is still too early to assess their value. The clinical and laboratory aspects of both acute and chronic ITP are discussed in detail by Gardner (1965) and Baldini (1966) in two recent reviews.

Serology

It has at once to be admitted that even the most experienced workers have failed to demonstrate antibodies in anything like all the patients they have studied, and in most people's hands tests for the antibodies have given results which can only be described as equivocal, unrepeatable and unpredictable. It is easy to set out reasons for this: the difficulty is to decide whether a failure to demonstrate the presence of a platelet auto-antibody unequivocally is because

Table 29.7

Techniques for the demonstration of platelet auto-antibodies in ITP

1. *On the patient's own platelets*	
Direct antiglobulin tests	Difficult to carry out; interpretation of positive results uncertain
Direct antiglobulin-consumption tests	Sensitive, but difficult to carry out through lack of patient's platelets. Interpretation of positive results uncertain
Direct florescein-conjugated antiglobulin tests	Not very sensitive; interpretation of positive results uncertain
2. *In the patient's serum*	
Indirect antiglobulin tests	Difficult to carry out; confusion possible with iso-antibodies
Indirect antiglobulin-consumption tests	Sensitive. Often positive; confusion with iso-antibodies likely
Agglutinin tests	Techniques unreliable and confusion likely with iso-antibodies
Mixed cell agglutinin tests	Results usually negative
Complement fixation tests	Results usually negative
Tests for platelet lysis	Value disputed
Tests for inhibition of clot retraction	Results usually negative
Tests for inhibition of uptake of ^{14}C-labelled serotonin	Results usually negative

the antibody is not there or because the techniques used are clumsy and inadequate or perhaps basically unsound and misdirected.

Platelet auto-antibodies have been looked for in two main ways:

1. By carrying out direct tests on the patient's own platelets, and
2. By attempting to demonstrate free auto-antibody in the patients' serum. The main methods that have been used and the results that have been obtained by their use, are listed in Table 29.7.

Antiglobulin tests

The difficulties here seem to be insuperable. For one thing very few platelets are obtainable in severe cases of ITP and large amounts of blood are required in order to get enough platelets to work with. It is also impossible to wash the platelets free from plasma without bringing about some clumping, and it is indeed impossible to free their surfaces from protein in this way. For these reasons direct antiglobulin tests have been generally abandoned and the antiglobulin consumption test has been substituted.

Antiglobulin-consumption test

The principle of this test (see Chapter 1) is to add washed platelets to antiglobulin serum and to back-titrate the serum using anti-D-sensitized red cells as test objects. The test has to be very carefully controlled using normal platelets which are handled in exactly the same way as the patient's platelets. It has the virtue that it does not matter whether the platelets adhere to each other in the washing process or not. A 'substantial' (at least 2-tube) difference in the titre of the antiglobulin serum after absorption with the test and control platelets, respectively, is required before the test can be considered positive. Unfortunately, antiglobulin-consumption techniques have the major disadvantage that large volumes of blood are required (in a thrombocytopenic patient) in order to obtain enough platelets and that even if the result is positive it may be difficult to decide whether this is due to auto-antibody protein adsorbed to platelets or to (non-antibody) protein adhering 'non-specifically' to damaged platelets.

Daussett, Colombani & Colombani (1961) showed that 50% of patients suffering from ITP gave positive results when their platelets were used in the direct antiglobulin-consumption test. Clinically, however, there was no distinction between the patients who gave positive results and those who gave negative results. Negative results may perhaps be explained by technical difficulties or a wrong indicator system, and an indicator system capable of detecting the fixation of certain complement components might be useful. On the other hand, negative results may mean that the disorder is caused by a non-immunological mechanism in some patients.

Tests for platelet auto-antibodies in serum

A major difficulty in carrying out tests for platelet auto-antibodies has been the source of the antigen. Because of the patient's thrombocytopenia most workers have been forced to use samples of normal platelets. This has led to confusion because positive agglutination tests under such circumstances could indicate merely the presence of platelet iso-antibodies provoked by past transfusion, treatment which nearly all patients with ITP receive at one time or another.

The variety of techniques available emphasizes the difficulty that has been experienced in finding a method which gives reproducible results, and the

problem is by no means solved. A careful study using the mixed antiglobulin technique failed to yield a single positive result in ITP (Coombs *et al* 1956). Similar negative results using other techniques have been obtained by Bridges and co-workers (1963), Shulman, Marder & Weinrach (1965) and other workers.

The failure of these techniques to demonstrate an auto-antibody does not necessarily mean that the platelets are not destroyed by immunological mechanisms: many of the techniques have been shown to be relatively insensitive with iso- and hetero-antibodies, and high-avidity auto-antibody could be fixed to the platelets and not present in the serum. It is possible, too, that in some patients the damage is caused by auto-antibodies acting perhaps on the megakaryocytes in the bone marrow or on 'deep' platelet antigens not readily demonstrable by conventional tests. At present such attractive concepts are difficult to confirm or refute.

Platelet-survival studies

The first studies of platelet life-span in patients suffering from ITP were done by Hirsh and Gardner in 1952. They transfused polycythaemic and normal blood, collected into silicone-coated syringes, and followed the subsequent rise of the platelet count in the peripheral blood. They found this was much less well sustained in ITP than in thrombocytopenia due to aplasia and concluded that the thrombocytopenia of ITP was due to rapid destruction of the platelets. Since then these results have been confirmed and extended by many workers using methods of labelling the platelets with radioactive isotopes. From this work it seems that the normal survival time of platelets is about 10 days and that in all patients suffering from clinically active ITP the life-span of both autologous and isologous platelets is shortened. One interesting observation that has come out of the work of Najean and his colleagues (1963) is that the relationship between the platelet life-span and the level of circulating platelets is much closer in ITP than is the relationship between red cell life-span and the degree of anaemia in auto-allergic haemolytic anaemia. They calculated that in ITP the peripheral platelet count depended almost exclusively on the length of platelet survival and that there was little or no compensatory increase in platelet production. The abnormalities which have been described in the megakaryocytes in the marrow of patients suffering from ITP indicate that the platelet-destroying factor may also act on platelet production. The increased numbers of megakaryocytes which are often present presumably represent an ineffective attempt at compensation.

Allergic Drug-induced Thrombocytopenic Purpura

The thrombocytopenic purpura resulting from hypersensitivity to the hypnotic drug Sedormid and other drugs is discussed in Chapter 26. As with drug-induced haemolytic anaemia it is not strictly speaking an auto-allergic reaction; the platelet is involved for similar reasons to those given for the allergic

drug-induced anaemias (see p. 818). Unlike ITP, laboratory tests frequently demonstrate antibodies when the drug and the patient's serum are added to normal platelets.

AUTO-ALLERGIC SYNDROMES INVOLVING LEUCOCYTES

Although iso-antibodies against leucocytes and leucocyte iso-antigens have been extensively studied in recent years very little is known about auto-antibodies against leucocytes. Their significance in human pathology is in fact even less well established than that of platelet auto-antibodies. Two syndromes in which leucocyte auto-antibodies may be important can, however, be mentioned. They are 'idiopathic' granulocytopenia and drug-induced agranulocytosis, although in this latter condition the antibodies cannot be classed as true auto-antibodies. For details of the literature up to 1959 the reader is referred to the monographs of Walford (1960) and Killman (1960).

Demonstration of leucocyte auto-antibodies

Because leucocytes adhere to each other if attempts are made to wash them free from surrounding plasma or serum, it is not possible to carry out direct antiglobulin tests on the leucocytes in a case of leucopenia, although a direct mixed antiglobulin test may be performed (see Chapter 1). Agglutination methods using serum and leucocyte suspensions are more successful and these have been the basis for the extensive work that has been carried out on leucocyte iso-antigens and iso-agglutinins. As with platelets, however, it is seldom that auto-agglutination tests are practicable in patients with marked degrees of leucopenia.

Some samples of leucocyte antibody act as incomplete antibodies and for their demonstration antiglobulin-consumption techniques have been devised (van Loghem *et al* 1958). As with platelets, these techniques are exacting and demand relatively large volumes of leucocytes. They can be used as a direct method using the patient's own leucocytes or indirectly using the patient's or normal leucocytes and the patient's serum. In either case if the patient is seriously leucopenic 100–200 ml of his blood may be required to obtain sufficient leucocytes.

Reports of leucocytic auto-antibodies in cases of leucopenia

Both Walford and Killman deal critically with the many reports of the presence of leuco-agglutinins in the sera of patients suffering from blood diseases including those associated with leucopenia. They conclude that the vast majority of such antibodies are, in fact, iso-leuco-agglutinins which have developed in consequence of blood transfusion or pregnancy or even have formed 'spontaneously'. There is, however, little reason to doubt that leucocyte auto-antibodies are

produced from time to time and that they may be responsible for some cases of leucopenia. In most instances the auto-antibodies have been demonstrated by the antiglobulin-consumption method.

A study of the direct antiglobulin-consumption test using the patient's own leucocytes was reported by Dausset, Colombani & Colombani (1961) and Dausset (1965). They found that the leucocytes from about 40% of patients with 'primary leukothrombocytopenia' gave positive results. They showed that a γ-globulin could be eluted from the patient's own leucocytes and refixed on to normal leucocytes. This 'auto-antibody' also appeared to possess species-specificity and to react with leucocyte cytoplasm rather than nuclei and more particularly with the ribosomal sub-fraction of the cytoplasm. Like in ITP, however, there was no clinical difference between those patients who gave positive tests and those patients who gave negative tests.

Leucopenia and Agranulocytosis due to Allergy to Drugs

Moeschlin & Wagner (1952) were the first to demonstrate *in vitro* the presence of a leucocyte-agglutinating mechanism in the plasma of a patient sensitive to amidopyrine. Their observations have been amply confirmed by subsequent workers, chiefly in connection with amidopyrine itself, but other drugs such as sulphapyridine, gold, methylthiouracil and salicylazosulphapyridine may act similarly. The exact nature of the agglutinating mechanism is, however, still uncertain. Several hypotheses are reviewed by both Walford and Killmann.

There are some curious features associated with leucocyte agglutination due to drugs. One is that the leuco-agglutinins develop (in a sensitive subject) within a few hours of the ingestion of the noxious drug; secondly, that they apparently often disappear within a matter of hours or days. It appears that the drug must be present in the plasma for leucocyte destruction *in vivo* or leucocyte agglutination *in vitro* to take place. Possibly, the leucocyte agglutinating mechanism is based on the adherence of leucocytes to drug (antigen)-antibody complexes, which circulate for only a short time after ingestion of the drug, and that the original antigen is not the drug adsorbed to leucocytes but a drug-plasma protein complex. Although the attachment of antigen (drug) and antibody to leucocytes provides an attractive hypothetical mechanism there seems to be no incontrovertible evidence that this occurs; whatever the mechanism, it is unlikely to be a true auto-allergic reaction (see Chapter 26).

AETIOLOGY AND PATHOGENESIS

How and why an apparently healthy person should suddenly revolt against his own red cells, leucocytes or platelets, still remains a mystery. In recent years,

however, exciting new hypotheses have been advanced, many discussions have been held and some relevant experimental studies carried out.

There are two main hypotheses. First, it is thought that the red cells, leucocytes or platelets of the patient undergo some change rendering them antigenic. This, it is suggested, could be the result of hidden antigens becoming exposed or unmasked by the action of body or bacterial enzymes or from adsorption of virus or bacterial products. The antibodies then produced by the normal immunological mechanism cross-react with the antigens of normal circulating cells. There is, however, no support for this hypothesis from experimental studies in animals and nothing to suggest that the transfusion of circulating cells from patients suffering from auto-allergic blood disorders would cause the production of auto-antibodies in normal people.

The second hypothesis locates the primary fault in the antibody-forming tissues of the subject. Burnet (1959), in particular, has powerfully supported the idea that the auto-allergic blood disorders, particularly the haemolytic anaemias, could result from a failure of normal homeostatic mechanisms and the emergence in the body of 'forbidden clones' of antibody-forming cells which do not recognize the normal antigens of the blood cells as 'self' and in consequence form antibodies against them. The emergence of the clone(s), it is suggested, could be due to 'spontaneous' somatic mutation, or as the result of the action of virus or other infective agent, or it could be the consequence of malignant disease affecting cells of the lymphoreticular system.

The not infrequent development of auto-allergic haemolytic anaemia in the course of chronic lymphocytic leukaemia or reticulosarcoma is consistent with the somatic mutation hypothesis as is also the occurrence of the cold-antibody type of auto-allergic haemolytic anaemia predominantly in elderly subjects (Dacie 1962). However, the fact that the warm-antibody type of haemolytic anaemia and thrombocytopenic purpura affect subjects of all ages, even small children, is less easily explained on this basis, and the possibility of infection acting in some way as a trigger mechanism cannot be dismissed.

The auto-antibody production that may follow α-methyldopa administration might be explained in a similar way. The antibody appears to be against red cell antigens and not against the drug, so if the drug is altering normal red cell antigens it must be doing so in some subtle way perhaps by affecting the structure and configuration of the antigens as they are being formed in the red cell precursors. This hypothesis certainly might explain the length of time that treatment has to be given before the anaemia develops—usually 6 months or more—as such subtly altered cells would not necessarily be expected to have a decreased life-span and could not be expected to be antigenic until they had left the circulation, but it does not explain the rapid rise in haemoglobin level that occurs almost immediately after stopping the drug. This rapid restoration of the haemoglobin level, which seems to be the consequence of a definite decrease in the amount of

auto-antibody present, is also against the hypothesis that these antibodies are produced by an abnormal mutated clone of immunologically competent cells. Such a clone being insensitive (in theory) to normal homeostatic mechanisms would be expected to be self-perpetuating and no longer require the stimulus of the drug, unless it is assumed that the drug is repressing a mechanism that would normally eliminate such a clone. This is an attractive hypothesis, and as mentioned above auto-antibody formation does seem to diminish as soon as the patient is taken off the drug. The fact that the direct anti-globulin test may continue to be positive for many months does not necessarily indicate continuing antibody production; the studies of Costea, Constantoulakis & Schwartz (1963) showed that passively transferred iso-antibody, by passing from red cell to red cell, can persist in the peripheral blood for over 6 months. As already mentioned, the relatively rapid cessation of the haemolysis once the patient is taken off the offending drug differs from the usually long persisting course in cases of similar serology but of unknown origin. It is tempting to think that the auto-antibody formation persists in the latter group because of the continued presence of the abnormal event or events which initiated the auto-immunization.

Experimentally, haemolytic anaemia has been shown to be an important component of runt disease and homologous disease and it is quite clear, therefore, that living grafted antibody-forming cells can react against the host's blood cells. The development of spontaneous auto-allergic haemolytic anaemia in the NZB/BL inbred strain of mice, and the incidence of auto-allergic disorders in hybrids produced between this strain and other inbred strains of mice, show the importance of genetic factors (Howie & Helyer 1965). In man, a peculiar proneness to develop antibodies has often been postulated as at least a part explanation for the development of auto-antibodies. This may well be important, and it is in fact likely that several factors play a part in the genesis of an auto-allergic blood disease in any particular patient.

Pathogenesis

There is little doubt but that red-cell destruction in the auto-allergic haemolytic anaemias is due to damage to the cells resulting from the adsorption of antibodies. Less is known of the effects of antibodies on leucocytes and platelets but in all probability their life-spans are curtailed in analogous ways. In the case of the red cells at least, removal from the circulation is probably brought about by the antibodies affecting the surface properties of the cells and more indirectly perhaps by affecting injuriously the cells' metabolism. It should be added, however, that no abnormalities of metabolism could be detected by Jandl (1965). Both types of change, as well as stasis in the spleen, lead to spherocytosis. The incomplete type of auto-antibody causes removal largely by the spleen; agglutinating and complement-fixing antibodies lead to removal widely in the reticulo-endothelial system, the liver being particularly important by reason of

its size. An important factor in relation to the filtering action of the spleen and liver is the intensity to which the antibody-affected cells undergo auto-agglutination (Jandl, Simmons & Castle 1961). Antibodies which are demonstrably lytic *in vitro* may cause, if present in sufficient strength, actual lysis in the blood stream, i.e. intravascular haemolysis. Erythrophagocytosis is particularly important as a means of disposing of cells coated with complement-fixing antibodies.

REFERENCES

ACKROYD J.F. (1949) The cause of thrombocytopenia in Sedormid purpura. *Clin. Sci.* **8,** 269.

ACKROYD J.F. (1955) Platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura, with a note on platelet groups. *Brit. Med. Bull.* **11,** 28.

BALDINI M. (1966) Idiopathic thrombocytopenic purpura. *New Engl. J. Med.* **274,** 1245.

BRIDGES J.M., BALDINI M., FICHERA C. & DAMESHEK W. (1963) Effect of anti-platelet antibodies on *in vitro* uptake of 5-hydroxytryptamine by blood platelets. *Nature (Lond.)* **197,** 364.

BURNET M. (1959) Auto-immune disease. II. Pathology of the immune response. *Brit. med. J.* **ii,** 720.

CARSTAIRS K.C., BRECKENRIDGE A., DOLLERY C.T. & WORLLEDGE S.M. (1966) Incidence of a positive direct Coombs test in patients on α-methyldopa. *Lancet* **ii,** 133.

COOMBS R.R.A., MARKS JOHN & BEDFORD D. (1956) Specific mixed agglutination: mixed erythrocyte platelet anti-globulin reaction for the detection of platelet antibodies. *Brit. J. Haemat.* **2,** 84.

COSTEA N., CONSTANTOULAKIS M. & SCHWARTZ R. (1963) The *in vivo* behaviour of erythrocyte antibodies in man. *Proc. 9th Congr. int. Soc. Haemat., Mexico* **2,** 503. New York and London: Grune & Stratton.

DACIE J.V. (1962) *The Haemolytic Anaemias: Congenital and Acquired,* 2nd edn., Part 2—*The Auto-immune Haemolytic Anaemias.* London: J. & A. Churchill.

DAUSSET J. (1965) Auto-antileukocyte ribosomal fraction in leukoneutropenics. *Ann. N.Y. Acad. Sci.* **124,** 550.

DAUSSET J., COLOMBANI J. & COLOMBANI M. (1961) Study of leukopenias and thrombocytopenias by the direct antiglobulin consumption test on leukocytes and/or platelets. *Blood,* **18,** 672.

ENGELFRIET C.P., MOES M. & VAN LOGHEM J.J. (1966) Auto-immune haemolytic anaemia. Abstract of Paper to *11th Congr. int. Soc. Blood Trans., Sydney,* p. 125.

EVANS R.S., TAKAHASHI K., DUANE R.T., PAYNE R. & LIU C.-K. (1951) Primary thrombocytopenic purpura and acquired hemolytic anaemia: evidence for a common etiology. *Arch. intern. Med.* **87,** 48.

EVANS R.S., TURNER E. & BINGHAM M. (1965) Studies with radio-iodinated cold agglutinin of ten patients. *Amer. J. Med.* **38,** 378.

EVANS R.S. & WEISER R.S. (1957) The serology of auto-immune hemolytic disease: observations on forty-one patients. *Arch. intern. Med.* **100,** 371.

GARDNER F.H. (1965) Idiopathic thrombocytopenic purpura. In *Immunological Diseases,* p. 861, ed. SAMTER M. & ALEXANDER H.C. London: J. & A. Churchill.

HARRINGTON W.J., MINNICH V. & MOORE C.V. (1951) Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J. Lab. clin. Med.* **38,** 1.

HARRIS J.W. (1956) Studies on the mechanism of a drug-induced hemolytic anemia. *J. Lab. clin. Med.* **47,** 760.

HUBINONT P.O., MASSART-GUIOT T., BRICOULT A. & GHYSDAEL P. (1959) Immunological specificity of eluates from 'Coombs positive' erythrocytes. *Vox Sang. (Basel)*, **4,** 419.

JANDL J.H. (1965) Mechanisms of antibody-induced red cell destruction. *Series Haemat.* **9,** 35.

JANDL J.H. & CASTLE W.B. (1956) Agglutination of sensitized red cells by large anisometric molecules. *J. Lab. clin. Med.* **47,** 669.

JANDL J.H., SIMMONS R.L. & CASTLE W.B. (1961) Red cell filtration and the pathogenesis of certain hemolytic anemias. *Blood* **18,** 133.

JENKINS W.J., KOSTER H.G., MARSH W.L. & CARTER R.L. (1965) Infectious mononucleosis: an unsuspected source of anti-i. *Brit. J. Haemat.* **11,** 480.

JENKINS W.J., MARSH W.L., NOADES J., TIPPETT P., SANGER R. & RACE R.R. (1960) The I antigen and antibody. *Vox Sang. (Basel)* **5,** 97.

JENSEN K.G. (1959) Elution of incomplete antibodies from red cells. A comparison of different methods. *Vox Sang. (Basel)* **4,** 230.

KARAKLIS A., VALAES T., PANTELAKIS S.N. & DOXIADIS S.A. (1964) Thymectomy in an infant with autoimmune haemolytic anaemia. *Lancet* **ii,** 778.

KILLMAN S.-A. (1960) *Leukocyte Agglutinins: Properties, Occurrence and Significance.* Oxford: Blackwell Scientific Publications.

LEDDY J.P. (1966) Immunological aspects of red cell injury in man. In *Seminars in Haematology* **3,** 48.

LEVINE M.D., CALANO M.J. & FALOWSKI F. (1963) The specificity of the antibody on paroxysmal cold haemoglobinuria (P.C.H.) *Transfusion* **3,** 27.

LOGHEM J.J. VAN JR., HART M. VAN DER, HIJMANS W. & SCHMIT H.R.E. (1958) The incidence and significance of complete and incomplete white cell antibodies with special reference to the use of the Coombs consumption test. *Vox Sang. (Basel)* **3,** 203.

MARSH W.L. (1961) Anti-i: a cold antibody defining the Ii relationship in human red cells. *Brit. J. Haemat.* **7,** 200.

MOESCHLIN S. & WAGNER K. (1952) Agranulocytosis due to the occurrence of leucocyte-agglutinins (pyramidon and cold agglutinins). *Acta haemat. (Basel)* **8,** 29.

NAJEAN Y., ARDAILLOU N., CAEN J., LARRIEU M.J. & BERNARD J. (1963) Survival of radiochromium-labelled platelets in thrombocytopenia. *Blood* **22,** 718.

SCHUBOTHE H., BAUMGARTNER W. & YOSHIMURA H. (1961) Makroglobulinvermehrung und lymphoide Zellproliferation bei der chronischer Kälteagglutininkrankheit. *Schweiz. med. Wschr.* **91,** 1154.

SHULMAN N.R. (1963) Mechanisms of blood cell destruction in individuals sensitized to foreign antigens. *Trans. Ass. Amer. Phycns* **76,** 72.

SHULMAN N.R., MARDER V.J. & WEINRACH R.S. (1964) Comparison of immunologic and idiopathic thrombocytopenia. *Trans. Ass. Amer. Phycns* **77,** 65.

SHULMAN N.R., MARDER V.J. & WEINRACH R.S. (1965) Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia or idiopathic purpura. Physiologic, serologic and isotopic studies. *Ann. N.Y. Acad. Sci.* **124,** 499.

WALFORD R.L. (1960) *Leukocyte Antigens and Antibodies.* New York and London: Grune & Stratton.

DE WECK A.L. (1964) Penicillin allergy: its detection by an improved haemagglutination technique. *Nature (Lond.)* **202,** 975.

WEINER W. & VOS G.H. (1963) Serology of acquired hemolytic anemias. *Blood* **22,** 606.

WIENER A.S., GORDON E.B. & GALLOP C. (1953) Studies on the autoantibodies in human sera. *J. Immunol.* **71,** 58.

WIENER A.S., UNGER L.J., COHEN L. & FELDMAN J. (1956) Type-specific cold auto-antibodies as a cause of acquired hemolytic anemia and hemolytic transfusions reactions: biologic test with bovine red cells. *Ann. intern. Med.* **44,** 221.

WILMERS M.J. & RUSSELL P.A. (1963) Autoimmune haemolytic anaemia in an infant treated by thymectomy. *Lancet* **ii,** 915.

WORLLEDGE S.M. (1965) Serological aspects of autoimmune haemolytic anaemia. In *Autoimmunity, a Symposium of the 5th Congress of the International Academy of Pathology*, p. 52, ed. BALDWIN R.W. & HUMPHREY J.H. Oxford: Blackwell Scientific Publications.

WORLLEDGE S.M., CARSTAIRS K.C. & DACIE J.V. (1966) Autoimmune haemolytic anaemia associated with α-methyldopa therapy. *Lancet* **ii,** 135.

WORLLEDGE S.M. & ROUSSO C. (1965) Studies of the serology of paroxysmal cold haemoglobinuria (P.C.H.) with special reference to its relationship with the P blood group system. *Vox Sang.* (*Basel*) **10,** 293.

CHAPTER 30

RHEUMATIC FEVER

L.E.GLYNN

INTRODUCTION

Rheumatic fever, a relatively uncommon disease in the economically most advanced countries, is still a major problem in many other parts of the world. Apart from infancy and early childhood no age is exempt; but the vast majority of cases first appear in late childhood and adolescence, and the incidence is significantly higher in females than in males.

The disease is characterized by a polyarthritis associated with fever; hence the name. Large joints tend to be affected more often than small joints and although the affected ones may be obviously inflamed and extremely painful, the transience of the inflammation in any one joint and its tendency to flit from one joint to another are features of diagnostic significance.

Accompanying the arthritis there may be some degree of cardiac involvement revealing itself by tachycardia, changing heart sounds and cardiac enlargement. In severe cases inflammation involves the pericardium and the accompanying pericardial effusion can occasionally further embarrass the heart's action. The frequency with which the heart is involved cannot be assessed with accuracy by clinical methods alone, but in all patients dying during an acute attack the typical

changes of rheumatic fever are seen in the heart at autopsy. These are a fibrinous pericarditis and a highly characteristic form of endocarditis in which the somewhat thickened valves are studded along the closure line by translucent vegetations 2–5 mm in diameter. The mitral valve is almost invariably involved and the aortic valve somewhat less frequently, but the valves on the right side of the heart are rarely affected, at least macroscopically, in a first attack. Histologically the so-called Aschoff bodies are generally regarded as diagnostic. They are seen most typically in the perivascular connective tissue of the myocardium but are also present in the endocardium and in the valves where they usually underlie the accumulations of platelets which constitute the vegetations.

The nature of the Aschoff body is still a subject of much controversy and its interpretation is undoubtedly influenced by preconceived notions as to its pathogenesis. In what are regarded as its earliest stages there is an accumulation of large cells, mononuclear as well as multi-nuclear, with deeply basophilic cytoplasm, which are apparently infiltrating a focus of eosinophilic material. Controversy is mainly concerned with the origin of this eosinophilic material which most observers interpret as fibrinoid degeneration of collagen. Murphy, however (1960), has presented considerable evidence that the eosinophilic material as well as the multinucleated cells of the Aschoff body are derived from necrotic muscle fibres.

Whether or not the Aschoff body is primarily a focus of necrotic muscle there can be no doubt that rheumatic fever is a systemic disease involving many tissues. In addition to the heart and joints, skin involvement is not rare. The commonest rash is the highly characteristic erythema annulare, a transient but frequently recurrent rash, which derives its name from the annular areas which result from central fading associated with peripheral spreading of the individual lesions. The subcutaneous tissues may also be involved but, unlike the rash, the subcutaneous lesions in the form of nodules can rarely be detected before the 3rd week of a first attack.

Undoubtedly the most important feature of rheumatic fever is the tendency to produce permanent cardiac injury. A very small proportion of cases die of congestive failure in the first attack; the majority recover without permanent cardiac damage; but in a significant minority cardiac damage is not only permanent but also progressive, culminating in fully developed mitral stenosis often associated with combined stenosis and incompetence of the aortic valve. The great majority of these progressive cases are associated with recurrent attacks of rheumatic fever, each attack leaving the heart more impaired. In some patients, however, progressive cardiac damage occurs without obvious clinical relapse and indeed some 30% of all patients with mitral stenosis fail to give any history of an acute episode diagnosable as rheumatic fever.

Some light upon the problem of continued rheumatic activity has been thrown by the study of cardiac tissue removed during surgical treatment of mitral

valves. Candidates for this type of treatment are invariably screened to exclude those with overt clinical activity. Nevertheless, a high proportion of these patients show typical Aschoff bodies in the excised tissue, which can only be interpreted as evidence of continuing activity despite the absence of clinical signs. It must therefore be concluded that the clinical methods available are too insensitive.

Of the other tissues of the body the most commonly affected are the blood vessels, the lungs and the central nervous system. Vascular lesions ranging from small foci of intimal hyperplasia to extensive medial necrosis have been described, especially in the small vessels of the heart (Gross, Kugel & Epstein 1935). Lung lesions, usually a widespread consolidation by a fibrin-rich exudate, are most probably the result of salicylate therapy in patients with incipient congestive failure. The substitution of corticosteroids for salicylates in the treatment of this type of patient has largely eliminated this pulmonary complication (Bywaters & Thomas, 1961).

The involvement of the nervous system as indicated by the clinical manifestations of chorea is rarely seen in patients with overt rheumatic fever and its rheumatic nature was long overlooked. The associated cardiac injury, indistinguishable from that of classical rheumatic fever, and many other features of the disease have, however, clearly revealed it as part of the rheumatic process.

THE RELATIONSHIP OF RHEUMATIC FEVER TO INFECTION BY β-HAEMOLYTIC STREPTOCOCCI

The aetiological relationship between β-haemolytic streptococci and rheumatic fever rests upon three independent lines of inquiry, epidemiological, serological and prophylactic. One of the first recognized features of the disease was its tendency to appear some 1–3 weeks after a febrile sore throat; and with the development of clinical bacteriology it was soon established that β-haemolytic streptococci of group A could be isolated with high frequency from the throat during this prodromal phase.

The Epidemiology of Rheumatic Fever and Streptococcal Infection

A rough parallel between the seasonal incidence of streptococcal infection and rheumatic fever has been noticed by many observers, but the remarkable figure published by Coburn & Young (1949) based on the incidence of these diseases amongst U.S. Navy personnel over a 4 year period provides some of the strongest evidence for their aetiological relationship. Similar observations by Rammelkamp, Denny & Wannamaker (1952) not only afford the strongest support to the aetiological role of the streptococcus but suggest that many of the factors predisposing to rheumatic fever, e.g. poverty, overcrowding, latitude

and season, operate largely through their influence on the incidence of streptococcal infection itself.

β-haemolytic streptococci of group A have been classified into over fifty types by the serological reaction of their M protein, and several studies have sought to detect whether any types are especially associated with rheumatic fever. Despite a few exceptions, the general consensus is that no specifically rheumatic types exist and that virtually any type can on occasion lead to development of the disease.

The incidence of rheumatic fever following a streptococcal infection of the upper respiratory tract varies from about 3% in streptococcal epidemics in relatively closed communities to as low as 0.3% in endemic sporadic cases. This striking tenfold difference has been largely explained by the studies of Stollerman (1964) who has emphasized the important differences between the two forms of streptococcal pharyngitis, especially regarding clinical severity, cultural characteristics and typability of the infecting organisms, and immunological response of the host. In any two series, if the incidence of rheumatic fever is compared in patients comparable in so far as these other variables are concerned, the results will be remarkably similar.

The Immunological Response to Streptococcal Infection

In addition to the group-specific polysaccharide and the type-specific M protein, β-haemolytic streptococci synthesize a large number of soluble antigens which can be detected in their culture media. In an elaborate study of this subject making use of sera from patients with rheumatic fever to detect the antigens by immunoelectrophoresis Halbert and his colleagues (see Halbert 1964) have demonstrated twenty or more distinct antistreptococcal precipitin systems. Isolation and concentration of many of the antigens involved enabled some of them to be identified with known streptococcal products such as Dick toxin, streptolysin O, desoxyribonuclease B, proteinase precursor, diphosphopyridine nucleotidase, streptokinase and C-carbohydrate-protein complex.

The ability of the human subject to produce specific antibodies to various biologically active products of β-haemolytic streptococci has been known for many years, and the detection of these antibodies, especially in rising titre is, short of isolating the organism itself, the most reliable means of detecting recent streptococcal infection. It can, moreover, be argued that such a rising titre is a better indication of actual infection than the presence of the organisms whose presence could be transient and superficial and therefore unlikely to lead to any post-infectious complications such as rheumatic fever.

Of the several biologically characterized products of streptococci capable of inducing a specific immune response in man the antistreptolysin O is by far the best studied. From the extensive literature on this subject it is evident that an

antistreptolysin titre of under 200 Todd units is not evidence of a recent infection. Following such an infection titres frequently exceed 500 or even 1000 units. It is equally well established that a diagnostic titre is not an invariable consequence of streptococcal infection and that probably no more than 80% of individuals produce such a titre even after a quite unequivocal infection. As a means of establishing that a streptococcal infection preceded a given attack of rheumatic fever it is therefore evident that the A.S.O. titre cannot have more than an 80% reliability. Other antibodies against streptococcal products have therefore been studied but none has proved more reliable than the A.S.O. These include antihyaluronidase, antidesoxyribonuclease and antistreptokinase, all of which have the added disadvantage of greater technical difficulty than the A.S.O. estimation. It has, however, been quite clearly shown that the immune response to these streptococcal antigens can vary considerably in a single individual and consequently attempts to demonstrate a recent streptococcal infection by serological methods should make use of more than one of these immune responses. Thus although only 80% of cases of acute rheumatic fever show an A.S.O. titre indicative of recent streptococcal infection, 95% provide evidence of such an infection if the antistreptokinase titre is also measured, and if four tests are employed the figure rises to virtually 100% (McCarty 1952).

Prophylaxis

The natural sequel to the establishment of rheumatic fever as a complication of streptococcal infection was to attempt to prevent the former by prevention of the latter. The successful outcome of the many prophylactic regimes designed for this purpose provides further convincing evidence of the streptococcal nature of rheumatic fever. Prophylaxis can be aimed at the primary attack of rheumatic fever by the rapid termination of streptococcal infection by immediate short-term chemotherapy (Denny, *et al* 1950). It can also be aimed at the rheumatic recurrence by preventing casual streptococcal infection by means of long-term chemoprophylaxis (Kohn, Milzer & MacLean 1950). The results of the many studies of this kind have not only confirmed the streptococcus as the initiating aetiological agent but have thrown some light on the mechanism of the process (see below).

THE MECHANISM OF STREPTOCOCCAL ACTION

Direct Involvement

Claims to have isolated streptococci from the joints, cardiac valves or other lesions of rheumatic fever have been made from time to time; but the more stringently aseptic the technique the less frequent the organisms grown. In what is probably the most careful study of this kind no organisms were found

in the valves of eleven patients with acute rheumatic carditis although two cases were positive when precautions against contamination were less stringent (Watson, Hirst & Lancefield 1961).

The time relationships are also against direct participation of the infecting organisms in the rheumatic lesions. Typically an interval of several days or even weeks separates the throat infection from the first clinical manifestations of rheumatic fever. If direct bacterial participation is necessary it is difficult to understand the necessity for this so-called silent interlude. The alternative suggestion that streptococcal exotoxins are the effective pathogenic agents comparable to the role of diphtheria toxin in diphtheria myocarditis is also subject to the same criticism.

Finally the advent of really potent antibiotics has shown that even the most drastic antibiotic regime is without significant effect on the course of an acute attack unless begun within the first few days of the initiating throat infection. It is extremely doubtful, however, if any prevention of subsequent rheumatic fever is to be expected if antibiotic treatment is delayed beyond the 5th day, and it is probably essential to continue for at least 10 days to ensure complete eradication of the infecting organisms. The information now available of the results of antibiotic treatment of streptococcal infections on the subsequent development of rheumatic fever strongly supplements the other evidence already presented of the aetiological role of β-haemolytic streptococci in the development of rheumatic fever. The time relationships also indicate that the critical phase is in the first few days of infection and although the eradication of the organisms is desirable at all stages, the process leading to rheumatic fever is irreversibly launched in the first 5 days.

Indirect Action

We have already seen that there is no convincing evidence that rheumatic fever results from the direct action of the micro-organisms on the affected tissues. On the contrary, all the facts point against such direct participation. There is similarly no evidence for direct toxic injury attributable to secretory products of the organisms, despite the large number of biologically active materials they produce both *in vitro* and *in vivo*. The key to the problem lies in the so-called silent interlude, between the upper respiratory infection and the clinical onset of the disease, and it is the rising titre of antibodies against various streptococcal products occurring during this period which draws attention to the possible importance of immunological processes in the pathogenesis.

The innumerable studies of the various antibodies to streptococcal antigens in patients with streptococcal infections, with and without subsequent rheumatic fever, have revealed in a remarkably consistent fashion that, as a group, the patients with rheumatic fever show stronger and longer lasting immune responses. There is, however, no sharp demarcation between the two groups

despite the statistically highly significant difference between them (Rantz, Randall & Rantz 1948; Rantz, Maroney & DiCaprio 1952). This increased response to streptococcal antigens is not part of a general immunological hyper-reactivity. An investigation, for example, of the antibody response to pneumococcal polysaccharide Type I and of delayed hypersensitivity to tuberculin after BCG vaccination showed no significant difference between controls and patients with rheumatic fever (Quinn, Seastone & Dickie 1952). This suggests that there is some special factor responsible for the characteristically raised response to streptococci. The best known factor leading to an increased immunological response is of course previous exposure to the antigen and this may well account for the results in rheumatic fever. Resistance to infection by β-haemolytic streptococci appears to be entirely a function of the immune response to the type-specific M protein. Although it is therefore unlikely for reinfection to occur with a streptococcus of the same type, reinfections with one of the fifty or more other types is commonplace. Since many of the streptococcal antigens, apart from the M protein, are common to most of the types it is evident that the sort of response to these antigens seen in rheumatic fever (i.e. higher and more persistent titres) could well be the result of such repeated infection (Rantz, Maroney & DiCaprio 1950).

If the response to streptococcal antigens released into the tissues is an essential part of the pathogenesis of rheumatic fever, there are two possible sequences by which tissue injury could arise. The first is the formation of complexes of antigen and antibody and the ensuing lesions would be essentially those of serum sickness. The second is the development of auto- or cross-reacting antibodies which would result in lesions essentially confined to the tissues in which the auto- or cross-reacting antigens are to be found.

The work of Rich & Gregory (1943) in which cardiac lesions somewhat resembling those of acute rheumatic fever were produced in rabbits by massive injections of foreign serum proteins does at least show that cardiac lesions approximating those of the human disease can result from a non-specific antigen-antibody reaction provided the antigen is given in sufficiently massive dosage. In view of the sheer weight of antigen used in these experiments it is difficult to see how a similar situation could arise from a natural bacterial infection. From the work of Dixon, Feldman & Vazquez (1961), Ishizaka (1963) and others, however, it is known that the pathogenicity of antigen-antibody complexes is largely associated with the soluble forms dependent upon an excess of antigen, and it is therefore conceivable that similar complexes could arise even when antigen was limited in amount provided the antibody was similarly limited. A mechanism of this kind could, moreover, explain the overlap of hyper-responders between the rheumatic and non-rheumatic groups since the rheumatic lesions would only arise in those patients in which the response, whether high or low, was 'out of step' with the amount of antigen available.

Three major facts still unaccounted for by this hypothesis are the restriction of rheumatic fever to the post-streptococcal state, the highly characteristic Aschoff body and the remarkable tendency of the lesions to select the heart in contrast to the more widespread involvement of the tissues in typical serum sickness. All of these could perhaps be explained if more were known of the influence of the antigen both on the toxicity and the sites of predilection of antigen-antibody complexes. That some influence is exerted by the antigen was shown 20 years ago by Hawn & Janeway (1947) who, in experiments similar to those of Rich and Gregory found that with albumin as the exciting antigen an arteritis was the predominant lesion found, whereas with serum globulin as the antigen the lesions were predominantly glomerular.

A more attractive hypothesis, since it accounts more readily for the characteristic localization of the lesions, is the development of auto-antibodies as a consequence of the initiating streptococcal infection. Here again two possibilities must be considered. The first envisages the genesis of autoantigenic substances as a result of tissue injury, brought about by the infection. The second envisages the existence in some streptococci of antigens that cross-react with antigens in the patients' tissues. There is evidence in support of both possibilities.

The presence of material in mammalian cardiac tissue capable of acting as an autoantigen was first suggested by Brockman, Brill & Frendzell (1937), when they reported a high incidence of complement-fixing antibodies to a saline extract of human heart in the sera of children with rheumatic fever. It was the Caveltis, however, (Cavelti & Cavelti 1945) who first claimed that the significant factor about β-haemolytic streptococci was their ability to react in some way with tissue antigens to render them autoantigenic. Their earlier work related to glomerulonephritis, but later Cavelti (1947) reported that animals immunized with a mixture of killed streptococci and an emulsion of homologous heart developed antibodies capable of reacting with extracts of autologous heart. Since animals injected with the heart extract alone did not produce these antibodies it was concluded that the effective antigen resulted from some interaction between the organisms and the organ extracts. Unfortunately attempts to reproduce this work have failed (Peck & Thomas 1948; Middleton & Seegal 1953). Nevertheless, it is now well established that human heart tissue does contain antigenic material capable of inducing autoimmunization. Humoral antibodies capable of reacting specifically with extracts of human heart have been repeatedly found in patients with the so-called post-commisurotomy syndrome and in a number of patients following a coronary thrombosis with myocardial infarction. It is presumably the traumatic release into the circulation of antigen normally retained within the myocardial fibres that stimulates the immunological tissues.

The presence of antigens in bacteria capable of cross-reacting with some human antigens has been known for many years. The type XIV pneumococcal

polysaccharide and human blood group substance are a typical example. But it was only as recently as 1962 that Kaplan and Meyeserian published their work showing the presence in a Type 5 strain of group A β-haemolytic streptococci of an antigen capable of cross-reacting with human heart tissue. This was revealed both by immunofluorescent staining of fresh frozen sections of myocardium as well as by complement fixation. Confirmation of these results has been obtained by Nakhla (1965), Lyampert, Vvedenskaya & Danilova (1966), and by Zabriskie & Freimer (1966). Lyampert *et al* found that the cross-reacting antigen in the myocardium differed with different types of streptococcus. With antisera to Types 5 and 29, for example, myocardial staining was sarcolemmal and subsarcolemmal as illustrated by Kaplan & Meyeserian (1962), but with Type 1 antisera staining was principally of the intercalated disks. The findings of Zabriskie and Freimer, although confirming the original observations concerning the distribution of staining, differ in some details from those of Kaplan. They find a much wider distribution of the cross-reacting antigen in β-haemolytic streptococci and localize the bacterial antigen in the cell membrane, not the cell wall. Nakhla also found a wide distribution of the antigen in group A streptococci, being present in nine of the ten types examined, but absent in other gram-positive cocci with the exception of a single strain of group G streptococci.

The distribution of the antigen is not confined to the myocardial fibres. All the investigators are agreed that a probably identical antigen is also present in voluntary muscle and in the smooth muscle of small arteries. In contrast to this fairly narrow organ or tissue specificity the antigen is apparently widely distributed amongst mammalian species where, in addition to man, it has been reported in guinea-pig, rabbit and rat.

It is at present impossible to assess the significance of this cross-reacting cardiac antigen in pathogenesis. Nakhla found no significant cardiac lesions in any of his animals, rabbits or guinea-pigs, despite the presence of the appropriate antibody. Nor were lesions found in those animals in which the immunizing streptococci were given with Freund's complete adjuvant intradermally to ensure the development of delayed hypersensitivity. It appears, therefore, that something more than an immune reaction, whether of humoral antibody or of the cell-mediated variety, is needed for the production of cardiac lesions by means of the cross-reacting antigen. Somewhat greater success has been achieved with immunization with cardiac tissue itself. Jaffe & Holtz (1948) reported that cardiac lesions obtained in rabbits by immunization with homologous heart tissue could be considerably enhanced by adding some foreign antigen, e.g. horse serum, to the immunizing injection. These more extensive lesions obviously involved the myocardial fibres as well as the interstitial tissue. Similar results have been reported by other workers (Kaplan & Craig 1963; Davies, Laufer & Gery 1964). In general a greater immune response is obtained with heteroimmunization and

both with homologous and heterologous antigens the incidence of cardiac lesions is increased if complete Freund's adjuvant is used. It must, however, be emphasized that none of the lesions obtained in these ways is in any way comparable to those of the rheumatic heart either histologically or in its natural history.

It is evident from the considerable literature of the subject that mammalian hearts contain one or more organ-specific antigens common to most if not all of the species studied. It is also evident that this antigen can, in appropriate circumstances, stimulate the autologous production of antibodies. What is not evident, however, is the relationship between the antibodies so produced and any cardiac lesions simultaneously observed. The absence of a direct correlation between antibody titre and severity of lesions weighs against any simple cause-and-effect relationship in either direction in the various experimental animals. There is even some suggestion that humoral antibody may exert a protective influence (Davies *et al* 1964). It is also imperative not to extrapolate from results with heteroimmunization to the autoallergic state. *In vitro* the cytotoxic effect of hetero-antibody contrasts sharply with the apparent innocuousness of the corresponding auto-antibody (Gery, Davies & Ehrenfeld 1960). Nevertheless, some recent experiments from this same unit suggest that the complexities of the *in vivo* situation can entirely alter the result. They found that a relatively non-toxic dose of isoproterenol given to rats previously immunized with rat cardiac antigen results in severe and extensive myocardial injury, presumably because the relatively minor damage produced by the drug permits the access of the antibody to the corresponding antigenic determinants (Gazenfeld, 1966).

With possibly one major exception, all the experimental attempts to produce rheumatic carditis in laboratory animals have failed as judged by their inability to reproduce the Aschoff nodule, the accepted hallmark of this disease. The one exception is the work of Murphy & Swift (1949), who showed that repeated injections of living streptococci into the skin of rabbits could lead to the development of a myocarditis in which the interstitial lesions were virtually indistinguishable from some varieties of Aschoff body. The novelty of these experiments undoubtedly lay in the use of a different type of group A streptococcus for each reinfection. This is of course essential for reinfection to occur, since immunity to streptococci is essentially directed against the M antigen, which differs with each type. Animals were examined after two to ten infections over a period of 3–20 months. Nineteen of a total of fifty-four animals receiving two or more injections showed the myocardial lesions. This work is of exceptional interest, not only because of the histological appearance of the lesions but because the repeated injection corresponding with the suspected importance of repeated throat infections in man strongly supports the hypothesis that the immunological response is of fundamental importance. The identification of these lesions with true Aschoff bodies has been adversely criticized by many workers,

notably Wagner (1960) and Ehrlich (1960), because of the obvious involvement of the myocardial fibres, necrotic fragments of which are a conspicuous constituent of the Aschoff body-like lesions. Murphy, however, has reacted to this criticism by an elaborate study of the human lesion from which he has concluded that, far from disproving the 'rheumatic' nature of his experimental lesions, the human lesions are also primarily the result of myocardial injury (Murphy 1960). In view of the frequent occurrence in rheumatic fever of humoral antibodies to human myocardial fibres (see below) it is obvious that Murphy's views cannot be lightly set aside.

ANTIMYOCARDIAL ANTIBODIES IN RHEUMATIC FEVER

The concept that myocardial damage in rheumatic fever is mediated by antibodies against myocardial antigens is supported by two pieces of evidence; the presence of fixed γ-globulin in auricular biopsies of the cardiac tissues of patients dying of acute rheumatic fever, and the presence of humoral antimyocardial antibodies in the serum of patients with this disease.

Kaplan & Dallenbach (1961), using the immunofluorescent technique, found fixed γ-globulin in 18 out of 100 auricular biopsies on patients undergoing surgery for rheumatic disease of the mitral valve. The sites of deposition were mainly sarcolemmal and subsarcolemmal, but deposits were also occasionally found in segments of arteries, veins and arterioles. No similar deposition of fibrin or albumin was found, thus excluding a non-specific deposition of vascular exudate. The later demonstration of the complement components β1C and β1E in the same areas adds further support to the specific immunological nature of the deposits. Evidence of injury was surprisingly slight, as shown by the absence of cellular reaction, but the affected sites could frequently be identified by their altered histological and histochemical reactions, which were generally those of so-called fibrinoid degeneration. In the five fatal cases of rheumatic fever included in the study by Kaplan and Dallenbach the extent of the γ-globulin deposition was indeed striking. The myocardium was massively infiltrated and all four chambers were involved. This discovery of fixed γ-globulin in the myocardium obviously led to the search for corresponding antibodies in the circulation and these were soon found. Kaplan, Meyeserian & Kushner (1961) reported their presence in about three-quarters of a small series of rheumatic fever patients but also in a number of other conditions. The most comprehensive study to date is that of Hess, Fink, Taranta & Ziff (1964). They also used the immunofluorescent method and distinguished two types of staining, a subsarcolemmal sometimes accompanied by intermyofibrillar staining on the one hand, and a diffuse form on the other. Only the former was seen with rheumatic sera. In 171 acute cases positive results were given by 71 (41.5%) but

in inactive cases the incidence in 201 patients was only 16%. Only 4 positives were found in a miscellaneous group of 107 patients and 2 amongst 66 normal individuals. Amongst the acute cases the incidence of positive reactions rose to 63% in the presence of clinical carditis. Here again, as in most other instances where humoral antibodies are found in association with organ lesions, it is not possible to say which is cause and which effect, or even if they are causally related. Hess *et al* (1964), however, point out that the antibodies in rheumatic fever are generally distinguishable from those found with myocardial infarction, the latter producing the diffuse, not the subsarcolemmal type of staining.

Humoral antibodies reactive with extracts of human heart have also been demonstrated in rheumatic fever by other methods. Employing his Coombs-consumption method Steffen (1957) found a high proportion of positive sera from cases of acute rheumatic carditis, but positives were by no means uncommon in other unrelated conditions, and the significance of these observations is therefore doubtful.

The relationship of the anticardiac antibodies found in rheumatic fever to the cross-reacting streptococcal antibodies obtained from immunized rabbits has also been investigated by Kaplan (Kaplan & Svec 1964). Two methods were employed to show the cross-reactive nature of the antibodies in the patients with rheumatic fever. In the first, a precipitin line obtained on immunodiffusion of the human sera against the streptococcal cross-reacting antigen was inhibited by previous absorption of the serum with cardiac extract. In the second the positive immunofluorescent test obtained with the sera and human heart was completely inhibited by previous absorption by streptococci or the extracted streptococcal antigen. There can therefore be little doubt that the antimyocardial antibodies in the sera of patients with rheumatic fever are the result of an immune response to a streptococcal infection. Since similar antibodies were found in seventeen of seventy-two recent cases of uncomplicated streptococcal infection, there can be equally little doubt that for the development of rheumatic carditis something more is required than the presence of these circulating antibodies.

GENETIC FACTORS AND SUSCEPTIBILITY TO RHEUMATIC FEVER

Despite the widespread distribution of streptococci and frequency of streptococcal infections, the incidence of rheumatic fever rarely exceeds 3% even in severe epidemics; but individuals who have once developed a rheumatic attack show a very much increased incidence of further attacks after each subsequent streptococcal infection. It seems, therefore, highly probable that the factors determining whether or not rheumatic fever supervenes reside in the host rather than in the infecting organisms. So much is generally conceded. What is still the

subject of controversy is how much of the host factor is environmental and how much genetic. Several reports of familial aggregation can no doubt be explained either on an environmental or genetic basis. The observation by St Lawrence (1922) that 14.8% of the members of a family exposed to one of its members with rheumatic fever also developed the disease speaks strongly in favour of a genetic predisposition. Even more striking are the observations of May Wilson (1940) on the incidence of rheumatic fever in the children of parents, one or both of whom have a history of the disease. From her results she concludes that hereditary susceptibility is a major factor and is based on a single autosomal recessive gene with 68% penetrance.

The mechanism of any genetic susceptibility is at present completely obscure. A possible clue has been suggested by the studies of Glynn, Glynn & Holborow (1956, 1959). They were impressed by the apparent association between the site of the initial streptococcal infection and the subsequent development of rheumatic fever. Thus all observers are agreed that streptococcal infections other than those of the throat are rarely, if ever, followed by rheumatic fever. This suggests that there is something peculiar about the throat environment which is lacking in other common sites of infection, e.g. the skin (erysipelas) and the uterus (puerperal sepsis). Since many of the constituents of the throat secretions are under genetic control, e.g. the blood group substances, this seemed a possible site of operation for genetic factors influencing the incidence of rheumatic fever. A study of the ABO blood groups in rheumatic fever showed no significant difference in their distribution from appropriately matched controls, but the secretion of the blood group substances in the saliva was significantly deficient. Secretion can be detected by several methods, the simplest being the precipitation of Lewis^a (Le^a) substance by means of a specific rabbit antiserum. By this means the incidence of non-secretors amongst 611 rheumatic children was 27.5% compared with the incidence of 21.2% amongst 890 healthy schoolchildren, a difference significant at the 0.01 level. Similar results were obtained by, amongst others, Clarke, McConnell & Sheppard (1960) in Liverpool, and Buckwalter, Neifeh, Auer & Edwards (1962) in Iowa. Dublin *et al* (1964), however, found similar results only amongst females. Taking all the published figures together it seems well established that rheumatic fever subjects show a small but significant excess of non-secretors above the normal incidence.

One interpretation of these results proposed by Glynn *et al* (1959) was that if rheumatic fever could only occur in individuals homozygous or heterozygous for non-secretion, then the incidence of non-secretors in rheumatic fever would not differ significantly from the observed figure of about 29%. If this hypothesis were correct, then all secretors with rheumatic fever should be heterozygotes. To test this hypothesis it was necessary to be able to distinguish the heterozygote from the homozygote secretor, and this was achieved by determining the ratio of Le^a determinants to H determinants on the salivary blood group macromole-

cules. The application of this test to ninety-four rheumatic fever secretors and ninety-eight normal secretors showed the expected incidence of sixteen heterozygotes amongst the controls but only seven amongst the rheumatics. Whilst these findings make the original hypothesis untenable, is still indicates some definite protection afforded by the homozygous secretor state (Kaklamanis, Holborow & Glynn 1964).

Although it is difficult to see how the non-secretor gene can affect the development of rheumatic fever, one possibility previously suggested by Glynn & Holborow (1952) was the conversion of salivary haptens to complete antigens by their absorption on to haemolytic streptococci. Reference has already been made to the presence of an antigen in several strains of β-haemolytic streptococci which cross-reacts with an antigen in human myocardium. In order to test for the presence in saliva of a hapten cross-reacting with myocardium which could be made antigenic by absorption on to a streptococcus, it was essential to obtain a strain that was itself lacking such a cross-reacting antigen. A Type 24 strain fulfilled this requirement. Rabbits immunized with this strain gave no detectable antibody reactive with human heart. The same strain of organism, however, previously incubated in human saliva, stimulated the production of cross-reacting antibody detectable by immunofluorescence, complement fixation and tanned cell agglutination. All these reactions, moreover, were inhibited by pretreatment of the serum with either saliva, heart extract or a culture of streptococci known to contain the cross-reacting antigen (Nakhla 1965).

An examination of saliva from forty normal subjects for the presence of the cross-reacting hapten by an inhibition test showed it to be present in all. The possibility that its presence would inhibit the production of the corresponding antibody in response to infection with a strain of streptococcus containing the corresponding antigen suggested the further possibility that individuals capable of developing rheumatic fever might be lacking in this salivary constituent. But eight samples of saliva from children with rheumatic fever all showed the presence of the cross-reacting material by an inhibition test. At present, therefore, it is impossible to assess the significance of its presence in saliva or in the positive strains of haemolytic streptococci. It is nevertheless extremely unlikely that its presence is purely coincidental and without significance in the pathogenesis of rheumatic fever or rheumatic heart disease.

REFERENCES

BROCKMAN H., BRILL J. & FRENDZELL J. (1937). Komplementablenkung mit Organextracten von Rheumatikern BBF-Reaktion bei sogennanten akutem Gelenkrheumatismus. *Klin. Wschr.*, **16**, 502.

BUCKWALTER J.A., NEIFEH G.S., AUER J.E. & EDWARDS J.H. (1962) Rheumatic fever and the blood groups. *Brit. med. J.* **2**, 1023.

BYWATERS E.G.L. & THOMAS G.T. (1961) Bed rest, salicylates and steroid in rheumatic fever. *Brit. med. J.* **1**, 1628.

CAVELTI P.A. (1947) Studies on the pathogenesis of rheumatic fever. I. Experimental production of autoantibodies to heart. *Arch. Path.* **44**, 1.

CAVELTI P.A. & CAVELTI E.S. (1945). Studies on the pathogenesis of glomerulonephritis. I. Production of autoantibodies to kidney in experimental animals. *Arch. Path.* **39**, 148.

CLARKE C.A., MCCONNELL R.B. & SHEPPARD P.M. (1960) ABO blood groups and secretor character in rheumatic carditis. *Brit. med. J.* **1**, 21.

COBURN A.F. & YOUNG D.C. (1949) *Epidemiology of Haemolytic Streptococcus.* Baltimore: Williams & Wilkins.

DAVIES A.M., LAUFER A. & GERY I. (1964) Organ specificity of the heart. III. Circulating antibodies and immunopathological lesions in experimental animals. *Arch. path.* **78**, 369.

DENNY F.W., WANNAMAKER L.W., BRINK W.F., RAMMELKAMP C.H. & CUSTER E.A. (1950) Prevention of rheumatic fever. Treatment of the preceding streptococcic infection. *J. Amer. med. Assoc.* **143**, 151.

DIXON F.J., FELDMAN J.D. & VAZQUEZ J.J. (1961) Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis. *J. exp. Med.* **113**, 899.

DUBLIN T.D., BERNANKE A.D., PITT E.L., MASSELL B.F.I., ALLEN F.H. & AMEZCUA F. (1964) Red blood cell groups and ABH secretor system as genetic indicators of rheumatic heart disease. *Brit. med. J.* **2**, 775.

EHRLICH J.C. (1960) Discussion of paper by WAGNER B.M. *Ann. N.Y. Acad. Sci.* **86**, 1006.

GAZENFELD E., ROSENMANN E., DAVIES A.M. & LAUFER A. (1966) Isoproterenol induced myocardial lesions in the immunized and non-immunized rat. *Immunology* **10**, 193.

GERY I., DAVIES A.M. & EHRENFELD E.N. (1960) Heart specific auto-antibodies. *Lancet* **i**, 471.

GLYNN A.A., GLYNN L.E. & HOLBOROW E.J. (1956) The secretor status of rheumatic fever patients. *Lancet* **2**, 759.

GLYNN A.A., GLYNN L.E. & HOLBOROW E.J. (1959) Secretion of blood group substances in rheumatic fever. A genetic requirement for susceptibility. *Brit. med. J.* **2**, 266.

GLYNN L.E. & HOLBOROW E.J. (1952) The production of complete antigens from polysaccharide haptens by streptococci and other organisms. *J. Path. Bact.* **64**, 775.

GROSS L., KUGEL M.A. & EPSTEIN E.Z. (1935) Lesions of the coronary arteries and their branches in rheumatic fever. *Amer. J. Path.* **11**, 253.

HALBERT S.P. (1964) Analysis of human streptococcal infections by immuno-diffusion studies of the antibody response. In *The Streptococcus, Rheumatic Fever and Glomerulonephritis*, p. 83, UHR J.W. Baltimore: Williams & Wilkins.

HAWN C.V. & JANEWAY C.A. (1947) Histological and serological sequences in experimental hypersensitivity. *J. exp. Med.* **85**, 571.

HESS E.V., FINK C.W., TARANTA A. & ZIFF M. (1964) Heart muscle antibodies in rheumatic fever and other diseases. *J. clin. Invest.* **43**, 886.

ISHIZAKA K. (1963) Gamma globulin and molecular mechanisms in hypersensitivity reactions. *Progress in Allergy* **7**, 32.

JAFFE R. & HOLTZ E. (1948) Experimental allergic myocarditis. *Exp. Med. Surg.* **6**, 189.

KAKLAMANIS E., HOLBOROW E.J. & GLYNN L.E. (1964) A method for differentiating homozygous from heterozygous secretors of ABH blood group substances. *Lancet* **i**, 788.

KAPLAN M.H. & CRAIG J.M. (1963) Immunologic studies of heart tissue. VI. Cardiac lesions in rabbits associated with autoantibodies to heart induced by immunization with heterologous heart. *J. Immunol.* **90**, 725.

KAPLAN M.H. & DALLENBACH F.D. (1961) Immunologic studies of heart tissue. III. Occurrence of bound γ-globulin in auricular appendages from rheumatic hearts. Relationship to certain histo-pathological features of rheumatic heart disease. *J. exp. Med.* **113,** 1.

KAPLAN M.H. & MEYESERIAN M. (1962) An immunologic cross-reaction between group A streptococcal cells and human heart tissue. *Lancet* **i,** 706.

KAPLAN M.H., MEYESERIAN M. & KUSHNER I. (1961) Immunologic studies of heart tissue. IV. Serologic reactions with human heart tissue as revealed by immunofluorescent methods; iso-immune, Wassermann and autoimmune reactions. *J. exp. Med.* **113,** 17.

KAPLAN M.H. & SVEC K.H. (1964) Immunologic relation of streptococcal and tissue antigens. III. Presence in human sera of streptococcal antibody cross-reactive with heart tissue. Association with streptoccoccal infection, rheumatic fever and glomerulonephritis. *J. exp. Med.* **119,** 651.

KOHN K.H., MILZER A. & MACLEAN H. (1950) Oral penicillin prophylaxis of recurrences of rheumatic fever. *J. Amer. med. Assoc.* **142,** 20.

LAWRENCE W.ST (1922) The family association of cardiac disease, acute rheumatic fever and chorea. A study of 100 families. *J. Amer. med. Assoc.* **79,** 2051.

LYAMPERT I.M., VVEDENSKAYA O.I. & DANILOVA T.A. (1966) Study on streptococcus group A antigens common with heart tissue elements. *Immunology* **11,** 313.

MCCARTY M. (1952) The immune response in rheumatic fever. In *Rheumatic Fever,* p. 136, ed. THOMAS L. Baltimore: Williams & Wilkins.

MIDDLETON E. JR., MIDDLETON E.B. & SEEGAL B.C. (1953) Effect of injecting rats with homologous renal tissue mixed with adjuvants or streptococci. *Arch. Path.* **56,** 125.

MURPHY G.E. (1960) Nature of rheumatic heart disease. *Medicine* **39,** 289.

MURPHY G.E. & SWIFT H.F. (1949) Induction of cardiac lesions closely resembling those of rheumatic fever in rabbits following repeated injections with group A streptococci. *J. exp. Med.* **89,** 687.

NAKHLA L.S. (1965) Antigenic relationship between group A β-haemolytic streptococci and the heart and other tissues, with special reference to rheumatic fever. Thesis for Ph.D., University of London.

PECK J.A. PECK J.A. & THOMAS L. (1948) Failure to produce lesions or autoantibodies in rabbits by injecting tissue extracts, streptococci and adjuvants. *Proc. Soc. exp. Biol.(N.Y.).* **69,** 451.

QUINN R.W., SEASTONE C.V. & DICKIE H.A. (1952) Antibody production and tuberculin sensitivity in individuals with a history of rheumatic fever. *J. Lab. clin. Med.* **40,** 930.

RAMMELKAMP C.H., DENNY F.W. & WANNAMAKER L.W. (1952) Studies on the epidemiology of rheumatic fever. In *Rheumatic Fever,* p. 72, ed. THOMAS L. Minneapolis: Univ. Minnesota Press.

RANTZ L.A., MARONEY M. & DICAPRIO J.M. (1950) Antibody response after haemolytic streptococcal respiratory infection in childhood. *J. clin. Invest.* **29,** 840.

RANTZ L.A., MARONEY M. & DICAPRIO J.M. (1952) Infection and reinfection by haemolytic streptococci in early childhood. In *Rheumatic Fever,* p. 90, ed. THOMAS L. Baltimore: Williams & Wilkins.

RANTZ L.A., RANDALL E. & RANTZ H.H. (1948) Antistreptolysin O. A study of this antibody in health and in haemolytic streptococcus respiratory disease in man. *Amer. J. Med.* **5,** 3.

RICH A.F. & GREGORY J.E. (1943) Experimental evidence that lesions with basic characteristics of rheumatic carditis can result from anaphylactic hypersensitivity. *Bull. Johns Hopkins Hosp.* **73,** 289.

STEFFEN C. (1957) Three years' experience of the use of the Coombs-consumption test in rheumatic carditis. *Wein Z. inn. Med.* **38,** 287.

STOLLERMAN G.H. (1964) The epidemiology of primary and secondary rheumatic fever. In *The Streptococcus, Rheumatic Fever and Glomerulonephritis*, p. 311, ed. UHR J.W. Baltimore: Williams & Wilkins.

WAGNER B.M. (1960) Studies in rheumatic fever. III. Histochemical reactivity of the Aschoff body. *Ann. N.Y. Acad. Sci.* **86**, 992.

WATSON R.F., HIRST G.K. & LANCEFIELD R.C. (1961) Bacteriologic studies of cardiac tissues obtained at autopsy from eleven patients dying with rheumatic fever. *Arthr. and Rheum.* **4**, 74.

WILSON M.G. (1940) *Rheumatic Fever*. New York: Commonwealth Fund.

ZABRISKIE J.B. & FREIMER E.H. (1966) An immunological relationship between the group A streptococcus and mammalian muscle. *J. exp. Med.* **124**, 661.

CHAPTER 31

RHEUMATOID ARTHRITIS

L.E.GLYNN

INTRODUCTION

Rheumatoid arthritis is a systemic disease, although as the name implies the joints are usually most conspicuously affected. No age is exempt, but the maximum incidence is in the fifth and sixth decades. Women are far more frequently affected than men in the ratio of approximately four to one.

It is highly probable that in many aetiologically distinct conditions a polyarthritis can occur more or less closely mimicking rheumatoid arthritis, e.g. in systemic lupus, sarcoidosis, ulcerative colitis and psoriasis. It was therefore felt necessary, if serious investigation was to be directed towards these arthritides, for a more precise classification to be made so that the studies of different groups be more comparable. This was finally achieved by the criteria laid down by the American Rheumatism Association and now generally accepted (Ropes *et al* 1959). Apart from classifying cases into classical, definite, probable and possible categories based upon the number of diagnostic features presented, the Association drew up an important list of criteria, any one of which made untenable a diagnosis of rheumatoid arthritis.

The synovial membrane of the affected joints is usually swollen, oedematous and hyperaemic, but the most striking features are the dense infiltrations of plasma cells and the aggregations of lymphocytes into follicular structures

sometimes complete with germinal centres (Allison & Ghormley 1931). The subcutaneous nodules, which develop at points of pressure or friction, are a characteristic feature with a histology that, apart from granuloma annulare, is almost pathognomonic. Involvement of the serous membranes is common and lesions in the heart and peripheral blood vessels are by no means rare. Splenomegaly and a generalized hyperplasia of lymphoid tissue may occur at all ages but is more frequent in Still's disease, i.e. rheumatoid arthritis in patients under 16 years of age. Weight loss, anaemia and a raised erythrocyte sedimentation rate are further evidence of the generalized nature of the condition. Although in many patients, especially those seen within 1 year of onset, the disease may soon become arrested, one of the main pathological problems it presents is its extraordinary chronicity with evidence of active inflammation persisting for 30 years or more.

A variant of the disease in which the enlargement of the lymph nodes and spleen is a major clinical feature, usually accompanied by such signs of hypersplenism as anaemia, leucopenia and thrombocytopenia, has long been recognized as Felty's syndrome.

INFECTIOUS AGENTS IN RHEUMATOID ARTHRITIS

The last 5 years have seen a remarkable resurgence of the infective hypothesis of rheumatoid disease. This has been stimulated by several claims to have isolated members of the mycoplasmataceae (sometimes known as PPLO) from the synovia of affected joints (Bartholomew 1965; Pease 1965). These organisms are closely related to the L forms which various bacteria can be induced to assume, but differ from them in their apparent stability, i.e. lack of any evident tendency to revert to a bacterial form. They are extremely difficult organisms to grow, but can reproduce extracellularly, which distinguishes them from the viruses with which they otherwise have much in common. Unfortunately these organisms exist widely in sera and cell cultures, and none of the positive findings have rigidly excluded the possibility of contamination. As a group they are highly susceptible to antibiotics, especially the tetracyclines, and the failure of treatment of rheumatoid arthritis with these substances throws further doubt on their possible aetiological role.

Advances in virological techniques have also stimulated further searches for these organisms in rheumatoid lesions, especially in cultures of synovial tissue. In a careful study of this kind no evidence of virus was found, either by the development of spontaneous degeneration even up to 5 months, or by demonstration of interference with another virus such as Newcastle disease virus (Ford & Oh 1965).

THE RHEUMATOID FACTOR

With the continuing failure to implicate living organisms in the genesis of rheumatoid arthritis, and the absence of any consistent nutritional deficiency or of any metabolic disturbance such as characterizes gout, it is not surprising that many students of the disease continue to give serious consideration to the possible role of autoimmunity in its pathogenesis. This view received its principal stimulus when Waaler (1940) and Rose, Ragan, Pearce & Lipman (1948) found that the sera of most of their patients with this disease could agglutinate sheep cells coated with a subagglutinating dose of a rabbit anti-sheep cell antiserum. Although at first sight this would appear to offer little tangible support to the 'autoimmune' hypothesis the widespread study of the reaction has clearly established it as due to an immune* reaction by the host to one or more specific determinants present in his own γ-globulin. As this conclusion is of far-reaching significance for immunology in general, and for the nature of rheumatoid disease in particular, the steps leading to this conclusion will be followed in some detail.

Early Methods for Detection of the Rheumatoid Factor

A property of the rheumatoid factor (RF) frequently quoted as evidence against its being an antibody is its wide species specificity. This soon became apparent owing to the numerous efforts to improve upon the results obtainable by the original Waaler-Rose test. As a result of such experiments it was established that the role of the sheep cells could be taken by other cells, e.g. human (Greenbury 1956), and that immune γ-globulin of a variety of species could take the place of the rabbit globulin used in the original test. Thus, Foz, Batalla & Arcalis (1953) found that positive results could be readily obtained if a guinea-pig anti-sheep cell serum were used in place of rabbit amboceptor, and Hobson & Gorrill (1952) were equally successful using an anti-sheep cell serum prepared in the horse. Of even greater interest was the discovery that human antisera could be used for sensitization: Foz & Batalla (1956) used Brucella coated with an incomplete agglutinin of human origin and Waller & Vaughan (1956) human group O Rh D+ cells treated with incomplete anti-D antisera. Furthermore, Lamont-Havers (1955) showed that the high titre agglutination of streptococci by rheumatoid sera was due to the action of the RF upon the organisms sensitized by low titre anti-streptococcal agglutinins present in most human sera. Still other modifications showed that the relationship of the sensitizing protein to the sensitized particle need not be that of antibody to antigen. Jacobson *et al* (1956) had devised an extremely satisfactory test using tanned sheep cells coated with human γ-globulin (Cohn's Fraction II) and in the same year Plotz & Singer published their results using particles of polystyrene latex pretreated

* Sc. allergic: Eds.

with human Cohn Fraction II as the test substrate (Singer & Plotz 1956; Plotz & Singer 1956).

A consideration of these various test procedures developed since the introduction of the Waaler-Rose test shows that they all depend for their success upon the presence in rheumatoid sera of a factor or factors capable of reacting with γ-globulins from various species, including man. It is therefore conceivable that a similar interaction between rheumatoid factor and reactant γ-globulin might occur *in vivo* and the precipitate observable in many rheumatoid sera on standing or dilution certainly supports this possibility. The study of the precipitin reaction between rheumatoid sera and human F II, as first undertaken by Epstein, Johnson & Ragan (1956), provided evidence to support the view that the reaction was that of an antigen with its antibody, the F II providing the antigen. Different batches of F II differed widely in their ability to precipitate with rheumatoid sera, and the subsequent demonstration that mild heat, e.g. 63°C for 30 min, or previous exposure to an acid pH considerably increases the precipitable fraction suggests that some alteration from the native state of the γ-globulin is necessary before it can react with the rheumatoid factor (Edelman, Kunkel & Franklin 1958).

Isolation of the Rheumatoid Factor

Two main lines have been followed for the isolation of the rheumatoid factor itself; column chromatography and ultracentrifugation. LoSpalluto & Ziff (1956), by means of column chromatography on DEAE cellulose, achieved a 500-fold concentration of the factor which on electrophoresis revealed only two components, both in the γ-region. Analytical ultracentrifugation of high titre rheumatoid sera by Franklin *et al* (1957) revealed an 'unusual protein component of high molecular weight' with a sedimentation coefficient of 22S, which contained virtually the whole of the RF activity. Similar work by Svartz *et al* (1958) isolated a heavy protein component with a sedimentation coefficient of 19S, in which again the RF activity was concentrated. This discrepancy in the sedimentation coefficients has now been resolved and is due to the existence *in vivo* of a combination of the RF with a sedimentation coefficient of 19S with several molecules of low molecular weight γ-globulin (sedimentation coefficient of 7), the sedimentation coefficient of the complex being 22S. Presumably in the technique used by Svartz *et al* this complex becomes dissociated. Electrophoretically the 19S protein has the mobility of a fast γ-globulin and has been shown by Edelman *et al* (1958) to contain 10% of polysaccharide. The molecule is composed of sub-units, each with a sedimentation coefficient of 7S, apparently held together by disulphide bonds, since it is readily dissociated into these units by reagents such as mercaptoethanol. Earlier work showed an irreversible loss of antibody as a result of this treatment but more recently Schrohenloher, Kunkel & Tomasi (1964) have shown that this was due to excessive reduction leading to

inactivation of the active centres and possibly to the presence of inactive 19S molecules in the original preparation whose fragments would then interfere, by random linkage, with the reunion of the potentially active fragments.

The Antibody Nature of Rheumatoid Factor

The view that the rheumatoid factor is an antibody against γ-globulin has received widespread but by no means universal acceptance. Aho (1961) quotes six arguments that have been advanced against the antibody hypothesis. These are given here together with the counter arguments that have been produced to rebut them:

1. Apart from the easily dissociated 22S complex referred to above it has proved difficult to demonstrate a reaction between the factor and unaltered γ-globulin. This is not evidence against the antibody nature of the RF but merely casts doubt upon the role of unaltered γ-globulin as the antigen.
2. RF reacts with induced antibodies but not with naturally occurring ones. This is almost certainly because most naturally occurring antibodies, e.g. the ABO agglutinins, are 19S γ-globulins whereas the RF reactant is associated with the 7S γ-globulin.
3. Low titre agglutination is often found with normal sera. There is little evidence that this is mediated by the same mechanism as is responsible for the high titre reactions of rheumatoid sera.
4. The serological reactions only become positive late in the disease. Although this was stressed in many of the early reports, it has more recently been found (De Forest, Mucci & Boisvert 1958; Wager & Lehtinen 1961) that the appearance of the RF in the circulation is an early and not a late phenomenon, and may even precede overt disease by many years.
5. The quantitative relationship between the reactant γ-globulin and the RF as expressed in a standard precipitation curve differs significantly from that of a typical antigen-antibody reaction; the difference is seen especially in the region of antigen excess where in the typical curve the amount of precipitate falls owing to its solubility in excess of antigen. Glynn & Holborow (1960) have, however, pointed out that such solubility in excess of antigen is not an invariable character of immune precipitin reactions and that it could be expected to be absent if the antigen were already denatured.
6. Intravenously injected human F II is not eliminated more rapidly from patients with rheumatoid factor than from controls. But Vaughan, Armato, Goldthwaite, Brachman, Favour & Bayles (1955) found in a similar experiment that the half-life of injected γ-globulin was about 25% less in their rheumatoid subjects than in their controls.

The Nature of the Rheumatoid Factor Reactant

Edelman *et al* (1958) in an extensive study of the precipitation of human γ-globulin by rheumatoid sera used several methods to aggregate the γ-globulin prior to its interaction with RF. These included heating at 62–65°C for 30 min and treatment with urea, with or without mercaptoethanol, and the formation of antigen-antibody complexes in solution in antigen excess. Since all these methods of aggregation yield a product precipitable by the RF Edelman *et al* inclined to the view that the aggregation is merely responsible for rendering visible the reaction between the factor and γ-globulin and not that it is necessary to unmask groups normally inaccessible in the native state. Presumably they regarded the existence of the 22S complexes as further evidence in favour of the former view. But in 1957 Glynn, Holborow & Johnson found that the sera of rabbits immunized with rabbit red cells coated with horse or sheep serum were able to agglutinate human group O D-positive cells, sensitized with incomplete anti-D serum to titres of over 1000 despite insignificant cross-reactions in precipitin tests. On the basis of this observation they suggested that several mammalian γ-globulins have specific groups in common but that they only become revealed when the molecule is unfolded in various denaturing processes. They further suggested that the various properties of the RF as known at that time could be most simply explained on the hypothesis that it was an antibody directed against one or more of these common groups. At about the same time Milgrom, Dubiski & Wozniczko (1956) had described the existence of human sera capable of agglutinating human group O D-positive red cells sensitized with incomplete anti-D, even in the presence of free human γ-globulin. From this latter property they concluded that the specificity of such sera was directed exclusively against the groups exposed in the γ-globulin constituting the incomplete anti-D antibody as a result of its interaction with its antigen on the red cell surface. Such sera were described by them as containing 'anti-antibody'.

Further evidence is now available to support the view that the RF is specifically directed against groups in human γ-globulin molecules that are inaccessible in their native state. One of the strongest arguments against this interpretation was afforded by the existence *in vivo* of the 22S complex of RF and presumably native, 7S γ-globulin as originally described by Edelman *et al* (1958). That this interaction between the RF and native 7S γ-globulin differs essentially from its reaction with aggregated γ-globulin is now strongly suggested by two observations. Firstly, little difference was detectable between the isolated 19S rheumatoid factor and the native complexed factor in their ability to precipitate aggregated γ-globulin. Secondly, with the native complex the 7S component of the complex is carried down in the precipitate, thus indicating that the groups of the RF responsible for the precipitation of the aggregated globulin are distinct

from those responsible for the combination with the 7S globulin (Edelman *et al* 1958). Christian (1959), however, found on the contrary that when the 22S complex was precipitated with increasing amounts of aggregated γ-globulin the 7S component of the complex was progressively displaced by the aggregate. This result would be expected if the union between the factor and the reactive site on the aggregate was stronger than the union between the factor and the sites on the native γ-globulin, but might equally result if the combining sites were the same and the equilibrium displaced in favour of the aggregate because precipitation removed the aggregate from solution.

According to this latter interpretation there is no need to postulate that the reactive groups of the aggregate differ in any way from those on the native γ-globulin. But observations on the nature of the reactant in the Heller F II test and in the latex test of Singer and Plotz are incompatible with this view. It has been known since the introduction of the Heller test that samples of human Fraction II (F II) differ considerably in their suitability for use in this test and it has now been established that it is the presence of aggregates in the F II sample that is essential (Oreskes, Singer & Plotz 1961). It is therefore evident that the tanning procedure is not itself adequate to induce a sufficient degree of aggregation. Since the cells coated with unsuitable samples of F II are readily agglutinated by a Coombs serum, failure of agglutination by rheumatoid sera cannot be due to absence of the γ-globulin from the cell surface, but most probably to the inaccessibility of the appropriate reactant groups. Studies on the latex test lead to a similar conclusion. Samples of F II do not differ significantly in their suitability for coating the latex particles. This implies that if the reactant groups are not accessible in the native state of the F II they must become so as a result of adsorption on to the particle surface. That this is indeed so is shown by the inability of free F II to inhibit the test whereas the heat-aggregated material is strongly inhibitory (Singer *et al* 1960). The only justifiable inference from these many observations is that the rheumatoid factor is specifically reactive with groups common to many mammalian species of γ-globulin but which are not easily accessible to it in their completely native state. Since free rabbit γ-globulin can inhibit the Rose-Waaler reaction, but free undenatured human γ-globulin cannot, it suggests that the reactive groups are more accessible in the heterospecific than in the homospecific protein.

Further work by Singer's group (Oreskes, Singer & Plotz 1963) has clearly differentiated the significance of denaturation from aggregation. Thus fourteen commercial preparations of human γ-globulin varied widely in their ability either to sensitize tanned red cells, to inhibit their agglutination by rheumatoid sera or to precipitate with it; but although in any one preparation these three properties showed excellent correlation, there was little between them on the one hand and the amount of aggregated globulin present on the other. Moreover, denaturation without aggregation, e.g. by treatment with alkali or urea, gave a

product suitable for detection of RF activity. They therefore conclude that denaturation is an essential prerequisite for converting pooled human γ-globulin into a reactant for rheumatoid factor and that aggregation merely serves to enhance the reactivity so produced.

Histochemical Demonstration of Rheumatoid Factor

Indirect evidence strongly supporting the antibody nature of RF has been provided by the histo-immunological studies of Mellors and his collaborators (Mellors *et al* 1959, 1961). Heat-aggregated human γ-globulin conjugated with fluorescein isothiocyanate was used as the histochemical reagent in the earlier work, but later a fluorescein conjugated antigen-antibody complex was also used. This complex consisted of egg albumin-rabbit anti-egg albumin dissolved in excess of the antigen. With these reagents staining indicative of the presence of the RF was clearly obtained in the synovial membrane, lymph nodes and subcutaneous nodules of rheumatoid arthritis. In all three situations it was the plasma cells that stained; in the lymph nodes staining was also found in about 10% of the germinal centres. Since the work of Fagraeus (1948), the plasma cell has become increasingly accepted as the site of antibody production and the participation of cells in the germinal centres has been clearly demonstrated more recently by White (1960). Partial confirmation that the cell-staining by Mellors' reagents was indeed due to the RF was obtained by showing that these same cells were included amongst those stained by a fluorescent antibody specific for 19S γ-globulin, although they constituted only a small proportion of all the cells containing this globulin. Staining with the heat-aggregated γ-globulin was consistently stronger than with the antigen-rabbit antibody complex. Moreover, inhibition by pretreatment with the non-fluorescent reagents was complete with the aggregated γ-globulin but only partial with the immune complex, a finding that agrees well with other work suggesting the presence of two factors, one specific for human γ-globulin, the other cross-reacting also with that of other animals. Amongst a large variety of control sections positive staining was found only once, in a case of Waldenstrom's macroglobulinaemia. It is of considerable interest that positive staining in this case was only obtained with the aggregated γ-globulin and not with the rabbit antibody-antigen complex, thus emphasizing once again that it is the species cross-reactivity of the rheumatoid factor that is the diagnostic feature for rheumatoid arthritis (cf. the latex test).

A study by Bonomo (1965) of the distribution of the RF in various tissues of patients with the factor in their serum showed a remarkable concentration of RF-positive cells in the main affected tissues, e.g. in the lymph nodes in Hodgkins disease, the bone marrow in macroglobulinaemia, the liver in hepatitis, the sputum in chronic bronchitis, and the synovial tissue in rheumatoid arthritis. He concluded that in all these instances the production of the RF was a response to

local antigenic stimulation, presumably in the form of appropriately denatured IgG.

The Nature of the Cross-Reactivity of Rheumatoid Factor

If the RF is an antibody then presumably the specific antigen responsible for its production is appropriately altered human γ-globulin and its ability to react with altered γ-globulins of other species is an example of cross-reactivity, a common phenomenon in immunology. The experimental results of Aho (1961) afford strong supporting evidence. Thus, he was not only able to confirm the results of Vaughan (1956) that immune precipitates of egg albumin-rabbit anti-egg albumin serum can absorb the factor responsible for the sensitized sheep cell test, but that they leave the titre against F II-coated latex particles almost unaffected. Nevertheless, eluates from the immune precipitates used in these absorption experiments were not only capable of agglutinating sensitized sheep cells to high titre but gave significant titres in the latex test; 2560 and 10,240 respectively. Like other cross-reacting antibodies therefore the RF can be fractionated by appropriate absorption and elution into specific and cross-reacting moieties. Further confirmation of the antibody nature of RF is provided by Aho's observations that as with other antibodies its cross-reactivity is an expression of its avidity. Thus all the RF activity eluted from immune precipitate made with a human antiserum and treated with a large excess of high-titre rheumatoid serum was absorbable by a rabbit immune precipitate and vice versa, a finding in marked contrast to the factor in whole serum or in eluates made from precipitates treated with only small amounts of RF. The greater avidity of the factor for human than for rabbit γ-globulin is also clearly shown by the effect of temperature on the corresponding reactions: at 45°C the agglutination of cells sensitized with rabbit amboceptor is completely inhibited whilst that of cells sensitized with human sera is virtually unaffected (Aho 1961).

It has been suggested by both Mellors *et al* (1961), and Milgrom, Witebsky, Goldstein & Loza (1962) that some of the RF molecules react with rabbit γ-globulin but not with human γ-globulin. This would of course throw some doubt upon the autoimmune nature of the RF. It should, however, be remembered that Milgrom & Witebsky (1960) have also shown that the serum of rabbits immunized with their own γ-globulin reacts far more intensely with human γ-globulin than with the immunizing antigen itself. It is thus apparent that γ-globulin is a somewhat unusual antigen and raises the interesting question of the possible existence of specific interfering groups as well as specifically reactive ones.

Allotypes of Human γ-Globulin: the Gm Groups

The use of human group O D+ cells sensitized with human incomplete anti-D

sera as a test for RF was introduced by Waller & Vaughan (1956). Not all anti-D sera are suitable; some will only detect a small percentage of rheumatoid sera, but others are able to detect as high a proportion as sheep cells sensitized with rabbit amboceptor. Anti-D sera of this latter reactivity are referred to by Harboe (1960) as diagnostic anti-D sera. As judged by the variety of absorption and elution tests performed by Aho (1961) the configuration of the γ-globulin molecules of these diagnostic anti-D sera when on the red cells approximates to that of the F II molecules on latex particles or when tanned on to sheep cells for the Heller test; and those components of the RF capable of reacting with them show similar cross-reactivity with rabbit γ-globulin, as in the Rose-Waaler test. For example, if a rheumatoid serum rich in RF be absorbed with an albumin rabbit-anti-egg albumin precipitate the eluate will not only give a high reading in the Rose-Waaler test but give comparable figures with human O D+ cells sensitized with such a 'diagnostic' anti-D serum. Incomplete anti-D sera of this type are not common; the majority of incomplete anti-D sera are of the kind that are only able to react with a small proportion of rheumatoid sera. This is, however, a remarkable phenomenon as it demonstrates the capacity of some rheumatoid sera to distinguish between the γ-globulins of certain other human individuals.

The whole question of immunological differences between homologous serum proteins of members of the same species, so-called allotypes (Oudin 1960), has been illumined by this differentiating capacity of certain rheumatoid sera. This capacity was first clearly demonstrated by Grubb & Laurell (1956) and led to their discovery of the Gm groups or allotypes of human γ-globulin. They found that with some incomplete anti-D sera and certain rheumatoid sera the agglutination of the sensitized red cells could be inhibited by some normal human sera, but not by others. The normal sera with this inhibitory capacity were designated Gm(a+) and those lacking it Gm(a−). They further established that this character is an attribute of the γ-globulin and that it is genetically determined by a single dominant autosomal gene. Amongst Europeans about 60% are Gm(a+) and 40% Gm(a−). Several similar genetically determined attributes of human γ-globulin have since been discovered by the use of combinations of appropriate rheumatoid or other sera and appropriate incomplete anti-D sera. These include the factors Gm(b), Gm(x), Gm-like and in accordance with the terminology used originally by Grubb & Laurell the rheumatoid sera used for their detection are described as anti-Gm(a), anti-Gm(b), anti-Gm(x), etc.

This terminology is that usually applied to antibodies and it has been tacitly assumed by almost all workers in this field that these anti-Gm sera do indeed contain antibodies specifically directed against a corresponding Gm antigen. Much evidence exists in harmony with this view. Thus Laurell & Grubb (1958) have reported that of twenty-one rheumatoid sera examined the eight that agglutinated the coated Rh-positive cells were derived from Gm(a−) individuals,

whilst the thirteen sera from Gm(a+) patients failed to agglutinate similarly coated cells. Conversely Harboe (1960) has found by Gm typing of incomplete anti-D sera that all those used for detecting Gm(a) are themselves Gm(a+), those for detecting Gm(b) are Gm(b+) and those for detecting Gm(×) are Gm(×+). Fudenberg & Kunkel (1961) have gone even further and have isolated the 7S γ-globulin from a number of rheumatoid sera used for Gm typing and found that in the four sera used for Gm(a) typing the isolated 7S γ-globulin was of the phenotype Gm(a− b+), in the one serum used for Gm(b) typing the phenotype was Gm(a+b−), and in two sera used for Gm(×) typing the phenotype was Gm(a+ b+ ×−). It would therefore appear that with those reagents suitable for Gm typing the rheumatoid serum must lack the factor being sought and the incomplete anti-D serum must possess it. If therefore the rheumatoid sera used for detecting Gm groups react by virtue of specific anti-Gm antibodies, at least these components of the sera must be regarded as iso-antibodies not auto-antibodies. Grubb himself (Grubb 1958), however, was reluctant to regard them as antibodies because anti-Gm titres could not be increased by injections of γ-globulin of the appropriate Gm group, nor was the catabolism of such γ-globulin increased by the presence of the corresponding anti-Gm factor.

Whether the anti-Gm factors are antibodies or not, absorption experiments with non-human serum proteins show that unlike other reactive components of rheumatoid sera their specificity is entirely species specific. Thus absorption of an anti-Gm rheumatoid serum by an egg albumin-rabbit anti-egg albumin precipitate does not significantly reduce the anti-Gm titre, and the eluate of such a precipitate although possessing considerable activity in the Rose-Waaler or latex tests is without significant anti-Gm activity. The same serum absorbed with a human immune precipitate loses all its anti-Gm activity which can, however, be subsequently eluted with the other RF activities (Aho 1961).

The Immunological Significance of the Gm Groups

Although the anti-Gm activities of rheumatoid sera are by definition Gm specific, this only applies to their reactions with native human sera or incomplete anti-D sera on appropriate red cells. Any human serum γ-globulin when heat aggregated or as an immune precipitate with its antigen can inhibit the specific anti-Gm effect of a rheumatoid serum, a finding difficult to reconcile with specific antigenicity of the Gm groups. Such a finding would be expected if the specific anti-Gm activity were carried on the same molecules that react with the γ-globulin in the immune precipitate. Antibodies, however, are invariably regarded as monospecific; bispecific antibodies are entities whose existence runs counter to general immunological experience. If, moreover, the loss of anti-Gm activity brought about non-specifically by absorption with human γ-globulin, either as a heat aggregate or an immune precipitate, were

attributable to such bispecific antibodies a similar loss of activity should be obtainable with aggregated rabbit γ-globulin. An alternative explanation of this phenomenon would be to regard all rheumatoid anti-Gm sera as directed against a single specifically human reactant group common to all human serum γ-globulin. The Gm phenomenon could then be attributed to a mutual interference between the RF and this specific human reactant if both proteins were obtained from individuals of the same Gm group. On this hypothesis the Gm group has the properties of a self marker, which is readily obliterated by the configurational changes undergone on heat aggregation or immune precipitation. On this hypothesis the specificity of the Gm groups is not itself an antigenic specificity and a rheumatoid anti-Gm serum has no specific anti-Gm antibody, as Grubb himself suspected in 1958.

Although the evidence therefore suggests that the rheumatoid anti-Gm reagents are not antibodies directed specifically against the Gm determinants, true anti-Gm reagents have been described in some non-rheumatoid sera obtained from patients after multiple blood transfusions. These have been designated SNagg sera (Steinberg, 1962) in contrast to the RAgg sera obtained from rheumatoid patients; and in contrast to the latter, SNagg sera are not inhibited non-specifically by immune precipitates or heat aggregates of human 7S antibodies.

We have already referred to the existence of some anti-D sera which, when coated upon appropriate red cells, react with rheumatoid sera irrespective of their Gm group. This could arise either because such so-called diagnostic anti-D sera possess all varieties of Gm determinants, or, according to our hypothesis, if the reaction between such anti-D sera and the red cells is unusual and results in the removal of the interfering Gm determinants. Study of such diagnostic anti-D sera has eliminated the first alternative; the demonstration that red cells treated with such antisera fix complement (Waller & Lawler 1962) strongly supports the second as it implies a configurational change not usually undergone by antibodies of the incomplete type.

The ability of rheumatoid sera to react specifically only with those Gm determinants absent from the individual's own γ-globulin has been regarded by Fudenberg & Kunkel (1961) as evidence against the autoimmune nature of rheumatoid factors in general. If our interpretation, however, is correct, the specific determinants against which the rheumatoid factors are directed are common to all human 7S γ-globulins although access to these determinants may be more or less concealed in the native state of the molecules. With the determinants responsible for the Rose-Waaler reaction considerable unfolding of the native molecule is necessary for their exposure, but those responsible for the apparent Gm reactions are accessible even in the native state, provided there is no steric hindrance from the adjacent Gm determinants. If this be so then the objection of Fudenberg & Kunkel to the autoimmune nature of the rheumatoid anti-Gm reagents falls to the ground.

A virtually identical interpretation of the nature of the rheumatoid anti-Gm was recently put forward by Williams & Kunkel (1965) who then concluded—'. . . little evidence now remains that can be cited against the concept of immunization by some form of autologous γ-globulin leading to the production of the wide assortment of anti-γ-globulins found in the sera of patients with rheumatoid arthritis'.

Serum globulins reacting with various determinant groups on IgG are not the only auto-antibodies found in rheumatoid arthritis. A significant number of these patients possess antinuclear factors as shown by the L.E. cell test and by immunofluorescent staining of nuclei in tissue sections. Here again the reactivity is not directed against a single nuclear component but may include DNA, DNA protein, nuclear histone or other proteins of the nucleoplasm.

It is difficult to determine the incidence of these various factors since the published figures show the most remarkable variations from 14 to 50%. It is most probable that technical differences are mainly responsible for the discrepancies between different investigators.

The presence of a positive test for LE cells or for other antinuclear factors inevitably raises the question of systemic lupus as the underlying disease. Close attention to the clinical manifestations has, however, clearly shown that the great majority of rheumatoid patients with antinuclear factors are not suffering from SLE. They do, however, tend to show a much higher incidence of the extra-arthritic manifestations such as arteritis, peripheral neuritis and pulmonary lesions.

Some Alternative Views on the Nature of the Rheumatoid Factor

Not all investigators of the RF regard it as an antibody. Wager (1950), for example, originally regarded it as a non-specific potentiator of agglutination comparable in its action to that of albumin in the agglutination of red cells by incomplete Rh antibodies. In the light of the many investigations already discussed this interpretation is clearly untenable. In view of the reactivity of RF with antigen-antibody complexes a relationship between it and complement appeared not improbable despite its stability at temperatures used for complement inactivation. Hobson & Gorrill (1952) even claimed to have identified the factor with the fourth component of complement. The careful study of Laurell & Grubb (1958), however, revealed no significant difference between rheumatoid sera with positive Rose-Waaler tests and normal sera in the titres of complement or its individual components.

Nanna Svartz has for several years supported an entirely different origin for the RF. In 1955 she reported (Svartz & Schlossmann 1955) that a similar factor could be obtained *in vitro* by the growth on media containing bovine or human collagen of organisms obtained from the throats of patients with rheumatoid

arthritis. More recently she has claimed (Svartz 1960) that the organisms responsible, group B streptococci (*Str. agalacteae*), are capable of inducing a chronic arthritis in rabbits, rats and pigs, and that in many of these animals a globulin fraction can be obtained by cold precipitation that is capable of giving a positive Rose-Waaler test. Its relationship to the RF of man is further suggested by its sedimentation coefficient of 18. Confirmation of these findings from other laboratories is still awaited.

The Diagnostic Value of Rheumatoid Factor

A question of considerable practical importance is the diagnostic accuracy of the RF as assessed by the incidence of false negatives, and its presence in conditions unrelated to rheumatoid disease, i.e. the incidence of false positives. Positive findings in RA have varied in different studies from 35% (Waaler 1940) to over 90% (Ziff *et al* 1956). Increasing sensitivity as would be expected is associated with a raised incidence of false positive reactions. Despite this there is general agreement that a test of average sensitivity will detect 70–80% of patients with rheumatoid arthritis and give positive reactions in only some 2–5% of non-rheumatoid subjects. Within the group of connective tissue diseases negative results are consistently found in ankylosing spondylitis, psoriatic arthritis and in the arthritis associated with ulcerative colitis. In patients with systemic lupus erythematosus the incidence of RF is about one-third that in rheumatoid arthritis. In juvenile rheumatoid arthritis results have been most conflicting but it has been claimed that even in children in whom the serum factor is not detected its presence can be revealed histochemically in plasma cells (Mellors *et al* 1961).

Concerning the relative merits of the different types of test, those making use of human γ-globulin as the reactant, notably the latex test, are less specific for rheumatoid arthritis than those in which rabbit γ-globulin is used, as in the original Rose-Waaler test. The reason for this paradoxical behaviour of what is widely regarded as an auto-antibody to human γ-globulin is probably to be found in the relationship between avidity and cross-reactivity (Aho 1961). Apparently it is mainly in the rheumatoid diseases that the antibody to human γ-globulin is sufficiently avid to cross-react significantly with the γ-globulins of other species. In syphilis (Peltier & Christian 1959), sarcoidosis (Kunkel, Simon & Fudenberg 1958), and several virus diseases, e.g. hepatitis (Dresner & Trombley 1959), in which positive tests with latex particles are common the avidity is low and the cross-reactivity insufficient for the agglutination of sheep cells sensitized with rabbit antibody.

There are nevertheless some rare rheumatoid sera that react with rabbit γ-globulin although failing to react with the human equivalent. This type of reaction is difficult to explain on classical immunological theory but it could

arise if specific inhibitory groups are present as already suggested in our discussion of the Gm allotypes. According to such a hypothesis the reacting determinants on the rabbit γ-globulin are also present on human γ-globulin but the failure to obtain a demonstrable reaction with these rheumatoid sera is due to mutually interfering specifically human groups on the rheumatoid factor and the human γ-globulin reactant. Whereas with most rheumatoid sera such mutual interference is avoided by mild denaturation of the γ-globulin reactant, with those reacting with rabbit but not with human γ-globulin the mild denaturation associated for example with adsorption to a latex particle is presumably insufficient.

The Pathogenic Significance of Rheumatoid Factor

Attempts to implicate the RF in the pathogenesis of the disease have met with little success. The only well-established clinical correlation is that between the presence of the factor and the presence of subcutaneous nodules (Ball 1952). Duthie *et al* (1957) have found, however, that the prognosis is far better in those patients without the RF than in those in whom it is present when first examined. Attempts to influence the clinical picture by the transfusion of high-titre rheumatoid sera into other rheumatoid patients failed to reveal any obvious effect of the factor (Vaughan & Harris 1959): nor could any amelioration of symptoms be induced by the transfusion of concentrates of inhibitors (Ziff *et al* 1956).

Serum Inhibitors of Rheumatoid Factor

Much study has been devoted to the nature of the serum factors capable of inhibiting the Rose-Waaler reaction since Ziff *et al* (1956) claimed that the absence of inhibitor from the euglobulin fraction was a more sensitive test for rheumatoid arthritis than the presence of the RF. From that study the authors attributed the failure of some 20–30% of rheumatoid arthritics to give a positive Rose-Waaler reaction to the presence in their serum of a factor responsible for inhibiting the rheumatoid factor.

Separation of these two components was effected by dialysis of the serum against a phosphate buffer at pH 6, the agglutinator being precipitated with the euglobulin, the inhibitor remaining in solution. By this means the presence of the RF was detected in 90% of rheumatoid subjects and in only 2–7% of controls. Examination of the euglobulin precipitate for its inhibitory activity revealed an even more striking difference between the rheumatoid and non-rheumatoid populations; it was present in 96% of the latter and absent from 100% of the former. Unfortunately in other hands this inhibitor test has given an excessive proportion of false positive results (Clark 1957). Dresner & Trombley (1959),

however, have claimed that for the latex agglutination test the absence of inhibitor is with few exceptions diagnostic of rheumatoid arthritis and that the exceptions themselves fall into a few easily recognized nosological entities such as syphilis or recent virus infections.

The euglobulin of normal subjects has been subjected to ultra-centrifugation in a density gradient in an attempt to separate the inhibitor for further study (Franklin 1960). Three fractions were obtained, 7S, 19S and aggregates of higher sedimentation rate. Since all three fractions possessed inhibitory activity it is apparent that, at least as far as the 7S fraction is concerned, ability to react sufficiently firmly with the factor to inhibit it can occur without previous aggregation of the inhibiting globulin. It would seem reasonable to conclude that human γ-globulin in the form in which it is capable of precipitating with RF would also inhibit its agglutinating activities, and inhibitory activity in the γ-fraction of human serum is no doubt largely due to free reactant. It is less easy to account for the much stronger inhibitor found by Rantz, Randall & Kettner (1959) in the α- and β-globulin but at present there is no indication as to whether this inhibition is of the specific or non-specific kind.

The widespread interest in inhibitors of the RF has a dual origin, firstly for the light they might throw on the nature of the reactant, and secondly for any therapeutic action they might possess assuming that the factor is itself harmful. Vaughan (1959) has, however, justifiably emphasized that 'although it is true that the chemical nature of substances inhibiting rheumatoid agglutination reactions may give accurate information as to the stereo-chemical nature of the configuration for which the rheumatoid factor has primary specificity, this is true only if all possible non-steric factors can be reasonably ruled out'. Failure to observe a simultaneous change in pH, for example, led to the claims that the agglutination reactions were specifically inhibitable by relatively simple chemical compounds such as glycyl-glycine (Williams *et al* 1956) or sulphonamides (Svartz & Schlossmann 1956).

Genetic Aspects of the Rheumatoid Factor

The RF, apart from its undoubted importance in drawing attention to the possible role of autoimmunity in the pathogenesis of rheumatoid arthritis, has proved an invaluable tool for the study of the role of genetic factors in this disease. Thus Ziff *et al* (1958) found a significantly higher incidence of the RF in the relatives of rheumatoid arthritics than in similar relatives of control subjects. An extensive survey of a general population by Ball & Lawrence (1961), using the sensitized sheep cell test, revealed a striking rise in incidence of positive reactors from 1% in the 15–24 years age-group to 12% in those over 65 years. They also found a significant correlation between the titres of propositi and those of their first-degree relatives. Since no significant correlation was found between the

titres of husbands and wives they concluded that the familial aggregation of positive titres was mainly genetically determined. A study of the agglutination titres of three pairs of identical twins to which they refer showed, however, that environmental factors must also be important. From the titre distribution curve based on their findings both in a rural and an urban district they inferred that the RF is a continuous variable present in some degree in 17–40% of adults. Even if we accept that the titres in all subjects were due to RF this does not disprove its antibody nature. If, as is not improbable, the RF is an antibody to denatured γ-globulin, this is an antigen to which most adults must have been exposed on many occasions. That antibody production to a given antigen is a continuous variable is only what one would expect from the quantitative study of antibody production both in man and animals; and that the level in the blood stream should rise with increasing stimulation and hence with age is also not surprising.

Evidence is now available that both in man and animals RF can indeed develop as an immune response to IgG which has become denatured as a result of interaction with specific antigen. Thus in man chronic bacterial infection, such as subacute bacterial endocarditis, can be associated with the presence of RF which disappears when the infection has been eliminated by appropriate antibiotic treatment (Williams & Kunkel 1962). In rabbits a similar situation has been obtained by repeated immunization with *E. coli* or *Bacillus subtilis* (Abruzzo & Christian, 1961; Christian 1963).

Recent studies have been highly critical of the earlier results which indicated a distinct familial aggregation both of clinical cases as well as raised levels of RF. O'Brien, Burch & Bunim (1963) from their own studies of Pima and Blackfeet Indians, which showed a random distribution of both RF and rheumatoid arthritis, concluded that all previous conclusions based upon hospital attendance were invalid because they overlooked the bias caused by the increased probability of ascertaining the proband the greater the number of affected individuals in the family. They therefore maintain that valid conclusions can only be drawn from studies of whole populations. It seems that the case for familial aggregation and for a positive genetic influence on both the incidence of the disease and of the RF is much less strong than was supposed 5 years ago. Nevertheless, even when allowance is made for the biased selection of the probands the evidence in its favour is not entirely negligible. Note, for example, the threefold greater incidence of concordance of chronic arthritis in monozygotic than in dizygotic twins (Marshall, Hutchison & Honisett, 1962).

The Significance of the Rheumatoid Factor

If RA is a disease based on autoimmunity it might reasonably be expected that its victims would be found mainly amongst that section of the population with the

greatest tendency to react immunologically to its own slightly altered proteins, i.e. amongst those with the higher levels of rheumatoid factor. The presence of the RF in any individual is then, according to this hypothesis, no more than indicative of a heightened degree of sensitivity of the immune mechanism by which it is enabled to detect as foreign a degree of change in autologous protein that is ignored by the less sensitive antibody mechanisms of normal individuals. The undoubted correlation between the presence of the RF and rheumatoid disease points strongly to a causal relationship between them. The increased incidence of the factor in the relatives of rheumatoid arthritics and its detection in the early stages of the disease, often indeed preceding overt clinical manifestations, point equally strongly to the factor being associated with cause rather than an effect of rheumatoid arthritis.

Reference has already been made to the failure of attempts to implicate the rheumatoid factor in the direct pathogenesis of the disease. The development of typical rheumatoid lesions in subjects with congenital agammaglobulinaemia (Gitlin, Janeway, Apt & Craig 1959) shows, moreover, that neither circulating antibodies nor the plasma cells which give rise to them can play the fundamental pathogenetic role. This role would appear, therefore, to be taken by a mechanism of delayed hypersensitivity comparable to that thought to underly such experimentally induced auto-immune diseases as allergic encephalomyelitis or thyroiditis. Circulating antibodies in these conditions, as in rheumatoid arthritis, may then be regarded as no more than indicators of a more fundamental immunological disturbance underlying them. The cause and nature of this disturbance remain to be elucidated but the increased incidence both of the RF and of rheumatoid disease in the relatives of rheumatoid arthritics may be confidently attributed to some genetic control of the immune reactivity.

Although the rheumatoid factors remain the strongest evidence for an auto-immune aetiology of rheumatoid arthritis, attempts to induce a comparable arthritis experimentally have had their chief success when employing some form of hetero-immune reaction. Nevertheless, as clearly shown in a recent review (Gardner 1960), none of the then available experimental forms of arthritis sufficiently resembled the rheumatoid condition to suggest a closely related pathogenesis. Dumonde & Glynn (1962) have, however, shown that an arthritis virtually indistinguishable from RA can be induced in rabbits by the intra-articular injection of fibrin if the animals have been previously made sensitive by intradermal injections of fibrin in Freund's complete adjuvant. In the most successful experiments active inflammation was still present 14 weeks after a single intra-articular injection.

The persistence of the reaction was attributed to two factors: firstly the particulate insoluble nature of the antigen; and secondly to the constant renewal of antigen in the inflammatory exudate because of the cross-reactivity between the human fibrin used and the rabbits' own fibrin. This cross-reactivity could be

readily shown by delayed hypersensitivity to intradermal injections of as little as 5–50 μg of autogenous fibrin. In some experiments a similar arthritis was obtained when the rabbits' own fibrin was used exclusively both for the initial immunization and subsequent intra-articular injection. From these and similar experiments, Dumonde and Glynn concluded that the rheumatoid state might well result from delayed hypersensitivity to one or more products of inflammation. Since the hypothetical antigens would be variously altered from the native state by the inflammatory process the breakdown of tolerance could be more readily envisaged. Moreover, sensitization to an inflammatory product provides a basis for a chain reaction which offers a plausible explanation of the extraordinary chronicity of the individual lesions. The characteristic histopathology of the synovial membrane with its diffuse infiltration of plasma cells and its follicular aggregations of lymphocytes around germinal centres would then appear as the immunological response to the local persistence of antigen, such as is typically seen for example in Hashimoto's thyroiditis.

The results of these and similar experiments with inflammatory exudate (Phillips, Kaklamanis & Glynn, 1966) suggest that the development of an autoimmune reaction to one or more products of tissue injury or inflammation could well be the cause of chronicity of an inflamed joint. They do not throw any light on the initiating agent. In our present state of knowledge this could be any cause of local inflammation, traumatic, metabolic or infective. The latter is the most probable because the immune response to it could then account for the appearance of the RF as a further immune response to the denatured γ-globulin of the locally formed antigen-antibody complexes. The importance of intra-articular γ-globulin in the presence of RF has been strikingly shown by Hollander, McCarty, Antorge & Castro-Marillo (1965), who induced a severe acute arthritis by the injection of the autochthonous protein into the knee joint of rheumatoid arthritics sero-positive for the RF.

Two hitherto puzzling features readily explicable on the basis of an immunological sensitivity to inflammatory products are the frequently observed relationship between trauma and the onset of overt joint involvement, and the remarkable tendency for localization to one part of a joint whilst the remainder appears relatively normal.

A similar relationship between trauma and the localization of lesions has long been suspected as accounting for the development of the characteristic subcutaneous rheumatoid nodules over the bony pressure points, especially the elbows. A comparison of the histology of these nodules with the reaction to an implant of foreign fibrin in rabbits (Banerjee & Glynn 1960) cannot fail to suggest the possibility that a common immunological reaction underlies the two lesions. This by no means implies that the offending antigen is identical in both instances nor is it necessary to assume that it must be identical in different individuals. But the similarity between both the experimental nodules and the

arthritis on the one hand, and their naturally occurring counterparts on the other, stresses not only a similarity in an auto-immune* pathogenesis but also in the inflammatory origin of the offending antigen. The rheumatoid factor as an auto-antibody to altered γ-globulin thus receives added significance as reflecting the fundamental immunological reactivity to altered inflammatory products which may underlie the rheumatoid process.

REFERENCES

ABRUZZO J.L. & CHRISTIAN C.L. (1961) The induction of a rheumatoid factor-like substance in rabbits. *J. exp. Med.* **114,** 791.

AHO K. (1961) The problem of the antibody nature of the rheumatoid factor. *Ann. Med. exp. Fenn.* **39,** Suppl. 7.

ALLISON N. & GHORMLEY R.K. (1931) *Diagnosis in Joint Disease: A Clinical and Pathological Study of Arthritis.* New York: William Wood.

BALL J. (1952) Sheep cell agglutination test for rheumatoid arthritis. *Ann. rheum. Dis.* **11,** 97.

BALL J. & LAWRENCE J.S. (1961) Epidemiology of the sheep cell agglutination test. *Ann. rheum. Dis.* **20,** 235.

BARTHOLOMEW L.E. (1965) Isolation and characterization of mycoplasmas from patients with rheumatoid arthritis, systemic lupus erythematosus and Reiter's syndrome. *Arthr. & Rheum.* **8,** 376.

BONOMO L. (1965) Immunofluorescence investigations in various tissues in rheumatoid arthritis. Presented at XI International Congress of Rheumatic Disease, Mar del Plata.

CHRISTIAN C.L. (1959) A study of rheumatoid arthritis sera: comparison of spontaneous precipitates and gamma globulin-induced precipitates. *Arthr. & Rheum.* **2,** 289.

CHRISTIAN C.L. (1963) Rheumatoid factor properties of hyperimmune rabbit sera. *J. exp. Med.* **18,** 827.

CLARK W.S. (1957) Conference on serological reactions in rheumatoid arthritis. New York: Arthritis & Rheumatism Foundation.

DEFOREST G.K., MUCCI M.B. & BOISVERT P.L. (1958) Two-year comparative study of serial haemagglutination tests done on groups of rheumatoid arthritis patients. *Arthr. & Rheum.* **i,** 387.

DRESNER E. & TROMBLY P. (1959) The latex fixation reaction in non-rheumatic diseases. *New Engl. J. Med.* **261,** 981.

DUTHIE J.J.R., BROWN P.E., KNOX J.D.E. & THOMPSON M. (1957) Course and prognosis in rheumatoid arthritis. *Ann. rheum. Dis.* **16,** 411.

EDELMAN G.M., KUNKEL H.G. & FRANKLIN E.C. (1958) Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated gamma globulin. *J. exp. Med.* **108,** 105.

EPSTEIN W., JOHNSON A.M. & RAGAN C. (1956) Observations of a precipitin reaction between serum of patients with rheumatoid arthritis and a preparation (Cohn Fraction II) of human gamma globulin. *Proc. Soc. exp. Biol. (N.Y.)* **91,** 235.

FAGRAEUS A. (1948) The plasma cellular reaction and its relation to the formation of antibody *in vitro*. *J. Immunol.* **58,** 1.

FORD D.K. & OH J.O. (1965) Use of 'synovial' cell cultures in the search for virus in rheumatoid arthritis. *Arthr. & Rheum.* **8,** 1047.

FOZ A. & BATALLA E. (1956) Auto-antibodies against human globulin in rheumatoid arthritis patients. In *Contemporary Rheumatology,* p. 166, ed. GOSLINGS J. & VAN SWAAY, H. Amsterdam: Elsevier.

*Sc. Auto-allergic. Eds.

FOZ A., BATALLA E. & ARCALIS L. (1953) Estudios acerca del macanismo de la aglutinacion de los hematies sensibilizados, por el suero de los pacientes de poliartritis crónica progresiva. *Rev. españ. Reum.* **5,** 8.

FRANKLIN E.C. (1960) Stabilizers and inhibitors of the sensitized sheep cell agglutination reaction. *Arthr. & Rheum.* **3,** 266.

FRANKLIN E.C., HOLMAN H.R., MÜLLER-EBERHARD H.J. & KUNKEL H.G. (1957) An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. *J. exp. Med.* **105,** 435.

FUDENBERG H.H. & KUNKEL H.G. (1961) Specificity of the reaction between rheumatoid factors and gamma globulin. *J. exp. Med.* **114,** 257.

GITLIN D., JANEWAY C.A., APT L. & CRAIG J.M. (1959) Agammaglobulinaemia. In *Cellular and Humoral Aspects of the Hypersensitive States*, p. 375. London: Cassell.

GLYNN L.E. & HOLBOROW E.J. (1960) Immunological aspects of rheumatoid disease: A review. *Ann. rheum. Dis.* **19,** 197.

GLYNN L.E., HOLBOROW E.J. & JOHNSON G.D. (1957) The nature of the Rose-Waaler factor. *Proc. roy. Soc. Med.* **50,** 469.

GREENBURY C.L. (1956) Elution of the rheumatoid arthritis factor from red-cell agglutinates. *Lancet* **ii,** 644.

GRUBB R. (1958) Discussion. *Rheumatoid Arthritis. Immunopathology*, p. 376. First International Symposium, Basel/Seelisberg. Basel: Benno Schwabe.

GRUBB R. & LAUREL A.B. (1956) Hereditary serological human serum groups. *Acta path. microbiol. scand.* **39,** 390.

HARBOE M. (1960) Interaction between red cells coated with incomplete anti-D and rheumatoid sera. *Acta path. microbiol. scand.* **50,** 383.

HOBSON D. & GORRILL R.H. (1952) Agglutination test for rheumatoid arthritis. *Lancet* **i,** 389.

HOLLANDER J.L., MCCARTY D.J. JR., ANTORGE G. & CASTRO-MARILLO E. (1965) Studies on the pathogenesis of rheumatoid joint inflammation. *Ann. intern. Med.* **62,** 271, 281 and 285.

JACOBSON A.S., KAMMERER W.H., WOLF J., EPSTEIN W.V. & HELLER G. (1956) The haemagglutination test for rheumatoid arthritis. *Amer. J. Med.* **20,** 490.

KUNKEL H.G., SIMON H.J. & FUDENBERG H. (1958) Observations concerning positive serologic reactions for rheumatoid factor in certain patients with sarcoidosis and other hyperglobulinaemic states. *Arthr. & Rheum.* **1,** 289.

LAMONT-HAVERS R.W. (1955) Nature of serum factors causing agglutination of sensitized sheep cells and group A haemolytic streptococci. *Proc. Soc. exp. Biol. (N.Y.)* **88,** 35.

LAURELL A.B. & GRUBB R. (1958) Complement, complement components, properdin and agglutination promoting factors in rheumatoid arthritis. *Acta path. microbiol. scand.* **43,** 310.

LOSPALLUTO J. & ZIFF M. (1956) Purification of the accessory agglutinating factor of the serum in rheumatoid arthritis. *Ann. rheum. Dis.* **15,** 382.

MARSHALL A.G., HUTCHINSON E.O. & HONISETT J. (1962) Heredity in common diseases. A retrospective survey in twins in a hospital population. *Brit. med. J.* **7,** 1.

MELLORS R.C., HEIMER R., CORCOS J. & KORNGOLD L. (1959) Cellular origin of rheumatoid factor. *J. exp. Med.* **110,** 875.

MELLORS R.C., NOWOSLAWSKI A., KORNGOLD L.& SENGSON B.L. (1961) Rheumatoid factor and the pathogenesis of rheumatoid arthritis. *J. exp. Med.* **113,** 475.

MILGROM F., DUBISKI S. & WOZNICZKO G. (1956) A simple method of Rh determination. *Nature (Lond.)* **178,** 539.

MILGROM F. & WITEBSKY E. (1960) Studies on the rheumatoid and related serum factors. I. Autoimmunization of rabbits with gamma globulin. *J. Amer. med. Ass.* **174,** 56.

MILGROM F., WITEBSKY E., GOLDSTEIN K. & LOZA U. (1962) Studies on the rheumatoid and related serum factors. II. Relation of anti-human and anti-rabbit gamma globulin factors in rheumatoid arthritis serums. *J. Amer. med. Ass.* **181,** 476.

O'BRIEN W.M., BURCH T.A. & BUNIM J.J. (1963) A genetic analysis of the occurrence of rheumatoid factor and rheumatoid arthritis in 485 matings and 1633 sibling pairs in Pima and Blackfeet Indians. *Arthr. & Rheum.* **6,** 785.

ORESKES I., SINGER J.M. & PLOTZ C.M. (1961) Studies of human gamma globulin reactivity with rheumatoid arthritis sera. *Atti del X Congresso della Lega Internazionale contro il Rheumatismo*, vol. II, p. 796. Rome.

ORESKES I., SINGER J.M. & PLOTZ C.M. (1963) Reactivity of rheumatoid arthritis sera with altered and aggregated human γ-globulin. *J. Immunol.* **90,** 107.

PEASE P.E. (1965) *L Forms, Episomes and Auto-immune Disease.* Edinburgh: Livingston.

PELTIER A. & CHRISTIAN C.L. (1959) The presence of the rheumatoid factor in sera from patients with syphilis. *Arthr. & Rheum.* **2,** 1.

PHILLIPS J.M., KAKLAMANIS, P. & GLYNN L.E. (1966) Experimental arthritis associated with auto-immunization to inflammatory exudates. *Ann. rheum. Dis.* **25,** 165.

PLOTZ C.M. & SINGER J.M. (1956) The latex fixation test. II. Results in rheumatoid arthritis. *Amer. J. Med.* **21,** 893.

RANTZ L.A., RANDALL E. & KETTNER D. (1959) Electrophoretic study of the serum factors responsible for the serological reactions in rheumatoid arthritis with demonstration of two inhibitors. *Arthr. & Rheum.* **2,** 104.

ROPES M.W., BENNETT G.A., COBB C., JACOX R. & JESSAR R.A. (1959) Diagnostic criteria for rheumatoid arthritis. *Ann. rheum. Dis.* **18,** 49.

ROSE H.M., RAGAN C., PEARCE E. & LIPMAN M.O. (1948) Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. *Proc. Soc. exp. Biol. (N.Y.)* **68,** 1.

SCHROHENLOHER R.E., KUNKEL H.G., & TOMASI T.B. (1964) Activity of dissociated and reassociated 19S anti-γ-globulins. *J. exp. Med.* **120,** 1215.

SINGER J.M., ALTMANN G., GOLDENBERG A. & PLOTZ C.M. (1960) The mechanism of particulate carrier reactions with rheumatoid sera. II. Sensitizing capacity of various human gamma globulins for latex particles. *Arthr. & Rheum.* **3,** 515.

SINGER J.M. & PLOTZ C.M. (1956) The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. *Amer. J. Med.* **21,** 888.

SVARTZ N. (1960) The rheumatoid factor, its origin and nature. *Acta med. scand.* **168,** 285.

SVARTZ N., CARLSON L.A., SCHLOSSMANN K. & EHRENBERG A. (1958) Isolation of the rheumatoid factor. *Acta med. scand.* **160,** 87.

SVARTZ N. & SCHLOSSMANN K. (1955) Cold precipitable haemagglutinating factor in serum from patients with rheumatoid arthritis. *Ann. rheum. Dis.* **14,** 191.

SVARTZ N. & SCHLOSSMANN K. (1956) Inhibition of the haemagglutination reaction in rheumatoid arthritis by means of sulfa compounds. *Acta med. scand.*, **155,** 131.

VAUGHAN J.H. (1956) Behaviour of the rheumatoid arthritis agglutinating factor with immune precipitates. *J. Immunol.* **77,** 181.

VAUGHAN J.H. (1959) Serum responses in rheumatoid arthritis. *Amer. J. Med.* **26,** 596.

VAUGHAN J.H., ARMATO A., GOLDTHWAITE J.C., BRACKMAN P., FAVOUR C.B. & BAYLES T.B. (1955) A study of gamma globulin in rheumatoid arthritis. *J. clin. Invest.* **34,** 75.

VAUGHAN J.H. & HARRIS J. (1959) Transfusion of rheumatoid plasma and cells. *Arthr. & Rheum.* **2,** 51.

WAALER E. (1940) On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. *Acta path. microbiol. scand.* **17,** 172.

WAGER O. (1950) On the factor producing agglutination of sensitized red cells and its relation to the agglutination of haemolytic streptococci in rheumatoid arthritis sera. *Ann. med. exp. Fenn.* **28** (Suppl. 8), 154.

WAGER O. & LEHTINEN L. (1961) Rheumatoid serology of patients with incipient joint symptoms. *XIII scand. Congr. Path. Microbiol., Turku* 1961.

WALLER M.V. & VAUGHAN J.H. (1956) The use of anti-Rh sera for demonstrating agglutination activating factor in rheumatoid arthritis. *Proc. Soc. exp. Biol. (N.Y.)*, **92**, 198.

WHITE R.G. (1960) The relation of the cellular responses in germinal or lymphocytopoietic centres of lymph nodes to the production of antibody. In *Mechanisms of Antibody Production*. Prague: Czechoslovak Academy of Sciences.

WILLIAMS R.C. & KUNKEL H.G. (1962) Rheumatoid factors and their disappearance following therapy in patients with subacute bacterial endocarditis. *Arthr. & Rheum.* **5**, 126.

WILLIAMS R.C. & KUNKEL H.G. (1965) Observations on anti-γ-globulin factors in human sera and antibodies against autologous γ-globulins produced in rabbits. *Ann. N.Y. Acad. Sci.* **124**, 413.

WILLIAMS R.R., STONE S.S., JENKINS J., EVANS R.L. & BUNIM J.J. (1956) Some characteristics of the inhibitor system for the sensitized sheep cell agglutination reaction. *Ann. rheum. Dis.* **15**, 61.

ZIFF M., BROWN P., LOSPALLUTO J., BADIN J. & MCEWEN C. (1956) Agglutination and inhibition by serum globulin in the sensitized sheep cell agglutination reaction in rheumatoid arthritis. *Amer. J. Med.* **20**, 500.

ZIFF M., SCHMID F.R., LEWIS A.J. & TANNER M. (1958) Familial occurrence of the rheumatoid factor. *Arthr. & Rheum.* **1**, 392.

CHAPTER 32

SYSTEMIC LUPUS ERYTHEMATOSUS, SCLERODERMA AND DERMATOMYOSITIS

E.J. HOLBOROW

SYSTEMIC LUPUS ERYTHEMATOSUS

INTRODUCTION

Systemic lupus erythematosus was early recognized clinically as a complex disorder, and histopathological examination amply demonstrates its disseminated nature. As well as skin changes, widespread involvement of other tissues—joints, serous membranes, heart, blood vessels, kidneys and the cells of the blood—are well-recognized features of the pathology of the disease.

Klemperer, Pollack & Baehr (1941) concluded that the lesions of SLE are primarily lesions of connective tissue showing the histological changes described as fibrinoid necrosis, and seen also, to a varying extent, in the lesions of other diseases of connective tissue such as rheumatic fever, rheumatoid arthritis and scleroderma. Although fibrinoid necrosis has been regarded as a hallmark of allergic response on the part of connective tissue, changes more or less resembling fibrinoid are seen in many different pathological situations apparently unconnected with hypersensitivity, and it is doubtful how far significance in this respect can be attached to it.

The connective-tissue changes in SLE give rise to lesions in, for instance,

kidney glomeruli and splenic arterioles that are characteristic in histological appearance, but in themselves give little clue to the nature of the disease process. Histochemical analysis of fibrinoid necrotic material in SLE and in other conditions has led to no agreement about its fundamental nature. There is some agreement, however, on the nature of another histological feature of lupus lesions—the haemotoxylin-staining (haematoxophil) bodies, which although not always demonstrable are believed to be pathognomonic of the condition (Gross 1932). These bodies, irregular aggregates of material varying in diameter from a few to several hundred microns, are often seen in the fibrinoid lesions themselves. There is little doubt that they contain altered nuclear material, because of their characteristic Feulgen-staining and ultraviolet adsorption properties, and they could well represent the tissue counterpart of the same process of alteration of cell nuclei that leads *in vitro* to the formation of 'LE cells'.

The discovery in 1948 of the 'LE cell phenomenon' and its dependence on serum factors present in lupus patients began the present change in emphasis in the investigation of the disease, and since then a wealth of immunological features has been uncovered in the condition. Among the most characteristic of these are the serum antinuclear factors, and auto-antibodies directed at other non-organ-specific cellular antigens. It is now widely held that these are signs of a fundamental disturbance of the immune system that leads in some way to the appearance of the disseminated lesions already mentioned. Nevertheless, that a causal relationship exists between the known immunological abnormalities and the pathological tissue changes in lupus is still, save for some inconstant haematological features, far from certainly established, and the nature, fundamental or otherwise, of the underlying immunological disturbance remains obscure.

Antinuclear Antibodies

The LE Cell Phenomenon

Hargraves, Richmond & Morton (1948) showed that in smears made from incubated bone marrow or clotted blood taken from SLE patients, leucocytes with inclusion bodies in their cytoplasm may be found, often in large numbers. These cells are called lupus erythematosus cells, or LE cells, and investigation shows that the inclusion bodies consist of altered nuclei (usually derived from other leucocytes) which have been ingested by phagocytes. It was soon found by Haserick & Bortz (1949) that equally characteristic LE cells could be obtained if normal leucocyte preparations were incubated with SLE plasma or serum, and Miescher & Fauconnet (1954) showed that absorption of lupus serum with isolated nuclei would remove its ability to promote LE cell formation. Subsequently it has been repeatedly shown by electrophoretic and chromatographic analysis that the activity of lupus serum lies in its γ-globulin protein fraction (Haserick,

Lewis & Bortz 1950; Holman & Kunkel 1957; Goodman, Malmgren, Fahey & Brecher 1959).

The process that gives rise to the characteristic morphology of the LE cells takes place in two stages. The first of these is a reaction between a damaged or dead leucocyte (in which the nucleus is therefore accessible) and the LE serum factor. Following this, the nucleus swells, loses its structure and becomes homogeneous. This is the specific phase of the reaction. The second stage is the phagocytosis of this altered nucleus by a living leucocyte, a process which requires complement, but no other specific factor.

The formation of LE cells is essentially a laboratory phenomenon, and they have seldom been found ready made in circulating blood. However, Klemperer (1952) concluded that the characteristic haematoxophil bodies of lupus lesions are the degenerated remnants of a similar reaction occurring in the tissues.

Ultracentrifugal analysis of SLE serum shows that the LE serum factor is a 7S component of the γ-globulin, of molecular weight about 150,000 (Holman & Kunkel 1957; Goodman *et al* 1959). It is, therefore, similar to many other human antibodies, and in pregnant lupus patients it passes the placenta and appears in the foetal blood stream (Bridge & Foley 1954), where it apparently has no ill effects on the foetus.

The difficulty of defining accurately the limits within which a clinical diagnosis of SLE may be made makes it impossible to assert that the LE cell phenomenon can be demonstrated in every case. Many clinicians require a positive LE cell test before making the diagnosis, and most would agree that in active lupus the test is positive in more than 90% of cases. The number of LE cells found in preparations from a given case vary with disease activity, and in remissions they may disappear. Technical variations in LE cell tests have been extensively explored, in attempts to improve their sensitivity and specificity. They include *direct tests*, using the patients' whole blood, *indirect tests*, in which normal white cells provide both nuclear substrate for the patients' serum factor and phagocytes, and two-stage tests in which white-cell nuclei are separately sensitized and then exposed to the phagocytic action of normal white cells. Reviews by Marmont (1959), Lachmann (1961) and Dubois (1966a) have re-emphasized the morphological characteristics that define the LE cell.

A property of the LE cell factor shared by many of the antibodies described below is lack of species discrimination. It will readily react with nuclear material from rabbit white cells, or the nucleated erythrocytes of chickens, and sensitize them to give rise to LE cells (Hijmans & Schuit 1959). It is thus possible to use nuclear materials of animal origin (such as calf thymus, a rich source of nuclei) to study the properties of LE cell factor. Absorption experiments have shown that the latter is completely removed from lupus serum by only two types of nuclear material—isolated whole nuclei, and desoxyribonucleoprotein, the natural complex of desoxyribonucleic acid (DNA) and histone which constitutes

the major nucleoprotein component of cell nuclei. DNA alone does not absorb LE cell activity from the serum, nor does nucleoprotein from which histone has been removed by HCl treatment. Nucleoprotein treated with DNAse to remove DNA is likewise ineffective as an absorbing agent (Holman & Deicher 1959). The LE cell phenomenon is thus mediated by a factor reacting with the DNA-histone nuclear complex as a whole, and not with either constituent alone. Lachmann (1961) found in absorption experiments that LE cell factor reacts best with nucleohistone extracted at low ionic strength, and suggests that the most reactive antigenic configuration is presented by native nucleoprotein. In the native three-stranded DNA-DNA-protein helix the specific base-pairs point inwards, and thus it is the sugar-phosphate chains of the DNA and the peptide backbone of the histone that are presented outwards to meet with the LE cell factor. This may explain the striking lack of species and organ specificity required in the substrate in the LE cell test.

Antinuclear Factor (ANF)

The affinity of lupus sera for nuclei is particularly well shown by Coons's immunofluorescent method. A section of any unfixed tissue, or a smear of blood or other nucleated cell suspension, is exposed to a drop of lupus serum, washed, and 'stained' with fluorescein-labelled rabbit antibody to human γ-globulin. By ultraviolet microscopy such a preparation shows marked fluorescence of the cell nuclei, due to their uptake of specific antinuclear serum factor, which in turn is specifically combined with the fluorescent antiglobulin reagent (Holborow, Weir & Johnson 1957; Holman & Kunkel 1957; Friou, Finch & Detre 1958).

Lupus serum usually gives nuclear fluorescence by this method with the nuclei of all types of cell present in tissue sections or cell smears, whether these come from the patients' own tissues, from those of other individuals, or from the tissues of many different animal species. The only animal cells so far encountered which do not regularly give specific nuclear fluorescence are the heads of mature mammalian spermatozoa. In these the protein component of the nucleoprotein is protamine, which replaces histone during spermatogenesis (Vendrely, Knobloch & Vendrely 1957). The nuclei of immature spermatocytes, in which histone has not yet been replaced, give the usual nuclear fluorescence of somatic cells (Holborow & Weir 1959). This finding accords with the conclusion that both constituents of the DNA-histone complex participate jointly in reacting with at least one of the antinuclear antibodies of lupus. It must be mentioned, however, that Lachmann (1964) has shown that treatment with heparin renders sperm-heads stainable by some lupus sera.

A number of different antibodies to different nuclear constituents are identifiable in lupus sera and these are described below. Sera whose nuclear affinities are demonstrable by Coons's technique may therefore contain multiple nuclear antibodies, and the term 'antinuclear factor' (ANF) is widely used to

describe any γ-globulins reacting with nuclear material in this immunofluorescent context. In SLE, there is close correspondence between the results of LE cell tests and of ANF tests, and these two procedures might be regarded as two different ways of detecting the same factor. However, the fact that ANF, unlike LE cell factor, may belong to more than one immunoglobulin class, shows that this is not necessarily the case. It was observed by Goodman *et al* (1959) that while the LE cell activity of serum was confined to the 7S globulin fraction, ANF was demonstrable in both 7S and 19S fractions. More recently, the use of specific anti-IgG, anti-IgA and anti-IgM conjugates in immunofluorescence has shown that in SLE sera the ANF activity may reside in one, two or all three of these immunoglobulin classes (Barnett, Leddy, Condemi & Vaughan 1956; Rothfield, Frangione & Franklin 1965).

Further, in patients with rheumatoid arthritis (discussed more fully below) ANF usually occurs without demonstrable LE cell activity. This might be attributable to a greater sensitivity of the ANF test, but we have found (Holborow & Johnson 1965) that in rheumatoid sera the ANF is often destroyed by heating at 65°C, while in lupus sera ANF withstands this treatment. In addition, heat lability of ANF at 65°C is usually associated with activity in the IgM fraction. Treatment with mercaptoethanol, which dissociates IgM macromolecules, may destroy the ANF activity of some rheumatoid sera (Weir & Holborow 1962). This is not so with lupus sera.

These observations lead to the conclusion that antinuclear activity as detected by immunofluorescence may be quite independent of LE cell activity.

It may be mentioned here that Rothfield *et al* (1965) found among fifty-three SLE sera eight in which antinuclear activity, although detected with specific anti-IgM fluorescent antibody, proved on gel-filtration analysis to be located in a slowly sedimenting, mercaptoethanol-resistant immunoglobulin fraction.

Absorption of sera with calf thymus nucleoprotein usually removes ANF activity, while absorption with DNA alone consistently fails to do so. Some lupus sera, however, continue to show antinuclear reactivity even after absorption with nucleoprotein, indicating the presence of an antibody against another nuclear antigen. The existence of different nuclear antigens is also revealed by the different patterns of nuclear staining seen with positive sera (Beck 1961). In calf thyroid sections or rat liver sections or imprints, for example, the nuclei may be uniformly stained in homogeneous fashion (Plates 32.a, b, c) or they may show 'speckled' staining (sometimes superimposed on the homogeneous type) so that the nuclei show numerous minute points of fluorescence (Plate 32d.). Speckled staining is prevented by first washing the tissue section well in 0.15 M isotonic saline, but is not abolished by absorbing the serum with nucleoprotein, DNA or histone. It appears to be due, therefore, to a soluble nuclear antigen, distinct from nucleoprotein, and similar to the nuclear antigen extracted with phosphate buffer from nuclei by Holman, Deicher & Kunkel

(1959). Homogeneous staining, on the other hand, apparently involves the nucleoprotein itself.

Another pattern of nuclear staining was recognized by Casals, Friou & Teague (1963) who found certain lupus sera giving diffuse irregular staining spreading from the margins of leucocyte nuclei in smears of human peripheral blood. This 'shaggy' pattern they attribute to staining of a slowly diffusing slightly soluble nuclear component, probably DNA, since sera showing it give also positive complement fixation tests with DNA (see below). This pattern of staining appears to be due to the same antibody that gives the 'peripheral' staining of Gonzalez & Rothfield (1965), or (Plate 32.f) the 'membranous' staining of Beck (1963).

Another type of nuclear staining, less common, is of nucleoli, and is seen well when liver cell nuclei are used as substrate (Plate 32.e). It may well be due, as Beck suggests, to a factor reacting with the structural ribonucleic acid of the nucleolus. We have seen it with sera from dermatomyositis, scleroderma and rheumatoid arthritis.

Antibodies to Separated Nuclear Constituents

In the LE cell test, and in the immunofluorescent ANF test, the nuclear substrate material is provided by cells damaged in various ways that render the nucleus accessible to the action of serum antibodies. Complement fixation and precipitation methods have shown that, in lupus, antibodies may also be distinguished that react with several different components of cell nuclei.

Polli, Celada & Cepellini (1957) and Deicher, Holman & Kunkel (1959) described and characterized in lupus sera an antibody giving specific precipitation with DNA, demonstrable by double diffusion by Ouchterlony's method in agar gel. When a positive lupus serum is tested against DNA in this manner a precipitin line forms in the intervening agar. At acid pH, DNA forms non-specific salt complexes with serum proteins, but at pH values in the range 7–8 these do not occur, so that in fluid media at controlled pH and salt concentration quantitative precipitin curves may be obtained with DNA and lupus serum. The

---

PLATE 32.1. Demonstration of serum antinuclear factors (ANF) by indirect immunofluorescence using 1/10 dilutions of patients' sera, standardized anti-human globulin conjugate, and cryostat sections of snap-frozen tissue—except (c).

(a) SLE serum, homogeneous pattern ANF. Substrate, calf thyroid section. ×180.

(b) SLE serum, homogeneous pattern ANF. Substrate, rat liver section. ×240.

(c) SLE serum, homogeneous pattern ANF. Substrate, rat liver imprint. ×240.

(d) Rheumatoid arthritis serum, speckled pattern ANF. Substrate, calf thyroid. ×240.

(e) Scleroderma serum, nucleolar ANF. Substrate, rat liver section. ×240.

(f) Lupoid hepatitis serum, ring or membranous pattern ANF. Substrate, rat liver section. ×240.

facing p. 876

antibody has been recovered from the precipitated specific complex by using DNAse to release it.

If lupus serum giving a positive precipitin reaction is absorbed with DNA until all the precipitating antibody is removed, little if any decrease is found in the ability of the absorbed serum to promote LE cell formation. The anti-DNA antibody is thus distinct from the LE cell factor, and the two do not necessarily coexist. Seligmann, Cannat & Hamard (1965) have reported that most lupus sera from untreated cases give positive precipitin and complement fixation tests with both DNA and nucleoprotein, and that during treatment or in remission these reactions tend to disappear. The 'shaggy' anti-DNA factor of Casals *et al* (1963) was also noted only in sera from acutely ill cases of SLE. More lupus sera react immunologically with thermally denatured DNA than with native DNA (Levine, Murakami, van Vunakis & Grossman 1960) and this effect is due to a complement fixing reaction between single-stranded DNA present in the denatured material, and an antibody reacting specifically with this form of the DNA molecule (Stollar, Levine & Marmur 1962). Such an antibody was present in 66% of thirty-five SLE sera tested by Sturgill, Carpenter, Strauss & Goodman (1964) and also in some sera for rheumatoid arthritis, Sjogren's syndrome and myasthenia gravis; it was always accompanied by other antinuclear antibodies.

Hijmans & Schuit (1959) found that lupus sera may fix complement with suspensions of whole nuclei, with nucleoprotein, with DNA, and with histone, and Holman *et al* (1959) describe four different factors in lupus sera that react specifically and respectively in complement fixation tests with nucleoprotein, DNA, histone and a soluble component extractable from nuclei with phosphate buffer. LE cell activity, and ANF activity, may nevertheless exist in lupus sera in the absence of demonstrable complement-fixing antibodies against any of these components.

The results of testing SLE sera with nuclei, nucleoprotein, DNA and histone are consistent with the interpretation that the activity of the specific LE cell factor in lupus serum is directed against the desoxyribonucleic acid-protein complex, in which both DNA and histone, present in their natural combination, participate jointly in presenting the specific reactive site or sites. The reactivity of DNA separately, or of histone, or of the soluble material extracted from nuclei, with lupus sera is attributable to serum factors independent of the LE cell factor.

Complement-fixing antibodies against these nuclear antigens are found less frequently in SLE than LE factors or ANF. According to Hijmans & Schuit (1959) the antibodies against whole nuclei, nucleoprotein DNA and histone are found in descending order of frequency; 50–75% of cases give complement fixation with whole nuclei, and only occasional sera are positive with histone.

It is usually stated that antinuclear activity as demonstrated by immunofluorescence is not organ specific in substrate requirement. However, Feltkamp (1966) found that when several different tissues were used as substrate to test (for

example) a panel of rheumatoid arthritis sera, the incidence of sera giving positive staining varied from 4 to 19%; the lower incidences were obtained with heterologous tissue, the higher with human tissue. It was shown by Faber, Elling, Norup, Manson & Nissen (1964) that human antinuclear factors are occasionally encountered that react only with an antigen present in the nuclei of blood granulocytes.

Antibodies Against Cytoplasmic Constituents of the Cell

Auto-immune Complement Fixation Reaction

During attempts to detect infectious hepatitis virus in the livers of patients dying with this disease, Gajdusek (1937) found that certain human sera gave positive complement fixation tests with saline suspensions of homogenized normal human or rat liver or other organs. This test is distinct from the Wassermann test (where the antigen is an alcoholic liver extract), although both tests may be positive in some sera from both lupus and syphilis. Positive tests were found in acute viral hepatitis and in chronic liver disease, but the highest incidence (40–75% of cases) and usually the highest titres were found in SLE and in lupoid hepatitis (Mackay & Gajdusek 1958; Mackay & Larkin 1958; Hijmans, Doniach, Roitt & Holborow 1961). High titres were also found in some macroglobulinaemic sera. This is the reaction Gajdusek called the 'auto-immune complement fixation test' (AICF), and when positive with liver it is usually positive also with homogenates of a wide variety of other organs, although the preferential organ reactivity differs widely from case to case. The antibodies may be either IgG or IgM. Asherson (1959), Deicher, Holman & Kunkel (1960) and Wiedermann & Miescher (1965) prepared subcellular fractions from liver by ultracentrifugation, and showed that in positive sera complement-fixing activity may be directed singly or severally at antigens of the mitochondria, lysosomes, microsomes or soluble protein components of the cell cytoplasm, and is independent of any antibody against nuclear constituents that may also be present. Like antinuclear factors, the AICF antibodies in general lack species specificity, and react in similar fashion with human, calf and rat organ antigens. When present in high titre, AICF antibodies may give immunofluorescent staining of (for example) rat liver sections. The most readily recognizable pattern of non-organ-specific cytoplasm staining, however, is given by antibody directed against cell mitochondria ('M' antibody) and is best demonstrated on tissues containing cells rich in mitochondria, such as distal kidney tubules or gastric parietal cells. Although not uncommon in SLE, 'M' antibodies are seen especially in primary biliary cirrhosis (Walker, Doniach, Roitt & Sherlock 1965).

Deicher *et al* (1960) have described also an antibody which they have found

only in SLE, reacting specifically with a trypsin-sensitive antigen of cell microsomes extracted with TRIS-buffer.

Antibodies Against Other Cytoplasmic Constituents

Asherson & Broberger (1961) investigating sera from ulcerative colitis patients, found antibodies giving a haemagglutination test with sheep cells coated with phenol-water extract of human colon or liver. Sera from a variety of other conditions were usually negative, but in some cases of SLE, rheumatoid arthritis, and liver disease, similar haemagglutinins were present. These workers were unable to demonstrate complement fixation with this antigen by the usual methods, and it evidently differs from those present in saline extracts of liver, since there is no significant correlation between the presence of haemagglutinating and AICF antibodies. Using an agar diffusion method, Anderson, Gray, Beck, Buchanan & McElhinney (1962) demonstrated in twenty-one out of twenty-eight SLE sera precipitins reacting with saline extracts of human tissues; one of the four cellular constituents distinguished was DNA, and the other three (designated SjD, SjT and Lup) were considered to be cytoplasmic components probably capable of participating in the AICF reaction.

Biological False-positive Test for Syphilis

It has long been known that some cases (10–20%) of SLE give a positive Wassermann test. According to Harvey & Shulman (1966) a majority of these are biological false-positive (BFP) reactors, i.e. give negative treponemal immobilization tests. Among BFP reactors in the general population, a significant proportion (8%) develop SLE; in this group a long interval is usual between the initial serological positive and the clinical onset of the disease. In Harvey & Shulman's study, all SLE cases with a BFP reaction, and all BFP cases with SLE, were females. In addition, a familial aggregation of the BFP was found, and relatives of BFP reactors had a higher incidence of SLE and rheumatoid arthritis than would have been expected by chance alone. The significance of these observations lies in the fact that the cardio-lipin antigen used in the Wassermann test is a tissue phospholipid present in the organs of many species, including man, so that antibodies giving rise to a positive result, whether or not associated with syphilis, are essentially autoimmune in character.

A circulating auto-antibody with anticoagulant effect observed in some cases of SLE is reported to be strongly associated with the presence of a BFP reaction (Margolius, Jackson & Ratnoff 1961).

Antibodies Against Blood Cells

Positive antiglobulin tests with erythrocytes, both direct and indirect, are

obtained in 5–10% of cases of SLE (Dubois 1952; Dameshek 1958; Dacie 1959), sometimes in association with haemolytic anaemia, which may indeed be a principle or an early feature. The globulin reacting with the patients' own red cells is usually of the incomplete 'warm antibody' type. Its specificity may be directed against blood group antigens of the rhesus system, but more commonly shows no apparent blood group specificity, reacting with any human red cells. The antiglobulin test in lupus is, in most cases, inhibited by pretreatment of the Coombs' serum with human γ-globulin, which indicates the γ-globulin nature of the antibody on the red cells. Cases of 'auto-immune haemolytic anaemia' of similar type, however, are known in which this procedure does not readily inhibit agglutination, and in these the reaction may be of the complement, or of the mixed, type.

The antiglobulin consumption test performed on platelets and leucocytes from SLE patients is often positive (van Loghem, van der Hart, Hijmans & Schuit 1958; Dausset, Colombani & Colombani 1961), but it is not certain whether auto-antibodies directed at these blood cells are significant causes of the thrombocytopenia and leucopenia that may occur in SLE. Dausset (1965) has shown that the leucocyte antibodies react with the nuclei or cytoplasm of lymphocytes but not granulocytes.

Rheumatoid Factor

In the Rose-Waaler test, in which agglutination of sheep cells coated with rabbit amboceptor is measured, most normal sera are negative. In systemic lupus about 30% of cases give positive reactions—the highest incidence found in any disease group other than rheumatoid arthritis (Ziff 1957). In a series of fifty lupus cases, Hijmans *et al* (1961) found that twenty gave a positive agglutination test with latex particles coated with human pooled γ-globulins.

Incidence of Non-organ-specific Auto-Antibodies in Other Conditions

SLE is the only condition so far known in man in which the LE cell phenomenon is at some time demonstrable in virtually every case, but it has long been known that positive LE cell tests are also seen, although less commonly, in discoid lupus, in the group of diseases of connective tissue (for scleroderma and dermato-myositis see below), in chronic active liver disease, and occasionally following drug administration.

The immunofluorescent method of detecting serum ANF provides a readily applicable test for antinuclear antibodies that is widely used for surveying clinical material. Published results make it obvious, however, that the incidence of positive findings is significantly influenced by the techniques used in carrying out immunofluorescent tests, and point to an urgent need for standardization of methods if useful comparisons are to be made. Figures quoted here from our

own surveys refer to results obtained using a standardized immunofluorescent technique (Holborow & Johnson 1967) giving less than 1% positives with control 'normal' sera.

In rheumatoid arthritis, positive LE cell tests are unusual, but 20–25% of the classical or definite cases (classified according to the criteria of the American Rheumatism Association) we have examined by immunofluorescence for ANF give a positive result at the first testing. When cases are tested repeatedly, a higher incidence (35–40%) of positives is found. A recent analysis (Holborow & Johnson, unpublished) shows that positive ANF tests in rheumatoid arthritis are less closely correlated with particular clinical features than with the presence of rheumatoid serum factor. In rheumatoid arthritis the ANF titre is lower than in SLE, the factor is usually predominantly IgM and it may exhibit the heat lability at 65°C mentioned above (Baum & Ziff 1962; Holborow & Johnson 1965). Antinuclear antibodies were also present in the sera of 13% of a large series of cases of Still's disease (juvenile rheumatoid arthritis) examined by us.

The incidence of ANF in patients with discoid lupus erythematosus without clinical evidence of visceral involvement is given as 35% by Beck & Rowell (1966) in a recent report. They conclude that in discoid lupus ANF is more frequent among patients with positive rheumatoid factor tests, and that the presence of ANF does not materially alter the prognosis (less than 5% progressing to systemic lupus). Their conclusions for discoid lupus thus resemble our own for rheumatoid arthritis.

Apart from SLE, the highest incidence of non-organ-specific auto-antibodies is seen in Sjogren's syndrome, especially in cases with the keratoconjunctivitis sicca syndrome alone, unaccompanied by rheumatoid arthritis or other systemic connective tissue disease. Bunim (1965) reported that in the uncomplicated sicca syndrome, ANF is present in 88%, and AICF antibodies in 79% of cases.

In myasthenia gravis, ANF has been found to a varying extent. In the largest series reported there is an incidence of 10% (Van der Geld *et al* 1963). Antinuclear factors are commonly present in cases of liver cirrhosis with chronic jaundice of hepatocellular type where biopsy shows the liver to be heavily infiltrated with lymphocytes and plasma cells. This group (chronic active hepatitis, post-infective cirrhosis, juvenile cirrhosis) includes those cases with positive LE cell tests originally distinguished under the heading 'lupoid' hepatitis by MacKay, Taft & Cowling (1956). Most cases of chronic active hepatitis have also an auto-antibody to smooth muscle (demonstrable by immunofluorescence using rat tissue sections); this smooth muscle antibody has not been found in SLE (Johnson, Holborow & Glynn 1966). Antinuclear antibodies are present also in many cases of primary biliary cirrhosis (in addition to the 'M' antibody mentioned above) and in 'cryptogenic' cirrhosis, but not in alcoholic cirrhosis or viral hepatitis (Bouchier, Rhodes & Sherlock 1964; Doniach, Roitt, Walker & Sherlock 1966).

Positive LE cell tests, or ANF tests, may occasionally accompany drug reactions —hydrallazine is the best known example, but penicillin and other antibiotics may also be mentioned. It has been suggested that the lupus-like hydrallazine syndrome is in reality a drug hypersensitivity superimposed upon a latent SLE, and there is some clinical evidence favouring this view (Alarcón-Segovia *et al* 1965). Cannet & Seligmann (1966) report that 20% of a group of patients with pulmonary tuberculosis after prolonged treatment with isoniazid developed serum ANF, but could find no evidence of an underlying lupus diathesis.

In all the conditions quoted here, it is unusual to find ANF in as high titre as in SLE (1/500 or more).

The AICF test gives highest titres and most positives in SLE, but has also a considerable incidence in the chronic liver diseases in which ANF is found, especially in primary biliary cirrhosis, as might be expected (Doniach *et al* 1966).

Familial Factors

The literature contains a number of reports of striking aggregation of systemic lupus and other connective tissue diseases in families, and of its occurrence in identical twins; these instances have been summarized by Leonhardt (1964) and Dubois (1966). Nevertheless, several careful studies have failed to produce evidence that SLE and apparently related disorders of connective tissue are usually inherited on a genetic basis; familial aggregation appears to be the exception rather than the rule.

Thus in a high completion-rate study of first-degree relatives of 57 cases of SLE in Sweden, Leonhardt (1964) found 'collagen disease' (defined as chronic polyarthritis, chronic hepatitis, systemic sclerosis or SLE) in 2 of 115 male relatives and 12 of 110 female relatives; among the control subjects (relatives of spouses of cases or their families) the figures were 0/180 and 2/144 respectively; a small but significant difference in the females. In an earlier study of white, negro and Puerto Rican subjects in New York, Siegal, Lee, Widelock, Reilly, Wise, Zingale & Fuerst (1962) had found a small increase (4.9%) of rheumatoid arthritis in 142 family members of SLE cases as compared with an equal number of matched controls (1.4%). A subsequent study by Siegal, Lee, Widelock, Gwon & Kravitz (1965) confirmed a similar tendency among female relatives of patients with either SLE or rheumatoid arthritis. Ansell & Lawrence (1963), however, found no SLE among 127 relatives of 46 SLE probands, and no more rheumatoid arthritis (4 female cases) than would have been expected in a random population sample.

The plan of studies such as these has been criticized recently by O'Brien, Burch & Bunim (1964) who pointed out that using affected individuals to ascertain families results in severe bias and that the familial picture is more truly represented when a population survey is used as the means of ascertaining cases. Clearly serological investigation of SLE families is subject to a similar bias, and the

reported results no doubt reflect this, for the incidence of ANF in SLE relatives has been put at 33% (Pollak 1964) and 4% (Holborow & Johnson 1964; Siegal *et al* 1965). In Leonhardt's study the incidence of ANF in the controls was about 10%, and in SLE relatives was increased about four-fold. These discrepancies draw attention again to the difficulties of making useful comparison when different techniques are used. Siegal *et al* (1965) also reported γ-globulin levels in the sera of their subjects. They found the distribution curve of levels in SLE relatives similar to that in normals, except for a small secondary peak in the relatives in the upper-value range. This observation appears to support a view gaining ground at present regarding familial factors in SLE, viz. in the families of most cases, neither clinical nor serological abnormalities are usually encountered; but in a few families, both cases and serological abnormalities cluster strikingly. Whether in these latter families genetic or environmental factors are predominant is unknown.

The Role of Auto-antibodies in SLE in Producing Disease

The serum auto-antibodies in SLE are not necessarily implicated in the production of tissue damage. Both clinical and experimental study of anaphylaxis shows that the union of antigen and antibody within tissues can lead to cell damage through the release of histamine and other pharmacologically active substances, and that antigen-antibody precipitates formed in the tissues can evoke an inflammatory cellular response. The antigens against which many of the defined auto-antibodies of lupus are directed are, as we have seen, intracellular in situation. They are therefore apparently not accessible to circulating antibody, and unless evidence is forthcoming that auto-antibody can enter living intact tissue cells and react with these intracellular antigens there seem to be small grounds for incriminating this type of auto-antibody in producing lesions. In biopsy material from patients there is certainly no evidence from immunofluorescent study that antinuclear factors gain access *in vivo* to the nuclei of healthy cells. The haematoxophil bodies of lupus seem to arise rather from the combination of ANF with the exposed nuclei of dead or damaged cells. It is possible, however, that some tissue damage of anaphylactic type may arise from the effects of this and other immunological reactions involving products of disintegrated cells.

Most workers have found little or no correlation between titres of auto-antibody against cell constituents and the symptoms or severity of the disease, but there are one or two exceptions that may be important. Townes, Stewart & Osler (1963), for example, have used a quantitative complement fixation test involving rabbit anti-human γ-globulin to estimate antinucleoprotein antibodies in SLE and other diseases. They find that antibody levels are highest in SLE sera, and are usually associated with positive LE cell tests if they exceed 10 μg antibody N per ml. Their tests in thirty-five lupus patients showed a correlation

between high antinuclear antibody levels, low serum complement levels and the presence of renal disease as evidenced by renal biopsy as well as by urinary abnormalities and impaired renal function. In patients without evidence of kidney damage, antinucleoprotein antibody levels were lower, and serum complement higher, even though LE cell tests were positive. These observations, and others mentioned above, relating to the presence of anti-DNA antibodies in exacerbations of SLE underline the possibility that circulating antigen-antibody complexes, especially soluble complexes, may arise from autoimmune reactions in lupus patients and contribute to the pathogenesis of their disease. This possibility is strengthened when we consider together some other observations. It was shown by Tan, Schur & Kunkel (1965) that DNA may be demonstrated by double diffusion in agar in the sera of some SLE patients during febrile periods; in one patient, for example, they found that the decline of DNA antibody in the serum was followed by the appearance and persistence of free DNA. Further, the γ-globulin bound to affected glomeruli in lupus nephritis (Mellors, Ortega & Holman 1957) includes antinuclear antibody (Freedman & Markowitz 1959); and immunologically bound complement may be demonstrated at the same sites on the glomerular basement membrane (Lachmann, Muller-Eberhard, Kunkel & Paronetto 1962). It is therefore arguable that, in the absence of demonstrable specific antiglomerular antibodies, the renal lesions (and perhaps the vascular lesions) of lupus arise from the cytotoxic effects of deposited antigen-antibody complexes. On the other hand, binding of both γ-globulin and complement has also been demonstrated at the dermo-epidermal junction in the skin of LE patients (Kalsbeek & Cormane 1964) by an immunofluorescent method, and sthe fact that specific staining was as bright in sections from unaffected as from affected areas of skin appears to weaken the argument, at least in this case, that what is being demonstrated is a cytotoxic immune system.

One group of auto-antibodies in lupus—the haemolytic antibodies directed against red cells—has been shown to have an unequivocally destructive action, and these are antibodies reacting with cell surfaces. It is arguable that cytotoxicity of lupus serum may depend upon the presence of antibodies against cell membranes, rather than against inaccessible subcellular components, and a search for antibodies against tissue cell surfaces might be relevant to the problems of pathogenesis. The low complement levels in SLE (Williams & Law 1958; Ellis & Felix-Davies 1959), suggest that hidden antigen-antibody reactions may well be taking place.

Mention must be made here of delayed hypersensitivity to human tissue. It has been claimed that in SLE and discoid lupus intradermal injection of the patient's own leucocytes gives rise to a significant delayed reaction (Friedman, Bardawil, Merril & Hanan 1960; Bennett & Holley 1961), but some have failed to show this (Gerstein & Knox 1963; Nikolic & Holborow, unpublished). Varelzidis & Turk (1966) found no skin reaction to autologous separated blood

mononuclear cells in SLE cases with ANF. They point out that positive results obtained with leucocytes may be due to hypersensitivity to drugs or bacterial products carried by phagocytic cells. In discoid lupus with systemic involvement, however, they found some delayed skin reactions to separated blood monocytes.

The Nature of the Immunological Disorder in SLE

The serum factors which may be present in systemic lupus are remarkable in at least two respects—their multiplicity and the nature of the antigens with which they react. They have a strong claim to be considered as antibodies, since they are γ-globulins with immunochemical characteristics indistinguishable from those of many more familiar iso- and hetero-antibodies of human origin, and exhibit a degree of specificity which is not less striking because in many cases it cuts across the limits of species. Antigenic specificities common to several species were adumbrated by Landsteiner's work on serum proteins, and it is not surprising that macromolecular components of cells of different species may possess some determinant groups in common as a consequence of the structural and functional needs of cellular existence. Just as antigens may be organ specific rather than species specific, so some determinants may be common to cell components rather than more narrowly to organs or species. It is characteristic of lupus that antibodies are produced that react with nuclear and cytoplasmic antigens of this sort. Two questions thus arise—do these non-organ-specific antibodies represent a normal immune response to macromolecular components of nuclei or cytoplasm, liberated from damaged tissue cells, and remaining or becoming antigenic through some defect or aberration of catabolism? Or does the production of antibodies of this type signify an inherited or acquired abnormality of lymphoid cell function leading to over-production of plasma cells and the production of γ-globulin molecules with random combining configurations, some of which match nuclear or cytoplasmic determinants?

There is considerable evidence that antibodies reacting with nuclear constituents, especially DNA, may be induced experimentally by appropriate immunization with bacterial vaccines (Miescher, Cooper & Benacerraf 1960; Christian, De Simone, Abruzzo 1965), bacteriophage (Levine *et al* 1960), or with purines (Butler, Beiser, Erlanger, Tanenbaum, Cohen & Bendich 1962), pyrimidines (Tanenbaum & Beiser 1963) or nucleotides (Erlanger & Beiser 1964) conjugated to serum proteins.

Injection of DNA does not result in antibody production, but if DNA is complexed with a suitable protein and injected into rabbits, anti-DNA antibodies may be produced (Plescia, Braun & Palczuk 1964). Recently Barnett & Vaughan (1966) showed that rabbits suitably immunized with human serum produce antinuclear antibody demonstrable by immunofluorescence but specific for human

leucocyte nuclei, and attributed this result to the presence of protein-bound DNA in human serum. When anti-DNA antibodies are obtained by these methods they usually react better with denatured (single-stranded) than with native DNA, but they may, as in Barnett & Vaughan's instance, show a narrow specificity for one nuclear antigen, unlike the antinuclear factors characteristic of SLE. No lesions have been found in rabbits producing anti-DNA antibody, but the experiments support the idea that immunization with tissue breakdown products could account for the appearance of ANF in human disease. The marked clustering of ANF in the disease groups discussed above, however, seems to imply also an unusual responsiveness on the part of these patients to antigenic stimuli of this sort. It is known that SLE patients are more likely than others to exhibit drug and blood transfusion reactions (Leonhardt 1964) but it is doubtful whether they show increased immune reactions in general.

At present there is no clear evidence towards answering the second question—whether the lymphoid tissues of patients with SLE are functionally deranged, and whether the production of non-organ-specific auto-antibodies is a primary consequence of this derangement. MacKay (1966) reports a 'thymic dysplasia' in nine cases of SLE in which the thymus was small, depleted of lymphoid tissue, and contained accumulations of spindle epithelial cells. The periarticular fibrosis in the Malpighian corpuscles of the spleen is well known, but its significance and its effect on immune function is unknown. Cruikshank (1958) has drawn attention to the large numbers of plasma cells in the medullary tissue of the lymph nodes in SLE, and to the lack of follicular hyperplasia; in contrast, leucopenia, often with lymphopenia, is a well-recognized feature of the disease. However, to what extent, if any, these changes in SLE have their counterpart in abnormal immune function of the lymphoid tissues is so far conjectural.

SCLERODERMA AND DERMATOMYOSITIS

Scleroderma (systemic sclerosis) is another generalized disorder of the connective tissue. It is characterized by marked swelling and deposition of collagen in the affected skin and tissues, but shows little histological evidence of involvement of the lymphoid tissues; the synovial membranes of affected joints may show a mild degree of infiltration by lymphocytes and plasma cells. Nevertheless, serological abnormalities are common. In about half the cases the serum γ-globulin is moderately raised (up to 2 g%) and positive LE cell tests have been reported (Rodnan 1963). ANF is present in the sera of a majority of cases of scleroderma—in 22 of 27 cases reported by Fennel, Rodnan & Vazquez (1962) in 25 of 32 cases reported by Beck, Anderson, Gray & Rowell (1963), and in 30 of 33 cases reported by Burnham, Fine & Neblett (1966). The latter claim that nucleolar and speckled patterns of nuclear fluorescence are an important distinguishing characteristic of the ANF of scleroderma, but this is not our

experience (Johnson & Holborow, unpublished) nor that of Beck & Hughes (1966); both these patterns are seen in other conditions.

In dermatomyositis, an uncommon inflammatory disease of the skin muscles and blood vessels, muscle fibre degeneration and necrosis is accompanied by interstitial infiltration of lymphocytes and plasma cells, sometimes focal and sometimes diffuse. No evidence is yet forthcoming, however, of anti-muscle antibodies in this condition (Pearson 1966), whether of the type found in myasthenia gravis directed at A-bands of striated muscle (Strauss, Seegal, Hsu, Burkholder, Nastuk & Osserman (1960) or of the anti-myosin type demonstrated by Caspary, Gubbay & Stern (1964) in a proportion of cases of polymyositis, muscular dystrophy and muscular atrophy.

ANF has been found in several cases of dermatomyositis (Burnham *et al* 1966; Pearson 1966); of 13 cases we have tested, 8 were ANF positive (Holborow & Johnson, unpublished).

REFERENCES

ALARCÓN-SEGOVIA D., WORTHINGTON J.W., WARD L.E. & WAKIM K.G. (1965) Lupus diathesis and the hydrallazine syndrome. *New Eng. J. Med.* **272,** 462.

ANDERSON J.R., GRAY K.G., BECK J.S., BUCHANAN W.W. & MCELHINNEY A.J. (1962) Precipitating auto-antibodies in the connective tissue diseases. *Ann. rheum. Dis.* **21,** 360.

ANSELL B.M. & LAWRENCE J.S. (1963) A family study in lupus erythematosus. *Arthr. & Rheum.* **6,** 260.

ASHERSON G.L. (1959) Antibodies against nuclear and cytoplasmic constituents in systemic lupus erythematosus and other diseases. *Brit. J. exp. Path.* **40,** 209.

ASHERSON G.L. & BROBERGER O. (1961) Incidence of haemagglutinating and complement fixing antibodies. *Brit. med. J.* **1,** 1429.

BARNETT E.V., LEDDY J.P., CONDEMI J.J. & VAUGHAN J.A. (1965) Antinuclear factors in rheumatoid arthritis. *Ann. N.Y. Acad. Sci.* **124,** 896.

BARNETT E.V. & VAUGHAN J.H. (1966) Antinuclear antibodies in rabbit antisera. *J. exp. Med.* **123,** 733.

BAUM J. & ZIFF M. (1962) 7S and macroglobulin antinuclear fluorescence factors in systemic lupus erythematosus and rheumatoid arthritis. *Arthr. & Rheum.* **5,** 632.

BECK J.S. (1961) Variations in the morphological patterns of 'auto-immune' nuclear fluorescence. *Lancet* **1,** 1203.

BECK J.S. (1963) Auto-antibodies to cell nuclei. *Scot. med. J.* **8,** 373.

BECK J.S., ANDERSON J.R., GRAY K.G. & ROWELL N.R. (1963) Antinuclear and precipitating auto-antibodies in progressive systemic sclerosis. *Lancet* **2,** 1088.

BECK J.S. & HUGHES P. (1966) Classification of systemic lupus erythematosus (letter). *Lancet* **2,** 442.

BECK J.S. & ROWELL N.R. (1966) Discoid lupus erythematosus. *Quart. J. Med.* **35,** 119.

BENNETT C. & HOLLEY H.L. (1961) Intradermal hypersensitivity in systemic lupus erythematosus. *Arthr. & Rheum.* **4,** 64.

BOUCHIER I.A.D., RHODES K. & SHERLOCK S. (1964) Serological abnormalities in patients with liver disease. *Brit. med. J.* **1,** 592.

BRIDGE R.G. & FOLEY F.E. (1954) Placental transmission of the lupus erythematosus factor. *Amer. J. med. Sci.* **227,** 1.

BUNIM J.J. (1965) The frequent occurrence of hypergammaglobulinaemia and multiple tissue antibodies in Sjogren's syndrome. *Ann. N.Y. Acad. Sci.* **124,** 852.

BURNHAM T.K., FINE G. & NEBLETT T.R. (1966) The immunofluorescent tumor imprint technique. II. The frequency of antinuclear factors in connective tissue diseases and dermatoses. *Ann. Int. Med.* **65,** 9.

BUTLER V.P., BEISER S.M., ERLANGER B.F., TANENBAUM S.W., COHEN S. & BENDICH A. (1962) Purine-specific antibodies which react with desoxyribonucleic acid (DNA). *Proc. Nat. Acad. Sci.* **48,** 1597.

CANNAT A. & SELIGMANN M. (1966) Possible induction of antinuclear antibodies by isoniazid. *Lancet* **i,** 185.

CASALS S.P., FRIOU G.J. & TEAGUE P.O. (1963) Specific nuclear reaction pattern of antibody to DNA in lupus erythematosus sera. *J. Lab. clin. Med.* **62,** 625.

CASPARY E.A., GUBBAY S.S. & STERN G.M. (1964) Circulating antibodies in polymyositis and other muscle-wasting disorders. *Lancet* **2,** 941.

CHRISTIAN C.L., DE SIMONE H.R. & ABRUZZO J.L. (1965) Anti-DNA antibodies in hyperimmunized rabbits. *J. exp. Med.* **121,** 309.

CRUIKSHANK B. (1958) Lesions of lymph nodes in rheumatoid disease and in disseminated lupus erythematosus. *Scot. med. J.* **3,** 110.

DACIE J.V. (1959) Acquired haemolytic anaemias. *Brit. med. Bull.* **15,** 67.

DAMESHEK W. (1958) Systemic lupus erythematosus: a complex auto-immune disorder? *Ann. intern. Med.* **48,** 707.

DAUSSET J. (1965) Auto antileukocyte ribosomal fraction in leukoneutropenias. *Ann. N.Y. Acad. Sci.* **124,** 550.

DAUSSET J., COLOMBANI J. & COLOMBANI N. (1961) Study of leukopenias and thrombocytopenias by the direct antiglobulin consumption test on leucocytes and/or platelets. *Blood* **18,** 672.

DEICHER H.R.G., HOLMAN H.R. & KUNKEL H.G. (1959) The precipitin reaction between DNA and a serum factor in systemic lupus erythematosus. *J. exp. Med.* **109,** 97.

DEICHER H.R.G., HOLMAN H.R. & KUNKEL H.G. (1960) Anti-cytoplasmic factors in the sera of patients with systemic lupus erythematosus and certain other diseases. *Arthr. & Rheum.* **3,** 1.

DONIACH D., ROITT I.M., WALKER J.G. & SHERLOCK S. (1966) Tissue antibodies in primary biliary cirrhosis, active chronic (lupoid) hepatitis, cryptogenic cirrhosis and other liver diseases and their clinical implications. *Clin. & Exp. Immunology* **1,** 237.

DUBOIS E.L. (1952) Acquired haemolytic anaemia as the presenting syndrome of lupus erythematosus disseminatus. *Amer. J. Med.* **12,** 197.

DUBOIS E.L. (1966a) The lupus erythematosus cell test. In *Lupus Erythematosus*, chap. 12, ed. DUBOIS E.L. New York: McGraw-Hill.

DUBOIS E.L. (1966b) The clinical picture of systemic lupus erythematosus. In *Lupus Erythematosus*, chap. 9, ed. DUBOIS E.L. New York: McGraw-Hill.

ELLIS H.A. & FELIX-DAVIES D. (1959) Serum complement, rheumatoid factor, and other serum proteins in rheumatoid disease and systemic lupus erythematosus. *Ann. rheum. Dis.* **18,** 215.

ERLANGER B.F. & BEISER S.M. (1964) Antibodies specific for ribonucleosides, and their reaction with DNA. *Proc. Nat. Acad. Sci.* **52,** 68.

FABER V., ELLING P., NORUP G., MANSA B. & NISSEN N.I. (1964) An antinuclear factor specific for leucocytes. *Lancet* **ii,** 344.

FELTKAMP T.E.W. (1966) Idiopathic autoimmune diseases. Academisch proefschrift, Amsterdam University.

FENNELL R.H., RODNAN G.P. & VAZQUEZ J.J. (1962) Variability of tissue localizing properties of serum from patients with different disease states. *Lab. Invest.* **11,** 24.

FREEDMAN P. & MARKOWITZ A.S. (1959) Immunological studies in nephritis. *Lancet* **2,** 45.

FRIEDMANN E.A., BARDAWIL W.A., MERRILL J.P. & HANAN C. (1960) 'Delayed' cutaneous hypersensitivity to leukocytes in disseminated lupus erythematosus. *New Engl. J. Med.* **262,** 486.

FRIOU G.F., FINCH S.C. & DETRE K.D. (1958) Interaction of nuclei and globulin from lupus erythematosus serum demonstrated with fluorescent antibody. *J. Immunol.* **80,** 324.

GAJDUSEK D.C. (1957) An 'auto-immune' reaction against human tissue antigens in certain chronic diseases. *Nature (Lond.)* **179,** 666.

GERSTEIN W. & KNOX J.M. (1963) Intradermal leucocyte test in discoid lupus erythematosus. *Brit. med. J.* **2,** 901.

GONZALEZ E.N. & ROTHFIELD N.F. (1965) The relation of immunoglobulin class of antinuclear factor, pattern of nuclear fluorescence and clinical manifestations in patients with systemic lupus erythematosus. *Arthr. & Rheum.* **8,** 444.

GOODMAN H.C., MALMGREN R.A., FAHEY J.L. & BRECHER G. (1959) Separation of factors in lupus erythematosus serum reacting with components of cell nuclei. *Lancet* **ii,** 382.

GROSS L. (1932) Contributions to the med. Science in Honor of Emanuel Lipton *et al.* Vol. 2, p. 537. New York: International Press.

HARGRAVES M.M., RICHMOND H. & MORTON R. (1948) Presentation of two bone marrow elements: the 'tart' cell and the 'L.E.' cell. *Proc. Staff Meet. Mayo Clin.* **23,** 25.

HARVEY A.M. & SHULMAN L.E. (1966) Systemic lupus and the chronic biological false-positive test for syphilis. In *Lupus Erythematosus*, chap. 7, ed. DUBOIS E.L. New York: McGraw-Hill.

HASERICK J.R. & BORTZ D.W. (1949) Normal bone marrow inclusion phenomenon induced by lupus erythematosus plasma. *J. Invest. Derm.* **13,** 47.

HASERICK J.R., LEWIS L.A. & BORTZ D.W. (1950) Blood factor in acute disseminated lupus erythematosus. I. Determination of gamma globulin as specific plasma fraction. *Amer. J. med. Sci.* **219,** 660.

HIJMANS W., DONIACH D., ROITT I.M. & HOLBOROW E.J. (1961) Serological overlap between lupus erythematosus, rheumatoid arthritis and thyroid auto-immune disease. *Brit. med. J.* **ii,** 909.

HIJMANS W. & SCHUIT H.R.E. (1959) Studies on the L.E. cell phenomenon. III. Complement fixation with nuclear substances and the L.E. factor. *Vox Sang.* **4,** 376.

HOLBOROW E.J. & JOHNSON G.D. (1964) Antinuclear factor in systemic lupus erythematosus. A consideration of the immunofluorescent method of detecting antinuclear antibodies, with results obtained in a family study. *Arthr. & Rheum.* **7,** 119.

HOLBOROW E.J. & JOHNSON G.D. (1965) The nature of antinuclear immunoglobulins. *Ann. N.Y. Acad. Sci.* **124,** 833.

HOLBOROW E.J. & JOHNSON G.D. (1967) Immunofluorescence. In *Handbook of Experimental Immunology*, p. 571, ed. WEIR D.M. Blackwell Scientific Publications.

HOLBOROW E.J. & WEIR D.N. (1959) Histone: an essential component for the lupus erythematosus antinuclear reaction. *Lancet* **i,** 809.

HOLBOROW E.J., WEIR D.M. & JOHNSON G.D. (1957) A serum factor in lupus erythematosus with affinity for tissue nuclei. *Brit. med. J.* **ii,** 732.

HOLMAN H.R. (1959) Systemic lupus erythematosus—disease of an unusual immunologic responsiveness? *Amer. J. Med.* **27,** 525.

HOLMAN H.R. & DEICHER H.R.G. (1959) The reaction of the lupus erythematosus (L.E.) cell factor with deoxyribonucleoprotein of the cell nucleus. *J. clin. Invest.* **38,** 2059.

HOLMAN H. & DEICHER H.R.G. (1960) The appearance of hypergammaglobulinaemia, positive serological reactions for rheumatoid arthritis, etc. in the sera of relatives of patients with systemic lupus erythematosus. *Arthr. & Rheum.* **3,** 244.

HOLMAN H.R., DEICHER H.R.G. & KUNKEL H.G. (1959) The L.E. cell and L.E. serum factors. *Bull. N.Y. Acad. Med.* **35,** 409.

HOLMAN H.R. & KUNKEL H.G. (1957) Affinity between the lupus erythematosus serum factor and cell nuclei and nucleoprotein. *Science* **126,** 162.

JOHNSON G.D. & HOLBOROW E.J. (1961) Heat stability of antinuclear factor in systemic lupus erythematosus. *Lancet* **ii,** 663.

JOHNSON G.D., HOLBOROW E.J. & GLYNN L.E. (1966) Antibody to smooth muscle in patients with liver disease. *Lancet* **ii,** 878.

KALSBEEK G.L. & CORMANE R.H. (1964) Bound complement in the skin of patients with chronic discoid lupus erythematosus and systemic lupus erythematosus. *Lancet* **ii,** 178

KLEMPERER P. (1952) *Progress in Fundamental Medicine*, chap. 3, p. 51, ed. MCMANUS and FEBIGER, Philadelphia.

KLEMPERER P., POLLACK A.D. & BAEHR G. (1941) Pathology of disseminated lupus erythematosus. *Arch. Path.* **32,** 569.

LACHMANN P.J. (1961) An attempt to characterize the lupus erythematosus cell antigen. *Immunology* **4,** 153.

LACHMANN P.J. (1964) The reactivity of antinuclear factors with DNA-nucleoproteins. *Ann. rheum. Dis.* **23,** 311.

LACHMANN P.J., MULLER-EBERHARD H.J., KUNKEL H.G. & PARONETTO F. (1962) The localization of in vivo bound complement in tissue sections. *J. exp. Med.* **115,** 63.

LEONHARDT T. (1964) Family studies in systemic lupus erythematosus. *Acta med. Scand. Suppl.* **416.**

LEVINE L., MURAKAMI W.T., VAN VUNAKIS H., GROSSMAN L. (1960) Specific antibodies to thermally denatured desoxyribonucleic acid of phage T4. *Proc. Nat. Acad. Sci., U.S.* **46,** 1038.

MACKAY I.R. (1966) Histopathology of the human thymus. In *Thymus, Experimental and Clinical Studies: Ciba Foundation Symposium*, p. 449, ed. WOLSTENHOLME G.E.N. & PORTER R. London: J. & A. Churchill.

MACKAY I.R. & GAJDUSEK D.C. (1958) An auto-immune reaction against human tissue antigens in certain acute and chronic diseases. *Arch. intern. Med.* **101,** 30.

MACKAY I.R. & LARKIN L. (1958) The significance of the presence in human serum of complement-fixing antibodies to human tissue antigens. *Aust. Ann. Med.* **7,** 251.

MACKAY I.R., TAFT L.I. & COWLING D.C. (1956) Lupoid hepatitis. *Lancet* **ii,** 1323.

MARGOLIUS A., JACKSON D.P. & RATNOFF O.D. (1961) Circulating anticoagulants: a study of 40 cases and review of the literature. *Medicine (Baltimore)* **40,** 145.

MARMONT A. (1959) Nucleolytic phagocytosis (L.E. cell phenomenon) in systemic lupus erythematosus, rheumatoid arthritis, and systemic scleroderma. In *Immunopathology*, p. 479, ed. GRABAR P. & MIESCHER P. Basel. Schwabe & Co., Basle.

MELLORS R.C., ORTEGA L.G. & HOLMAN H.R. (1957) Role of gamma globulins in pathogenesis of renal lesions in systemic lupus erythematosus and chronic membranous glomerulonephritis, with an observation on lupus erythematosus cell reaction. *J. exp. Med.* **106,** 191.

MIESCHER P., COOPER N.S. & BENACERRAF B. (1960) Experimental production of antinuclear antibodies. *J. Immunol.* **85,** 27.

MIESCHER P. & FAUCONNET M. (1954) L'absorption du facteur 'L.E.' par des noyaux cellulaires isolés. *Experientia* **10,** 252.

PEARSON C.M. (1966) Polymyositis and Dermatomyositis. In *Immunological Diseases*, p. 758 ed. SAMTER M. London: J. & A. Churchill.

O'Brien W.M., Burch T.A. & Bunim J.J. (1963) A genetic analysis of the occurrence of rheumatoid factor (RF) in 485 matings and 1633 sibling pairs in Pima and Blackfeet Indians. *Arthr. & Rheum.* **6,** 785.

Plescia O.J., Braun W. & Palczuk N.C. (1964) Production of antibodies to denatured desoxyribonucleic acid (DNA). *Proc. Nat. Acad. Sci.* **52,** 279.

Pollak V.E. (1964) Antinuclear antibodies in families of patients with systemic lupus erythematosus. *New Engl. J. Med.* **271,** 165.

Polli E., Celada F. & Ceppelini R. (1957) A proposito di un fattore serico, del lupus eritematoso sistemico, reagente con l'acido desossipentosnucleico. *Boll. Ist. sieroter. milan* **36,** 454.

Rodnan G.P. (1963) The natural history of progressive systemic sclerosis (diffuse scleroderma). *Bull. Rheum. Dis.* **13,** 301.

Rothfield N.F., Frangione B. & Franklin E.C. (1965) Slowly sedimenting mercaptoethanol-resistant antinuclear factors related antigenically to M immunoglobulins (γ-M globulin) in patients with systemic lupus erythematosus. *J. Clin. Invest.* **44,** 62.

Seligmann M., Cannat A. & Hamard M. (1965) Studies on antinuclear antibodies. *Ann. N.Y. Acad. Sci.* **124,** 816.

Siegal M., Lee S.L. Widelock D., Gwon N.V. & Kravitz H. (1965) A comparative family study of rheumatoid arthritis and systemic lupus erythematosus. *New Engl. J. Med.* **273,** 893.

Siegal M., Lee S.L., Widelock D., Reilly E.B., Wise G.J., Zingale S.B. & Fuerst H.T. (1962) The epidemiology of systemic lupus erythematosus: preliminary results in New York City. *J. Chron. Dis.* **15,** 131.

Stollar D., Levine L. & Marmur J. (1962) Antibodies to denatured desoxyribonucleic acid in lupus erythematosus serum. II. Characterization of antibodies in several sera. *Biochim. biophys. Acta* **61,** 7.

Strauss A.J.L., Seegal B.C., Hsu K.C., Burkholder P.M., Nastuk W.L. & Osserman K.E. (1960) Immunofluorescence demonstration of a muscle binding, complement-fixing serum globulin fraction in myasthenia gravis. *Proc. Soc. exp. Biol. Med.* **105,** 184.

Sturgill B.C., Carpenter R.R., Strauss A.J.L. & Goodman H. (1964) Antibodies in systemic lupus erythematosus and myasthenia gravis which react with thermally denatured DNA-coated bentonite. *Proc. Soc. exp. Biol. Med.* **115,** 246.

Tan E.M., Schur P.H. & Kunkel H.G. (1965) DNA in the serum of patients with systemic lupus erythematosus (SLE). *J. clin. Invest.* **44,** 1104.

Tanenbaum S. & Beiser S.M. (1963) Pyrimidine-specific antibodies which react with desoxyribonucleic acid (DNA). *Proc. Nat. Acad. Sci.* **49,** 662.

Townes A.S., Stewart C.R. & Osler A.G. (1963) Immunological studies of systemic lupus erythematosus. I and II. *Bull. Johns Hopkins Hosp.* **112,** 183, 202.

van der Geld H., Feltkamp T.E.W., van Loghem J.J., Oosterhuis H.J.G.H. & Biemond A. (1963) Multiple antibody production in myasthenia gravis. *Lancet* **ii,** 373.

van Loghem J.J., van der Hart M., Hijmans W. & Schuit H.R.E. (1958) The incidence and significance of complete and incomplete white cell antibodies with special reference to the use of the Coombs consumption test. *Vox Sang.* **3,** 203.

Varelzidis A. & Turk J.L. (1966) Significance of reactions to intradermal injection of autologous granulocytes, mononuclear cells and serum. *Brit. med. J.* **2,** 379.

Vendrely R., Knobloch A. & Vendrely C. (1957) An attempt of using biochemical methods for cytochemical problems: the desoxyribonucleoprotein of spermatogenetic cells of bull testis. *Exp. Cell. Res. Suppl.* **4,** 279.

Walker J.G., Doniach D., Roitt I.M. & Sherlock S. (1965) Serological tests in the diagnosis of primary biliary cirrhosis. *Lancet* **i,** 827.

WIEDERMANN G. & MIESCHER P.A. (1965) Cytoplasmic antibodies in patients with systemic lupus erythematosus. *Ann. N.Y. Acad. Sci.* **124,** 807.

WEIR D.M. & HOLBOROW E.J. (1962) Serum antinuclear factor: laboratory studies. *Ann. rheum. Dis.* **21,** 40.

WILLIAMS R.C. & LAW D.H. (1958) Serum complement in connective tissue disorders. *J. Lab. clin. Med.* **52,** 273.

ZIFF M. (1957) The agglutination reaction in rheumatoid arthritis. *J. chron. Dis.* **5,** 644.

SECTION IV

THE ALLERGIC STATE AS RESPONSIBLE FOR CLINICAL HYPERSENSITIVITY AND DISEASE

B. ALLERGIC DISEASES PRIMARILY AFFECTING INDIVIDUAL ORGANS

CHAPTER 33

SKIN AUTOSENSITIZATION

W.E.PARISH & A.ROOK

INTRODUCTION

Whitfield (1921, 1922, 1931) was the first to suggest that an individual may become sensitized to his own skin, as a result of which a generalized eruption may develop about 10 days after local trauma to intact skin or to a circumscribed area of dermatitis. He proposed the concept, solely on the basis of clinical observation, that sensitization to some component of skin in a local area would result in the formation of some type of antibody to skin capable of causing widespread dermatitis.

Autosensitization dermatitis has remained a clinical concept and the term has been applied loosely to the secondary dissemination of chronic eczema usually of the lower leg. It is not a diagnosis, for the secondary dissemination is rarely due to auto-allergy to skin.

Several accounts of eruptions of this type have been published (Brown 1939; Hopkins & Burky 1944; Smith 1945; Haxthausen 1955; Young 1958) but with little agreement as to the nature of the process involved. It has been difficult to isolate or demonstrate the antigen responsible for the sensitization (Parish 1960), and without good evidence that some component of the body's own tissues provides the antigenic stimulus, a sensitivity to some allergen of the environment which has become closely associated with the skin may be overlooked.

DEFINITION OF AUTOSENSITIZATION TO SKIN

General Features of Autosensitization Reactions

Autosensitization is the sensitization of the body, by circulating antibody, or a delayed-type reaction, or both, to constituents of its own tissues. There is first: (*a*) autostimulation—the formation of antibody stimulated by antigen that is a component of the individual's tissues, and then (*b*) autoreaction—this antibody combines specifically with tissue antigen which may or may not produce a cytotoxic effect.

Auto-allergy is a more appropriate term than autosensitization, particularly if disease results, but since Whitfield's (1921, 1922) original description, autosensitization is the term generally used by dermatologists.

The two forms of allergic response that may result from autosensitization to skin are Type II, in which a circulating antibody to skin may be developed that is capable of causing a cytotoxic effect when reacting with cells of skin containing the specific antigen, and Type IV, in which a similar effect may be mediated by immunologically active leucocytes.

However, a Type II response may also follow the reaction between a circulating antibody and an antigen which was not part of the normal structure of the cell but had become intimately associated with or incorporated within it. Unless such an antigen was some constituent of the body, this reaction would not be an autosensitization.

The skin is constantly exposed to a large number of antigens of the environment, some of which are capable of becoming intimately associated with its cells, e.g. micro-organisms and their toxins, cosmetic preparations, medicaments and some materials used in clothing. An antibody to any of these may cause degeneration of the cells of the skin without autosensitization. This occurs

more often when the barrier function of the dermo-epidermal junction is impaired or destroyed in eczema, psoriasis and some other skin disorders; thus substances such as lanoline, which rarely sensitize when applied to normal skin, become antigenic, especially as they are applied over long periods when treating chronic disease. Such sensitization is overlooked if these substances are not included in the skin tests, and the secondary dissemination of eczema is attributed to autosensitization because the exogenous sensitizing antigen is not found.

Before it can be accepted that a cutaneous reaction is due to auto-allergy to skin, it is essential to demonstrate that the individual has become sensitized to a skin antigen and that the auto-antibody can induce a reaction resembling that already present in the patient.

Antigens of Skin

The skin is a complex organ containing a large number of different tissues that are potentially antigenic, some contain substances, e.g. keratin, that are organ-specific antigens.

Keratin is a protein not found normally in any other part of the body, and is formed and situated on the external surface of the epidermis where it is sufficiently far removed from the antigen recognition cells that it or its precursors could conceivably be treated as foreign material. This may be observed particularly in rupture of the hair follicles in the dermis which results in a severe local inflammatory reaction. Several soluble substances can be extracted from stratum corneum and even from callus, nail and hair (Matoltsy & Matoltsy 1963). Three antigens believed to be skin specific have been extracted from skin taken after death (Aoki 1965), and four antigens extracted from callus and psoriatic scales and three from normal stratum corneum were distinct from serum proteins (Fisher 1965). These antigens must be identified before they can be accepted as organ specific. An electrophoretically fast pre-albumin found in extracts of callus (Fisher 1965) and normal stratum corneum (Parish, unpublished) is believed to be synthesized locally in the skin (Neuberger 1957).

The A and B blood group antigens have been found on human epidermal cells (Coombs *et al* 1956; Szulman 1960), but though other cells of the body have been found to contain further blood group antigens, it has not been possible to demonstrate their presence in cells of the epidermis (Ashhurst *et al* 1956). The modified cells of the epidermis present in the hair or nails also contain the A and B antigens (Thoma 1954; McWright 1961). These blood group antigens are in no way known to be involved in autosensitization to skin, but they could be of importance in an allergic response to dander.

Autosensitization to Dander

The dander that is continually being shed from the surface of the body contains

a wide variety of potentially antigenic materials, including desquamated epithelial cells, fragments of hair, micro-organisms, dust and other substances that may be retained in the sweat or sebum on the surface.

It is to be expected that such material may cause an allergic response in susceptible individuals, and the majority of the descriptions of sensitization to dander are based on tests using dander obtained from other individuals which may cause an immediate or delayed type of reaction (Parish 1960). This material therefore elicits a Type I response in which the antigen reacts with antibody passively sensitizing certain cells of the skin, or a Type IV response.

Individuals may become sensitized to their own dander (Simon 1947; Cormia & Esplin 1950; Esplin & Cormia 1951) but this could well be due to fungi or bacteria contained within it. To eliminate the effects due to contaminating micro-organisms, Simon (1948) used the epidermal scales obtained from a newborn infant to demonstrate sensitivity to dander in adults, and though this should be regarded as foreign material being derived from another individual, it does indicate that epidermal cells may contain a potent allergen.

These tests carried out on sensitized patients demonstrate the difficulty of identifying the antigens causing the reaction, and though the majority of patients appear to be sensitive to some extraneous antigen, there remains the possibility that some individuals may be sensitized to the keratinized epidermal cells of their own skin, which are sufficiently remote from the tissues to have become 'foreign antigens'.

Allergic Response of the Skin to Micro-organisms

Antigens of micro-organisms in dust or dander may induce a Type I reaction on contact with sensitized skin, or occasionally a Type IV reaction if they contaminate materials applied to the skin. A Type I chronic urticarial reaction was investigated by Shelley & Florence (1961) in which the fungal antigen was bound to the erythrocytes and reacted with the skin after trauma. Bacterial antigens also have been detected on human erythrocytes (Weedon *et al* 1960) though not associated with skin disease. This could however cause disorders apparently auto-allergic in origin.

The possibility that bacterial antigens are responsible for a Type II cytotoxic reaction is being investigated. Fibroblast-type cells from human skin and Hela cells can adsorb bacterial protein and polysaccharide *in vitro*, so that the subsequent addition of antisera to the acquired antigens, and complement, causes lysis of the cells (Parish, unpublished). Thus bacterial antigens acquired by cells *in vivo* may predispose them to destruction by antibody or by a cell-mediated response in a manner similar to the reaction of auto-antibody to cellular antigen.

AUTOSENSITIZATION TO SKIN

In his original description, Whitfield (1921) described three lesions which may have resulted in autosensitization of the patient to his own skin. The first was a traumatic haematoma beneath the unbroken skin; 10 days later the patient developed a generalized erythematous and urticarial eruption which Whitfield believed to be due to absorption from the site of injury of products to which the patient became sensitized.

In the second patient, an area of chronic eczema of the leg was rubbed, which caused further inflammation followed 11 days later by a generalized haemorrhagic papular eruption.

In the third case, fluid from vesico-bullous eczema provoked urticarial wheals and vesicles as it flowed over the skin in the vicinity of the lesion, though the same fluid failed to induce a reaction on Whitfield's own arm.

The clinical picture presented by Whitfield's second case, in which exacerbation of a local area of eczema was followed by generalized dermatitis, is of common occurrence. In the more detailed descriptions (Smith 1945; Haxthausen 1955; Young 1958), the initial localized lesion was usually persistent and chronic, and because of the situation on the lower leg it was usually regarded as varicose eczema. Further inflammation of the lesion from exercise, trauma, particularly scratching, or overtreatment, was followed in a few days to a week or more by a generalized dermatitis. Sometimes small vesicles around the original lesion preceded or developed concomitantly with the generalized reaction, though in a few patients this reaction occurred without any evident increase in inflammation of the local area.

Certain parts of the body tend to be more frequently and severely affected by the generalized reaction, particularly the face, upper trunk, arms and thighs, though the whole body may become involved. Patchy erythema and wheals are soon followed by papules and vesicles which may become confluent to form plaques of eczema. The histological changes in these affected areas resembled those found in primary acute eczema (Haber 1954; Haxthausen 1955; Young 1958).

The cause of the generalized reaction is not yet certain, and the eczematous changes may result from different stimuli in different patients.

In those individuals in whom the reaction is due to sensitization, the responsible antigen may be foreign material which has entered the broken surface of the original lesion. Bacteria were believed to be the cause of the sensitivity in the patients investigated by Hopkins & Burky (1944) and Yamamoto (1963) and in one patient of Templeton, Lunsford & Allington (1949).

Contact dermatitis to topical medicaments very readily develops on the lower leg; if the application of the sensitizing chemical is continued the acute local eczematous dermatitis is soon followed by secondary dissemination to other

areas of the body. A great many of the cases loosely attributed to autosensitization are of this nature. They can be identified by careful patch-testing (see Chapter 19) after the eruption has subsided. Nevertheless, there are many patients in whom no exogenous factor can be incriminated and who appear to become sensitized to some component of their own skin.

Histological Changes in Eczema in Relation to Autosensitization

The histological changes of eczema are strikingly uniform in their essential features, but show minor variations according to the mode of action of the excitant, primary irritant or allergen, and the route by which it gains access to the epidermis. The earliest change is a necrosis or degeneration of small groups of cells of the stratum Malpighii. The affected cells are distorted by intercellular oedema. Progression of this state of spongiosis leads to the formation of small vesicles which may coalesce to form large bullae. The vesicles may rupture with escape of serum and crust formation. Simultaneously the papillary layer of the dermis is oedematous and infiltrated with inflammatory cells which migrate into the affected epidermis. In allergic reactions lymphocytes are numerous, but in primary irritant reactions polymorphonuclear leucocytes predominate.

The mode of formation of the vesicles is still disputed (Civatte 1925; Miescher 1952). The fluid is derived partly from lysis of epidermal cells but may also contain serum from dilated capillaries and perhaps the products of cutaneous glands. Percival & Hannay (1949) compared the sizes of epidermal cells and vesicles in serial sections and suggested that liquefaction of cells must occur on a considerable scale. The outer wall of vesicles may include cells of every layer of the epidermis including those of the stratum corneum and stratum granulosum which may thus be bathed in fluid. Much of this fluid escapes to the surface, but external rupture of the vesicles is not invariable, and some must be reabsorbed by the dilated capillaries of the dermis and spread throughout the body, carrying with it antigen from cells normally isolated from the circulation.

The importance of these histological changes in a study of autosensitization is the evidence it provides of the degeneration and destruction of large numbers of epidermal cells with liberation of their contents, which probably contain material in a degraded state, potentially antigenic.

Experimental Evidence for Autosensitization to Skin in Eczema

Due to the difficulty in isolating the antigens responsible for the sensitivity, autosensitization to skin has remained a clinical concept rather than a proven entity, but recently evidence has been obtained that circulating antibodies to skin may be developed by a few patients with chronic dermatitis.

Antibodies to skin antigens have been found in patients with ulceration of the

skin (Chytilova & Kulhanek 1960), exfoliative dermatitis and eczema (Parish 1960, 1961; Parish, Rook & Champion 1965), chronic dermatitis (Wise, Shames, Derbes & Hunter 1961), pemphigus vulgaris (Beutner & Jordan 1964) and agglutinating antibody in nine patients with various skin diseases (Fisher 1965). The significance of most of these antibodies is still to be determined for they may combine with cellular antigen, i.e. auto-react without causing any pathogenic changes, and the specificity for skin was not always demonstrated, just as the globulin found in the dermo-epidermal junction in lupus erythematosus (Cormane 1964; Kalsbeek & Cormane 1964; ten Have-Opbroek 1966) was not demonstrated to be antibody specific for skin antigen or to be cytotoxic.

The Demonstration of Agglutinating and Cytotoxic Antibody in exfoliative Dermatitis and Generalized Eczema

In preliminary studies on a patient with exfoliative dermatitis, no agglutinating antibody by the tanned cell test was detected in the serum, but a factor believed to be antibody was eluted from the skin that would agglutinate skin antigen coated cells and fix complement in the presence of extracts of autologous and homologous skin; both these reactions were skin specific (Parish 1960, 1961).

Four of eighty-one patients investigated by Parish, Rook & Champion (1965) had auto-antibody in their sera to their own skin. All four had a history of recurrent generalized eczema over several years and had, or at one time had, a patch of chronic eczema which was of the varicose type. During these investigations all developed generalized eczematous reactions, but one, though developing some scattered papules, was essentially a case of exfoliative dermatitis.

During the periods of dermatitis, but not during convalescence, the sera of three of these patients contained antibody capable of agglutinating particles coated with extracts of skin and all had antibody causing cytotoxic effects on epidermal cells *in vitro* and a reaction on intradermal injection. The antibody producing the agglutination was not inactivated after heating at 56°C, whereas the cytotoxic antibody no longer caused changes in cultures of epidermal cells, though until the nature of the activity of such antibody is understood, there is insufficient reason to regard them as distinct antibodies.

The agglutinating antibody could be adsorbed out of the sera by epidermal cells or neutralized by extracts of them, but cells from other organs were unable to do this, and antigen extracts of other cells used to coat the particles did not result in agglutination.

When the sera taken during the period of generalized dermatitis, and demonstrated to be free of histamine-type activity, were injected intracutaneously into the patient from whom they were obtained, an immediate wheal and flare reaction was induced, reaching its maximum in about 30 min. This did not follow the injection of the samples of sera taken during convalescence. The same

results followed the injection of the samples into the skin of a normal subject. Adsorption of the serum with liver or spleen cells did not remove the antibody, but it was removed by adsorption with cells of the skin.

When sections of fresh skin were incubated in the sera at 37°C and then examined for histological changes, the sera of the three patients tested caused cytotoxic effects. The cells of the stratum granulosum enlarged and the cytoplasm became coarsely granular and developed small vacuoles. Incubation for longer periods produced further degeneration of these cells and pyknosis of the nuclei.

The sera from all four patients taken during periods of eczema caused cytotoxic changes in the stratum Malpighii of organ explants of skin taken from normal individuals, or from themselves during periods of remission. The severity of these changes depended upon the age of the culture and duration of exposure to the antibody. The majority of explants taken from the four patients during active eczema degenerated immediately after culture, probably because the antibody had already reacted with the skin *in vivo*, though skin from other patients with eczema did grow in culture.

Further evidence of the presence of a cytotoxic antibody resulted from the changes occurring in the cells proliferating from explants of human skin in tissue culture after addition of the sera. Only a small proportion of the cells were susceptible. These enlarged and the cytoplasm flowed out of the cell wall, and further degeneration resulted in ghost cells or fragments of the cells which had disintegrated.

Two other patients with generalized eczema have recently been found to have antibody to skin (Parish, Champion & Welbourn, to be published). The sera of both agglutinated red cells coated with skin antigens, and reacted in immunofluorescence tests with epidermis, though only samples of sera from one patient have proved to be cytotoxic *in vitro*.

Tests to Determine that the Cytotoxic Factor is Specific Antibody

Wheal and flare reactions after injection of material into the skin and cytotoxic effects on cells *in vitro* are not confined to the response to an antigen-antibody reaction, and cells probably have a limited means of responding to a number of noxious stimuli. It therefore becomes important to demonstrate that the factor in the serum causing the changes is an antibody, and that it is specific for some antigen in the skin.

One factor that is probably not specific antibody is the α_2- globulin that kills suspensions of epidermal cells and causes necrosis of skin organ explants without the presence of complement, which was found by Anderson (1966) in the sera of elderly people with chronic inflammatory disease. This globulin did not damage their dermal fibroblasts or lymphocytes, and thus resembles the

factor cytotoxic for epidermal cells in the serum of normal rabbits and rats by Terasaki & Chamberlain (1962).

The cytotoxic factor found in the four patients described by Parish *et al* (1965) was a heat-labile, complement-dependent, 7S γ-globulin which was active at 37°C and weak or inactive at lower temperatures.

It was specific for human epidermal cells, causing cytotoxic changes in the skin of the patient from whom the serum was taken, and in the skin of normal individuals. The adsorption of the sera with partially separated cells of other human tissues and sections of rabbit and guinea-pig skin failed to remove the cytotoxic factor, but it was removed by the adsorption of the sera with partially separated cells of human epidermis. Furthermore, it only caused changes in the susceptible cells of explants of human skin, and not in cultures of other tissues. Immunofluorescence tests confirmed the epidermal cell specificity. Addition of the cytotoxic sera to sections of skin from normal individuals or unaffected skin from the same patients resulted in bright staining of the epidermis, particularly in the stratum granulosum, hair follicles and sweat ducts, when the sections were subsequently treated with rabbit anti-human γ-globulin.

The specificity and properties of the cytotoxic factor are characteristic of antibodies, and though any one of them is insufficient to determine its antibody nature, together they provide strong evidence that the factor is cytotoxic antibody.

Detection of Auto-Antibody to Skin by Fluorescent Labelling

Two of fourteen patients with chronic dermatitis were found by Wise *et al* (1961) to have globulins in their sera which, when separated and labelled with fluorescein, were found to have an affinity for the dermis of punch biopsy specimens of their own skin. It was not possible to identify the antigen apart from the evidence that the reaction was confined to the dermis, and in the patient with atopic dermatitis only the affected areas of skin were stained by the globulin and not normal skin from the patient or skin from other individuals. The antigen is unlikely to have been material absorbed from or through the epidermis as some trace of this should have been revealed by the labelled globulin, but it is possible that such a reaction may not have resulted from autosensitization and was due to the antibody combining with extraneous antigen that had been disseminated previously by the blood and deposited widely in the skin or in a small area of vascular dilatation and oedema.

The cytotoxic antibody to skin found in four patients with generalized eczema reacted strongly with epidermis, especially the stratum granulosum as described above, and sections of affected skin from the patients contained aggregated γ-globulin or antigen-antibody complexes in the epidermis but no bound complement (Parish *et al* 1965).

In an investigation of two more patients with generalized eczema both had IgG antibody reacting with epidermis and one also had IgM antibody combining with occasional cells in the dermis; these antibodies fixed complement (Parish, Champion & Welbourn, to be published).

No specific skin antibodies were detected by immunofluorescence in several skin disorders apart from fixation of a small amount of globulin on the margin of the active lesions of one case of erythema annulare centrifigum (Raskin 1964). In pemphigus vulgaris, however, sera from eight of thirteen patients combined with an intercellular substance on the surface of epithelial cells which was present in human and monkey skin, monkey oral mucosa, oesophagus and anus (Beutner & Jordan 1964). Its significance is still to be determined.

AUTO-ALLERGY TO SKIN RESULTING FROM TYPE IV REACTIONS

Detection of Type IV cell-mediated responses in auto-allergic diseases of the skin has been limited by the techniques available, though this may prove to be a valuable study. It has been claimed that the addition of extracts of skin or lymphocytes to cultures of leucocytes from patients with infantile eczema results in mitosis (Hashem *et al* 1963; Hashem & Carr 1963) though the specificity of such a test requires further investigation, and it is premature to consider the destruction of human embryo fibroblasts in culture by lymphocytes from patients with systemic lupus erythematosus and scleroderma (Trayanova, Sura & Svet-Moldavsky 1966) as evidence that the diseases are cell-mediated auto-allergy.

Similarly the detection of cell-mediated auto-allergy in chronic discoid lupus erythematosus by injecting autologous leucocytes into the skin of the same patient (Tromovitch & March 1961) has proved unreliable, for as many positive reactions were obtained in control tests as in patients with this disease (Gerstein & Knox 1963).

EXPERIMENTS ON ANIMALS TO INVESTIGATE AUTOSENSITIZATION TO SKIN

Auto-allergy to skin has rarely been induced in experimental animals. No antibody, cytotoxic or otherwise, was detected after injecting homologous or autologous skin in guinea-pigs (Rosenthal, Baer & Hagel 1958; Moschos, Rosenthal & Schroeder 1964; Parish, unpublished) or homologous skin in rabbits (Allgöwer, Blocker & Engley 1952; Walzer, Einbinder & Nelson 1964), and though agglutinating and cytotoxic antibodies to skin of pigs have been

found in the sera of six animals with chronic dermatitis (Parish 1960) it was not possible to stimulate these antibodies or induce skin disease in normal pigs by causing local areas of skin degeneration, nor by the intradermal or intramuscular injection of pulped skin or saline extracts of normal skin, with or without adjuvants.

There is, however, slight evidence that animals may be sensitized to homologous skin. Hecht, Sulzberger & Weil (1943) injected rabbits with non-sterile extracts of rabbit skin in adjuvant and some were also injected with staphylococcal toxins. These rabbits were found to develop precipitating antibodies to extracts of skin, but only extracts that had been kept for more than 24 hr; thus both the antigen injected and the antigen used for the precipitation tests contained bacterial antigen. It is possible that antibody was produced to degenerate skin, and animals with circulating antibody were found to develop special kinds of lesions after skin trauma, but the possibility cannot be excluded that such lesions were due wholly or in part to antibody entering the damaged area and reacting with antigen of the resident flora.

In the course of skin-graft experiments Voisin & Maurer (1955) discovered that of six rabbits which had been injected with skin extracts and later rejected homografts from the same donor rabbits, four subsequently rejected autografts; this they believed to be due to autosensitization to skin.

Stimulation of sensitization to skin which resulted in dermatitis in rabbits and guinea-pigs has been claimed by Wilhelmj, Kierland & Owen (1962) who injected their animals with homologous or autologous skin with Freund's complete adjuvant and obtained evidence of sensitization to skin in about half their animals by means of skin tests, complement fixation tests and tanned cell agglutination tests. The majority of the sensitized animals developed changes in the skin and loss of hair. It was also shown that the antigens of dorsal and ventral skin were apparently distinct, which may be a possible explanation for the different patterns in which eczema may be disseminated in man. This report in which experimental sensitization to skin resulted in cytotoxic changes is an important advance if it can be confirmed, but attempts to repeat these experiments have been unsuccessful (Moschos *et al* 1964; Walzer *et al* 1964) though this could be due to variations in the antigens in the different skin extracts.

Autosensitization to skin has been claimed to be a normal phenomenon in rabbits and adult rats whose sera were found to be toxic to suspensions of autologous epidermal cells, whereas the sera of mice and newborn rats were harmless to their autologous cells (Terasaki & Chamberlain 1962). The toxic factor was present in low concentrations, was complement dependent and could be adsorbed by epidermal cells but not by lymph-node cells or erythrocytes. Though believed to be an auto-antibody, it could be a non-specific factor cytotoxic for several organs not normally exposed to serum, for testicular cells were also killed.

CONCLUSIONS

Many patients developing a generalized dermatitis of sudden onset may be sensitized to foreign extraneous antigenic material, but evidence has been obtained that some become sensitized to autologous skin antigens.

The significance of the circulating antibodies to skin in autosensitization is not yet known. Their formation may be the harmless result of an inflammatory process, without causing any further dermatitis, or they could initiate degeneration in areas of normal skin. In four patients studied by the authors, circulating cytotoxic antibody specific for epidermis was found which could cause a Type II allergic response resulting in degeneration of the cells of the epidermis, and in these four patients the recurrent generalized dermatitis of many years' duration may be due, or partly due, to autosensitization to skin.

REFERENCES

ALLGÖWER M., BLOCKER T.G. & ENGLEY B.W.D. (1952) Some immunological aspects of auto- and homografts in rabbits tested by *in vivo* and *in vitro* techniques. *Plast. Reconst. Surg.* **9,** 1.

ANDERSON P.C. (1966) Epidermal cell injuring factor in autologous serum. Presented at 17th Annual Meeting of Tissue Culture Association, San Francisco, California, June 1st. (Abstract.)

AOKI T. (1965) Soluble antigens of human skin as characterized by immunodiffusion and immunoelectrophoresis. *Hifu (Osaka)* **7,** 219. (In Japanese, English summary.)

ASHHURST D.E., BEDFORD D., COOMBS R.R.A. & ROUILLARD L.M. (1956) Blood group antigens on human epidermal cells. *Nature (Lond.)* **178,** 1170.

BEUTNER E.H. & JORDAN R.E. (1964) Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining. *Proc. Soc. exp. Biol. (N.Y.)* **117,** 505.

BROWN W.H. (1939) Some chemical manifestations of endogenous sensitization eruptions following local infection or injury. *Brit. J. Derm.* **51,** 197.

CHYTILOVA, MARIE & KULHANEK V. (1960) Skin autoantibodies and unsuccessful autotransplantation following immunization with freeze-dried autologous skin. *Plast. Reconstr. Surg.* **26,** 109.

CIVATTE A. (1925) Eczéma et eczématides. *Bull. Soc. franç. Dermat. Syph.* **32,** 134.

COOMBS R.R.A., BEDFORD D. & ROUILLARD L.M. (1956) A and B blood group antigens on human epidermal cells. Demonstrated by mixed agglutination. *Lancet* **i,** 461.

CORMANE R.H. (1964) 'Bound' globulin in the skin of patients with chronic discoid lupus erythematosus and systemic lupus erythematosus. *Lancet* **i,** 534.

CORMIA F.E. & ESPLIN B.M. (1950) Autoeczematization. *Arch. Derm. Syph. (Chicago)* **61,** 931

ESPLIN B.M. & CORMIA F.E. (1951) Further studies in autoeczematization. *Arch. Derm. Syph. (Chicago)* **64,** 31.

FISHER J.P. (1965) Soluble substances of human stratum corneum. I. Immunochemical and immunological study. *J. Invest. Derm.* **44,** 43.

GERSTEIN W. & KNOX J.M. (1963) Intradermal leucocyte test in discoid lupus erythematosus. *Brit. med. J.* **ii,** 901.

HABER H. (1954) Histopathology of eczema. In *The Eczemas,* p. 86, ed. LOEWENTHAL L.J.A. Edinburgh: E. & S. Livingstone.

HASHEM N. & CARR D.H. (1963) Mitogenic stimulation of peripheral lymphocyte cultures by autologous lymphocyte extracts in autoimmune diseases. *Lancet* **ii,** 1030.

HASHEM N., HIRSCHHORN K., SEDLIS E. & HOLT L.E. (1963) Infantile eczema. Evidence of autoimmunity to human skin. *Lancet* **ii,** 269.

HAXTHAUSEN H. (1955) Generalized 'ids' ('autosensitization') in varicose eczemas. *Acta derm. venereol. (Stockh.)* **35,** 271.

HECHT H., SULZBERGER M.B. & WEIL H. (1943) Studies in sensitization to skin. I. The production of antibodies to skin by means of the synergistic action of homologous skin antigen and staphylococcus toxin. *J. exp. Med.* **78,** 59.

HOPKINS H. & BURKY E.L. (1944) Cutaneous autosensitization. *Arch. Derm. Syph. (Chicago)* **49,** 124.

KALSBEEK G.L. & CORMANE R.H. (1964) 'Bound' complement in the skin of patients with chronic discoid lupus erythematosus and systemic lupus erythematosus. *Lancet* **ii,** 178.

MATOLTSY G.A. & MATOLTSY M.N. (1963) A study of the soluble proteins of normal and pathologic horny tissues by modified disc electrophoresis. *J. invest. Derm.* **41,** 255.

MCWRIGHT G.G. (1961) The study of group specific substances in keratinized tissues. *J. Genet. Sci.* **6,** 351.

MIESCHER G. (1952) Zur Histologie der ekzematösen Kontakreaktion. *Dermatologica* **104,** 215.

MOSCHOS T.D., ROSENTHAL S.A. & SCHROEDER H. (1964) Attempts at sensitization to skin in guinea-pigs. *Arch. Derm. (Chicago)* **90,** 620.

NEUBERGER A. (1957) Observations on the presence and metabolism of plasma proteins in skin and tendon. In *Connective Tissue*, p. 35, ed. TUNBRIDGE R.E. Oxford: Blackwell Scientific Publications.

PARISH W.E. (1960) Autosensitization to skin. In *Progress in the Biological Sciences in Relation to Dermatology*, p. 259, *ed.* ROOK A. Cambridge University Press.

PARISH W.E. (1961) Laboratory investigations of autosensitization to skin. *Brit. J. Derm.* **73,** 10.

PARISH W.E., ROOK A.J. & CHAMPION R.H. (1965). A study of auto-allergy in generalized eczema. *Brit. J. Derm.* **77,** 479.

PERCIVAL G.H. & HANNAY P.W. (1949) Observations on the structure and formation of bullae—I. *Brit. J. Derm.* **61,** 41.

RASKIN J. (1964) Fluorescent antibody studies of certain dermatoses. *Arch. Derm. (Chicago)* **89,** 569.

ROSENTHAL S.A., BAER R.L. & HAGEL B. (1958) Failure to sensitize to autologous skin. *Proc. Soc. exp. Biol. (N.Y.)* **97,** 279.

SHELLEY W.B. & FLORENCE R. (1961) Chronic urticaria due to mold hypersensitivity. A study in cross-sensitization and autoerythrocyte sensitization. *A.M.A. Arch. Derm.* **83,** 549.

SIMON F.A. (1947) The allergen of human dander present in skin of the general body surface. *J. invest. Derm.* **9,** 329.

SIMON F.A. (1948) The allergen of human dander. Its relative concentration in several skin areas and in appendages, products and derivatives of the epidermis. *J. invest. Derm.* **11,** 203.

SMITH S.W. (1945) Eczema autolytica. *Brit. med. J.* **i,** 628.

SZULMAN A.E. (1960) The histological distribution of blood group substances A and B in man. *J. exp. Med.* **111,** 785.

TEMPLETON H.J., LUNSFORD C.J. & ALLINGTON H.V. (1949) Autosensitization dermatitis. *Arch. Derm. Syph. (Chicago)* **59,** 68.

TEN HAVE-OPBROEK A.A.W. (1966) Demonstration of immunoglobulins and complement in the skin of patients with lupus erythematosus. *Acta derm-venereol.* **46,** 68.

TERASAKI P.I. & CHAMBERLAIN C.C. (1962) Destruction of epidermal cells *in vitro* by autologous serum from normal animals. *J. exp. Med.* **115,** 439.

THOMA K. (1954) Blood group determination from nails. *Int. Criminal Police Rev.* **77,** 107.

TRAYANOVA T.G., SURA V.V. & SVET-MOLDAVSKY G.J. (1966) Destruction of human cells in tissue culture by lymphocytes from patients with systemic lupus erythematosus. *Lancet* **i,** 452.

TROMOVITCH T.A. & MARCH C. (1961) Intradermal tests with autologous white blood cells in chronic discoid lupus erythematosus and control subjects. *J. invest. Derm.* **37,** 345.

VOISIN G. & MAURER P. (1955) Sur une dermatose experimentale apparue chez des lapins traités par des extraits de petite homologue. *Sem. Hôp. Paris* **33,** 1909.

WALZER R.A., EINBINDER J.M. & NELSON C.T. (1964) Response to immunization with homologous skin. *Arch. Derm. (Chicago),* **90,** 583.

WEEDEN A.R., DATTA N. & MOLLISON P.L. (1960) Adsorption of bacteria on to red cells leading to positive anti-globulin reactions. *Vox Sang.* **5,** 523.

WHITFIELD A. (1921) Some points on the aetiology of skin disease. Lecture II. *Lancet* **ii,** 122.

WHITFIELD A. (1922) The question of sensitiveness to non-bacterial toxins and proteins. *Brit. J. Derm.* **34,** 331.

WHITFIELD A. (1931) 'Autosensitization' in eczema. *Comptes rendus des Sciences,* p. 142. 8th Cong. Internat. Dermat., Copenhagen, 1930.

WILHELMJ C.M., KIERLAND R.R. & OWEN C.A. JR. (1962) Production of hypersensitivity to skin in animals. *A.M.A. Arch. Derm.* **86,** 161.

WISE L.J., SHAMES J.M., DERBES V.J. & HUNTER F.M. (1961) Fluorescent antibody studies in chronic dermatitis. *A.M.A. Arch. Derm.* **84,** 37.

YAMAMOTO K. (1963) Studies on autosensitization dermatitis. *Proceedings XII Int. Congr. Dermatology (1962), Washington,* vol. 2, 1260, ed. PILLSBURY D.M. & LIVINGOOD C.S. Amsterdam: Excerpta medica Foundation. (Abstract.)

YOUNG A.W. JR. (1958) Dynamics of autosensitization dermatitis. A clinical and microscopic concept of autoeczematization. *A.M.A. Arch. Derm.* 77, 495.

CHAPTER 34

THE KIDNEY IN ALLERGIC DISEASE

J. HARDWICKE

INTRODUCTION

That many forms of glomerulonephritis have an allergic basis has been postulated for many years. Before adequate antibiotic control of β-haemolytic streptococcal infections, clinical acute nephritis was common, and it was found to occur 10–15 days after a primary upper respiratory tract infection with these organisms. At this time very high circulating titres of antistreptolysin O antibodies were detected, and it was difficult to avoid postulating that the nephritis occurred at the time of maximum antibody response, and represented a form of allergic disease. In addition it had been found that proteinuria was an almost invariable accompaniment of acute serum sickness in man and animals, appearing at the time of maximum antibody response to the injected antigen.

Over the years many attempts have been made to induce nephritis in experimental animals by the injection of antigenic materials. These have had variable success, and are reviewed in this chapter.

Since the advent of renal biopsy (Kark *et al* 1955) the histological appearances of the various types and stages of human glomerulonephritis have been fairly well defined. Also evidence has accumulated to strengthen further the previous view that an immunological aetiology underlies most forms of human disease (Drummond *et al* 1964). However, as yet no specific antigens, whether autologous or heterologous, nor specific antibody, auto-immune or hetero-immune,

have been clearly defined in human disease; until this is achieved the precise aetiology of the various clinical types of nephritis will continue to evade medical science. In this chapter it is intended first to define the anatomical components of the glomerulus, and to consider how these may be modified in association with glomerulo-nephritis; second, to analyse the nature of the lesion seen in a variety of, more or less, well defined clinical types; thirdly, to consider the models of glomerulonephritis currently known to be experimentally induceable in animals; and finally to consider how closely these models relate to what we so far know of human disease.

A number of key references will be found at the end of the chapter, firstly to comprehensive review articles, and secondly to individual papers cited in the text. Through these the reader wishing to examine the subject in detail will be able to gain access to all the voluminous literature extant.

A. STRUCTURE OF THE GLOMERULUS (Rhodin 1962) (Plate 34.1)

The structural elements of the glomerulus consist of the afferent and efferent glomerular arteriole, the capillary tuft, and Bowmans capsule. The capillaries have an endothelial cell lining, and a central stalk consisting of mesangial cells; these, although remote from the capillary lumen, probably have some form of direct communication with it. The basement membrane consists of a relatively structureless glycoprotein matrix, without any clear fibrillary structure: chemically it resembles a modified collagen (Kefalides & Winzler 1966). Apposed to the outside of this membrane are modified epithelial cells or podocytes. Bowmans capsule also is formed of a glycoprotein matrix, continuous with the capillary tuft basement membrane, and lined by epithelial cells.

The changes in these structures which can be encountered in disease can be summarized as:

1. Loss of the foot processes of the podocytes.
2. Appearance of extraneous deposits, either inside, in the substance of, or outside the basement membrane. Such deposits may be composed of complexes of proteins involved in immune precipitates, fibrinogen or unidentified proteins such as amyloid (Vazquez & Dixon, 1956).
3. Proliferation of cellular elements, endothelial, mesangial or epithelial.
4. Infiltration by cellular elements, round cells or polymorphonuclear leucocytes.
5. Scarring and disorganization of structure consequent on the changes above.

These relatively few changes occur together or separately in different patients, and in different forms of renal disease; in the absence of any certain understanding of aetiological processes in glomerulonephritis, it is therefore not surprising that considerable confusion still persists. However, some characteristic

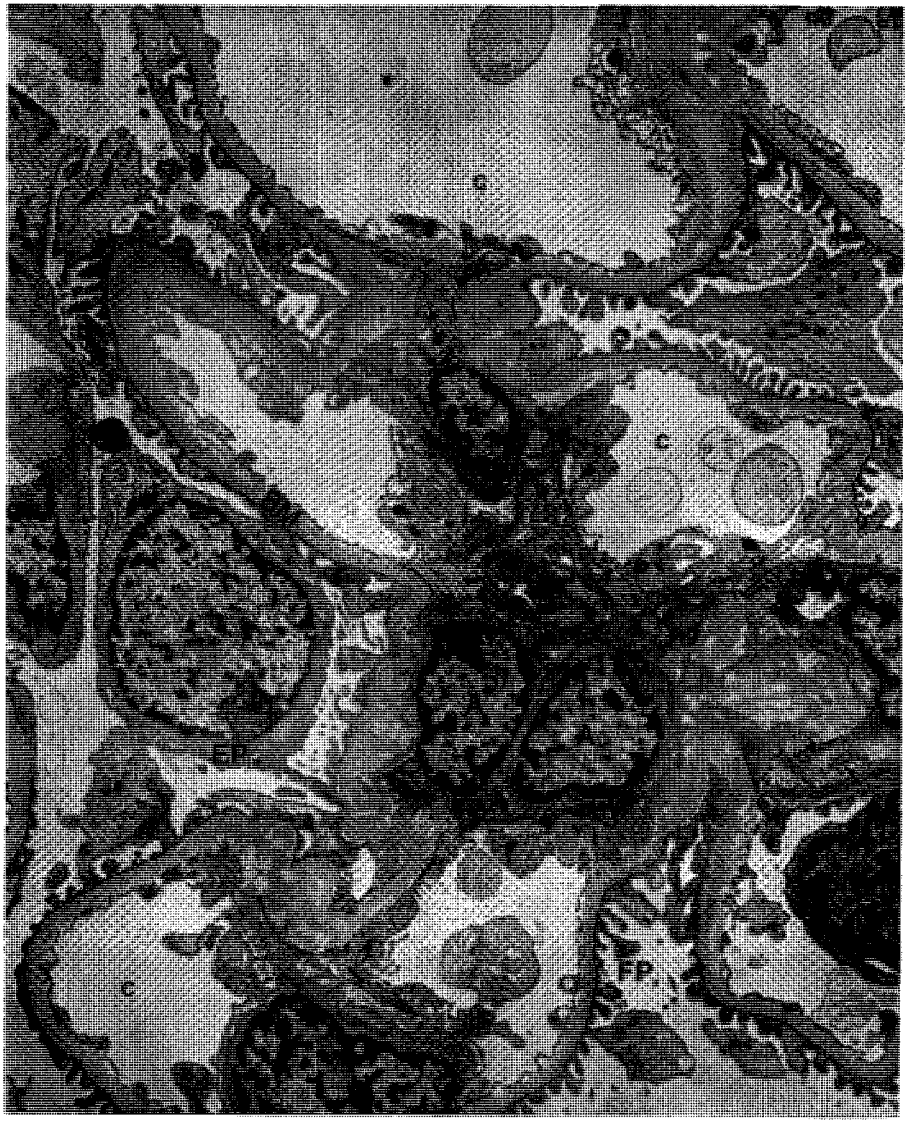


PLATE 34.1. Normal Human glomerulus.

Electron micrograph of normal human glomerulus (×9000). For description see page 910.

Key (Plates 34.1–34.7)

BM = Basement membrane.
C = Capillary lumen.
EN = Endothelial cell.
EP = Epithelial cell.
FP = Foot processes.
A = Axial cell (mesangial).

facing p. 910

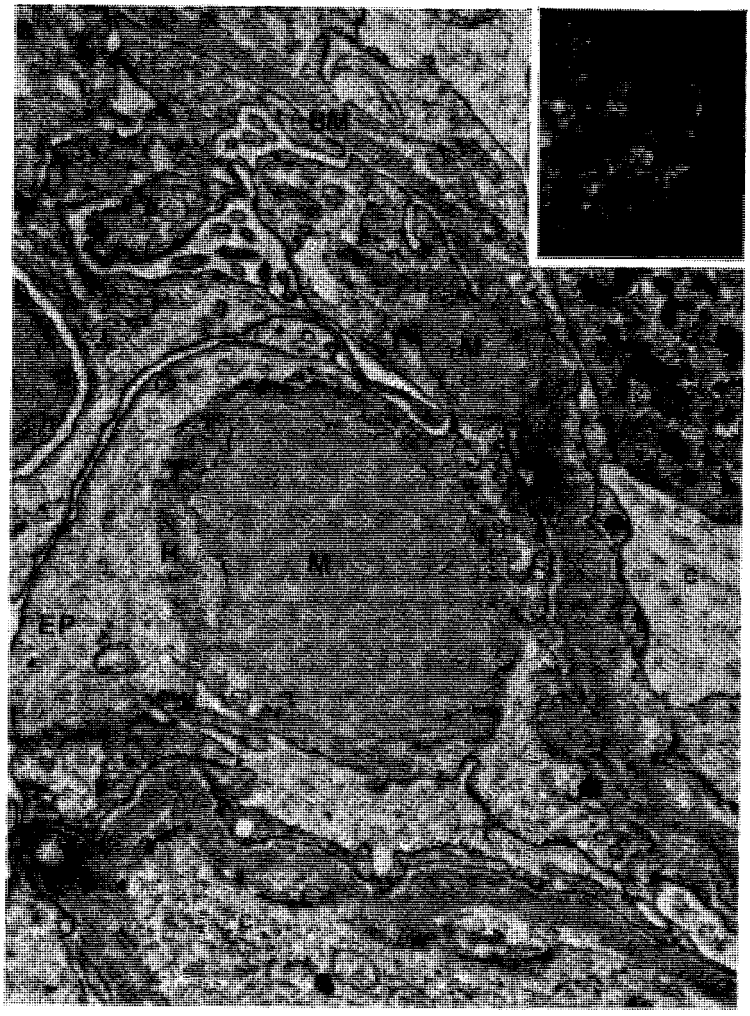


PLATE 34.2. Human acute glomerulonephritis.

Electron micrograph of a case of human acute glomerulonephritis (×16,500). Inset shows a portion of a glomerulus stained with fluorescent IgG from a similar case (×350). (From Figs. 2A and 5 in Michael *et al* (1966). By kind permission of Dr R.L.Vernier.)

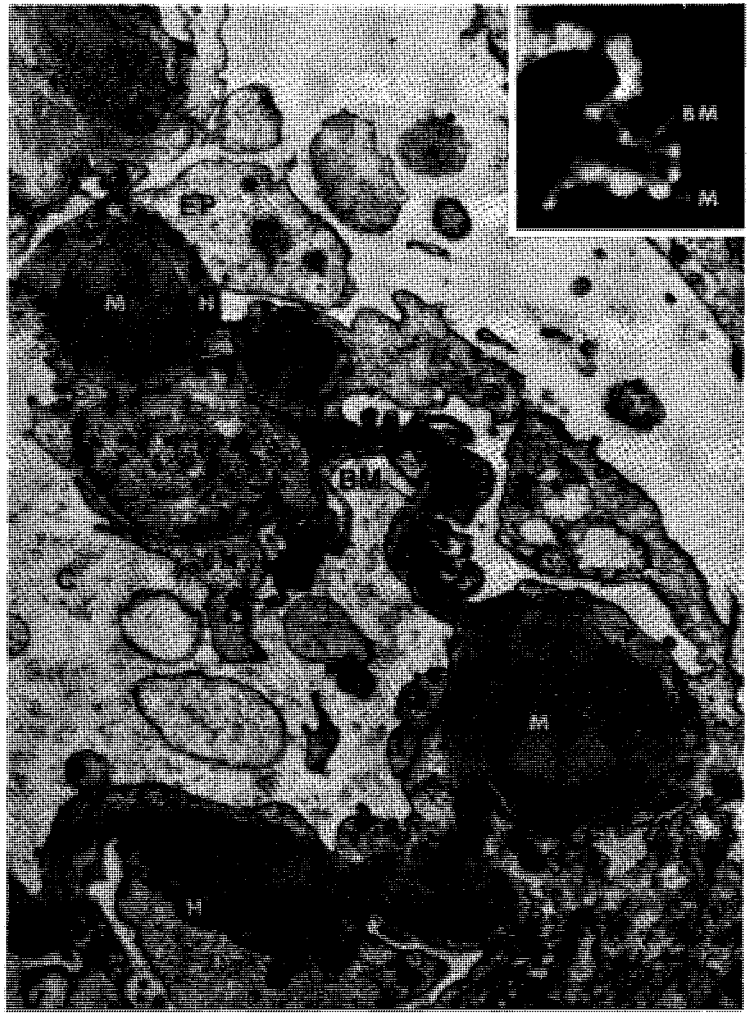
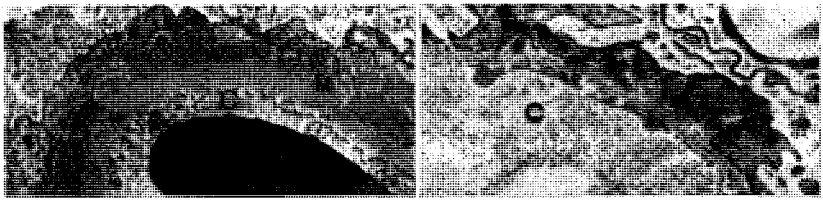


PLATE 34.3. Rabbit acute serum sickness.

Electron micrograph for a rabbit with acute serum sickness (× 17,000). Inset shows capillary loop for same animal stained with fluorescent anti-rabbit IgG (× 700).

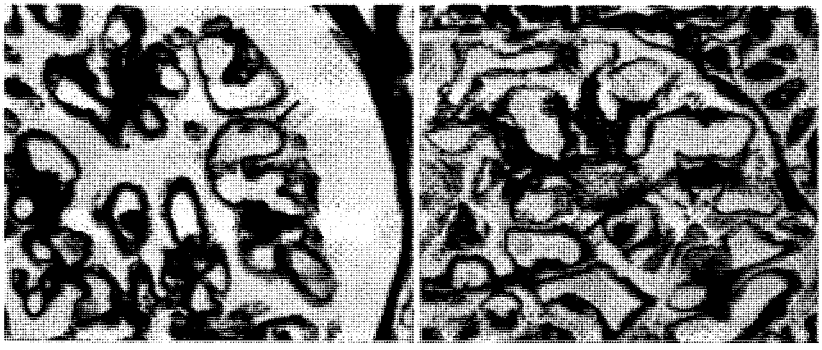
Both Plates 34.2 and 34.3 show deposits (M) outside the basement membrane, a dark halo (H) in the cytoplasm of the epithelial cell often surrounds these. Fluorescence microscopy shows patchy deposits of IgG with an apparently similar distribution.



(a) (b)

PLATE 34.4. Human membranous glomerulonephritis.

(a) Electron micrograph (× 5000), courtesy of Dr D.B.Brewer from Brewer (1960).
(b) PA silver stain (× 900).



(a) (b)

PLATE 34.5. Rat 'auto-immune' membranous glomerulonephritis (Heymann).

Rat immunized intraperitoneally to homologous azomitochondria in Freund's adjuvant. 'Heymann model' (1959).

(a) Electron micrograph (× 5000).
(b) PA silver stain (× 1200).

Human and rat glomeruli show striking similarities. Dense masses are present on the epithelial side of the basement membrane. In the PA silver preparation projections can be seen on the outer surface of the capillary loops (arrowed).

morphological patterns are beginning to emerge. Perhaps the most significant finding has been that nearly all glomeruli showing electron-dense deposits can also be shown to contain host immunoglobulins, often in association with complement components, the distribution of which parallels the electron-dense deposits. In addition fibrinogen has on occasions been demonstrated, particularly in those deposits found within the capillary or in the mesangial region.

Based on this finding of immunoglobulin deposits many forms of glomerulonephritis are now regarded more certainly as being of immunological origin. This evidence supplements previous studies, which have shown lowered serum complement levels (Gotoff *et al* 1966; Lagrue *et al* 1966) circulating antibody to kidney components (Lange *et al* 1955; Krammer *et al* 1961), and have pointed out associations with primary immunological stimuli, or accelerated responses following secondary stimuli, in such instances as streptococcal infections.

B. HUMAN GLOMERULONEPHRITIS

Human glomerulonephritis can be classified into those lesions in which the kidney is apparently the primary target, and those in which the renal lesion is secondary to a recognized systemic disease.

I. Primary Lesions

(A) Proliferative Glomerulonephritis

(Herdson *et al* 1966)

In the acute form of this condition there is, in the majority of cases, a clear history of antecedent infection, usually with a nephritogenic strain of streptococci. Haematuria follows after 10–15 days, and usually resolves spontaneously. Histologically the glomerulus shows polymorph infiltration and a striking proliferation of endothelial and mesangial cells, which may actually obliterate the capillary lumens. Host immunoglobulin has been demonstrated as a series of discrete nodules in the glomeruli; on electron microscopy these show again as discrete nodules, mainly deposited subepithelially outside the basement membrane, so-called 'lumpy' deposits. Variable amounts of complement components are found in these deposits, and fibrinogen may also be found in intracapillary situations, usually as an amorphous, non-fibrillary form. With healing, the deposits disappear rapidly, as does the endothelial cell proliferation; but mesangial zone thickening, with increased cellularity and marked irregularity of electron density, may persist for many months (Michael *et al* 1966) (Plate 34.2).

Systemic evidence of an immunological reaction includes very high antistreptolysin O titres, low levels of serum complement during the attack and high levels of serum γ-globulins.

In patients showing more persistent forms of diffuse proliferative glomerulitis the major cellular changes may be either endothelial or epithelial. Electron-

dense deposits may be found inside or outside the basement membrane, and immunoglobulin and complement are usually demonstrable by fluorescent techniques. Eventually disorganization of the membrane occurs with splitting and fibrillary thickening. In individual patients the serum complement may be low or normal, and ASO titres are also variable. Serum protein changes are frequently modified towards a nephrotic pattern by the heavy proteinuria that many of these patients show.

Focal glomerulonephritis (Heptinstall, 1964)
This is another form of proliferative lesion, the affected areas being confined to focal parts of the tuft. Proliferation is of endothelial or epithelial cells, most commonly of both, and fibrillary thickening of the basement membrane is seen in involved areas. Detailed electron microscope studies have been reported in recent literature (Lannigan & Insley 1965). Immunofluorescent studies are few, but these have shown immunoglobulin deposits in the lesions.

As in acute glomerulonephritis, the serum γ-globulin is usually raised. Serum complement levels are, however, only seldom low. Very high ASO titres are found in some patients, suggesting a streptococcal aetiology.

Lobular glomerulonephritis
In the glomeruli of patients with this condition there is only moderate proliferation confined to the mesangial cells, but there is gross infiltration of the mesangial zone with material with the staining characteristics of glycoprotein. With the PA silver techniques, this shows a spongy network of fibrils suggesting that it is relatively disorganized structurally compared with basement membrane. In many respects the lesion resembles the diabetic nodular lesion, but is more widely distributed throughout the glomeruli; in addition there is no evidence of diabetes. Electron microscopic and immunofluorescent studies have been reported in this condition (Anagnostopoulos & de Montera 1964).

(B) MEMBRANOUS GLOMERULONEPHRITIS
(Movat & McGregor 1959)
Clinically this lesion is found in some patients who develop oedema of insidious onset. There is seldom a history of episodes of acute infection. Heavy proteinuria (>0.15 g/kg/day) is almost the rule.

Histologically the lesion is quite characteristic (Brewer 1964), and appears to be specific for the syndrome. In the fully developed lesion there is thickening of the basement membranes on light microscopy; with the PAS stain this is diffuse, but with PA silver stain only a part of the membrane stains, giving a castellated appearance to the outer margin. On electron microscopy the membrane is seen to be grossly disorganized, being heavily infiltrated with deposits, usually less dense than the membrane itself. Splitting or fibrillary change in the membrane

is not seen. The deposits in the membrane stain strongly for immunoglobulin. Epithelial cell foot processes are sometimes lost over the lesions, but cellular proliferation and infiltration of the glomeruli is not seen. Characteristically the mesangial zone is not involved. Serum complement is not depressed, nor are there any other systemic signs of an immunological reaction (Plate 34.4).

(c) Minimal Changes (Lipoid Nephrosis)

(Plate 34.6)

In some patients with heavy proteinuria no lesion can be seen in the glomeruli on light microscopy. Electron microscopy shows only a diffuse loss of the foot processes of the epithelial cells. With successful treatment with steroids these foot processes rapidly return (Pollak *et al* 1958). This lesion is most commonly seen in childhood, though it can occur at all ages, and is found in 10–20% of adults presenting with a nephrotic syndrome. Most reports have failed to demonstrate immunoglobulin deposition in these cases (McCluskey *et al* 1966; Drummond *et al* 1966).

In most patients there is no evidence for systemic immunological process, though a few have been reported in association with a hypersensitivity to a variety of allergens (Hardwicke *et al* 1959). Serum complement values may be at the lower limit of the normal range, and these rise with successful treatment (Lange *et al* 1955).

II. Secondary Lesions

There are many occasions when the kidney is involved secondary to a well-recognized systemic disease; only a few of those which have been well investigated from an immunological viewpoint will be considered.

(a) Systemic Lupus Erythematosus

(McCluskey *et al* 1966)

The immunological nature of this disease is well authenticated, and glomerular lesions are commonly found. The type of lesion may vary widely, and can mimic almost any form of glomerulonephritis. Widespread deposition of immunoglobulin and complement components are found in any location within the glomeruli, subendothelially, within the membrane, or subepithelially (Koffler & Paronetto 1965). Fibrinogen is also often found deposited in intracapillary sites, and variable amounts of proliferation, necrosis and fibrillary thickening of the basement membrane are found.

(b) Goodpastures' Syndrome

(Duncan *et al* 1965)

In this syndrome, of haemoptysis associated with proteinuria, a severe form of progressive proliferative disease is usually found. Histologically there is an

increase in the basement membrane thickness, and on electron microscopy this is seen to be the result of linear deposition of electron-dense material subendothelially. This material sometimes shows a fibrillary structure, and fluorescence microscopy shows it to contain fibrinogen, in addition to immunoglobulin and complement. This immunofluorescent technique shows, characteristically, a linear deposition of IgG on the glomerular capillary walls.

(c) Diabetes (Lannigan *et al* 1964)

Fibrillary thickening of the basement membrane is the commonest lesion in this disease. In addition a marked infiltration of the mesangial zones with material which stains with the PAS stain, but not with PA silver, may be found; when fully developed this becomes the Kimmelstiel-Wilson lesion (Kimmelstiel & Wilson 1936). Immunofluorescence shows immunoglobulin in these deposits, though the glycoprotein components have not been identified.

III. Lesions Associated with Fibrin Deposition

In addition to the lesions described above, there are three conditions involving the kidney in man, which are associated with deposition intra-glomerularly, of components of the coagulation system. In many other forms of glomerulitis fibrin or fibrinogen are found in association with immunoglobulin precipitates. No such immunoglobulins are found in these conditions, but because of the experimental association between fibrinogen deposition and immunological reactions these are described briefly.

(a) Toxaemia of Pregnancy

(Vassali *et al* 1963)

Patients with toxaemia show a diffuse thickening of the basement membrane and mesangial zones with the PAS stain, together with a minor degree of endothelial cell proliferation and marked endothelial and epithelial swelling (Pirani *et al* 1965). With the PA silver stain the thickening is fibrillary. Fluorescence microscopy shows fibrillary deposition of fibrinogen on the membrane, and in addition fibrinogen reacting material in the mesangial zone and within the endothelial cells. Immunoglobulin and complement are rarely seen, and when present are in occasional nodular deposits unrelated to the main lesion.

(b) Acute Cortical Necrosis

In some cases of septicaemia acute necrosis of the glomeruli and the renal cortex is found, often in association with adrenal cortical necrosis. The lesion is one of deposition within capillaries and arterioles of platelet and fibrin emboli, with consequent ischaemic necrosis.

(c) The Haemolytic-uraemic Syndrome

(Gianantonio *et al* 1966, Habib *et al* 1967)

This syndrome is almost confined to infants. The histological lesions in the kidney are similar to those seen in acute cortical necrosis, namely fibrin and platelet deposition and embolization. In addition morphological changes in the erythrocytes, leading to 'burr' cells are found. The blood shows a hypercoagulable state, and scattered lesions are found in other organs. A number of viral infections are associated with the disease, and death or chronic renal damage is usual. Successful treatment with fibrinolytic agents and with heparin has been claimed.

(C) EXPERIMENTAL GLOMERULONEPHRITIS

It will now be apparent that, with the possible exception of minimal change lesions, most forms of glomerulonephritis are associated with immunoglobulin, or coagulation system components within the glomeruli; sometimes this is associated with systemic evidence of an immune response (Lange *et al* 1966). The next section will discuss some of the many experimental models which have been developed in an effort to reproduce the diseases seen in man.

I. Experimental Streptococcal Nephritis

In view of the body of evidence implicating nephritogenic streptococci in the production of acute nephritis in man, many attempts have been made to induce nephritis in animals using whole streptococci or a variety of streptococcal antigens. In the main these have been singularly unsuccessful (Uhr 1964).

In the rabbit and rat Type 12 streptococci grown intreperitoneally in Millipore chambers from which they cannot escape lead to renal tubular lesions only; streptolysin S is the effective agent (Tan *et al* 1961). Whole killed streptococci injected intraperitoneally with kidney homogenates (Cavelti & Cavelti 1945) have been shown to produce both tubular damage, due to streptolysin S, and glomerular proliferative lesions. The streptococcal M protein used as an antigen produces an antiserum (Kantor 1965) which cross-reacts immunologically with fibrinogen, and in addition the M protein itself will precipitate fibrinogen. In mice intravenous injection (Kantor 1965) of 3 mg of M protein will produce a glomerulitis, characterized by capillary thrombi and fibrin deposition within the glomeruli. Serum complement is not involved in this reaction.

It seems unlikely that these lesions are related to the human disease. Histologically they differ, the dose of antigen is too great and complement is not involved. It has been suggested that acute nephritis in man represents an acute toxic lesion similar to that due to M protein, followed by a secondary 'auto-immune' nephritis (Rammelkamp 1964) in those patients in whom the lesion is progressive. This implies a mechanism similar to that seen in the 'auto-immune

allergic disease' described below; while this could be so in the progressive proliferative lesions, the model of the primary lesion in no way resembles, histologically, acute glomerulonephritis in man.

II. The Role of Circulating Antigen-Antibody Complexes

(a) Acute Lesions

It has been known for many years that proteinuria was an accompaniment of the acute serum sickness that follows a single large dose of antigen intravenously. In the rabbit proteinuria appears 10–14 days after injection, and in most instances recovers spontaneously after a short period. The actual nature of the damage to blood vessels varies with the antigen used (Hawn & Janeway 1947), but little detailed work is available on the relative nephritogenicity of different antigens. Proteinuria appears, together with maximum systemic effects, at the time of increasing antibody production and consequent rapid antigen destruction (Feldman 1958); the peak of the renal damage just follows the time when a high proportion of the remaining intravascular antigen can be shown to be circulating as complexes with antibody in antigen excess (Talmage *et al* 1951). Histological damage on light microscopy of the glomeruli at this time is usually remarkably slight, at least with the antigens mainly studied (bovine serum albumin, human serum albumin, bovine IgG and human IgG) in spite of the large amounts of complexes disappearing daily from the circulation. There is a moderate proliferation of endothelial cells, with occasional polymorph infiltration, but this seldom approaches that seen in the human acute glomerulonephritis (Feldman 1958); red cells are not seen in the tubules, nor is haematuria commonly present.

Much more striking damage is seen on electron microscopy. There are numerous deposits in relation to the basement membrane; most of these are subepithelial in site, separated from the membrane itself, and typically small and nodular (the 'lumpy-bumpy' deposits). Antigen and antibody can be demonstrated in the nodules by immunochemical techniques. The lesions resolve rapidly within 14–21 days, leaving no sequelae (Fish *et al* 1966). Large amounts of antigen (>100 mg/kg) are required to produce the typical 'one-shot' lesions in rabbits (Plate 34.3).

In view of the suggestive evidence provided by acute serum sickness, many attempts have been made to induce renal lesions with intravenous injections of preparations of soluble antigen-antibody complexes. Such experiments do indeed produce renal lesions and proteinuria, but again large amounts of complex are necessary (McCluskey *et al* 1962). Both antigen and antibody are demonstrable in the lesions. The lesions resolve rapidly, and chronic renal damage has not been obtained. Preliminary damage of the vascular endothelium, by agents such as histamine or endotoxin, facilitate the deposition of complexes on the capillary walls and accentuate the lesions produced (Cochrane 1963).

(B) CHRONIC LESIONS

Repeated injections of small amounts of heterologous serum proteins over many months have been reported to produce a variety of renal lesions in rabbits (Longcope 1913; McClean *et al* 1951). Dixon *et al* (1961) have studied this model in detail in the rabbit, using simple antigens (BSA, human serum albumin and human IgG) and daily intravenous injections. By titration of the rabbit sera and adjusting the intravenous dose of antigen, an attempt was made to maintain the animals close to antigen-antibody equivalence over each 24 hr period. Animals producing large amounts of antibody produce an acute renal lesion analogous to that of 'one-shot' serum sickness. In these animals attempts to maintain antigen excess by increased dosage of antigen resulted in a high mortality from anaphylactic shock. In some animals little antibody was produced. In these immune paralysis appeared early, and no renal damage ensued. In a further group of intermediate antibody producers the dose given nearly balanced the antibody production, over long periods, and in these chronic renal lesions were induced after a period of 2–3 months. These results have been confirmed (Andres *et al* 1963), and the sequence of events in these animals appears characteristic. During an initial phase of 10–20 days the daily antigen injections exceeded antibody production, to the extent that antigen was detectable in the circulation 24 hr after injection, and preceding the next dose. Following this was a period during which antibody was found 24 hr after injection of antigen, but was absent immediately following each antigen injection. Proteinuria during this period was intermittent or absent. In the third phase antigen again appeared in excess before each injection, and it was during this period that proteinuria reappeared and became persistent. Severe and progressive renal lesions appeared at this time, and sometimes progressed following complete cessation of injections. Histologically the glomeruli show moderate proliferative changes, with marked membrane thickening with the PAS stain. Electron microscopy shows widespread deposition of electron-dense material, both subendothelially and subepithelially, and splitting and reduplication of basement membrane-like material.

Germuth and colleagues (1967) have recently reported the effects of constant daily intravenous injections of BSA into rabbits in a dosage varying from 0.1 to 25 mg. Only those receiving 12.5 or 25 mg were affected. At the higher dosage only five out of twenty-five animals produced significant antibody. Two of these died of anaphylaxis, while the remaining three developed renal failure. Fifty-six animals were given 12.5 mg daily; these animals mostly produced high antibody titres and in this group many developed chronic renal lesions. Once weekly the animals were bled, 24 hr following the last intravenous injection, and the excess of antibody or antigen titrated. Many of the animals passed from antigen to antibody excess at 14–21 days, and had an acute episode of proteinuria. They then remained in antibody excess for a variable period of time before again passing into antigen excess with the development of immunological

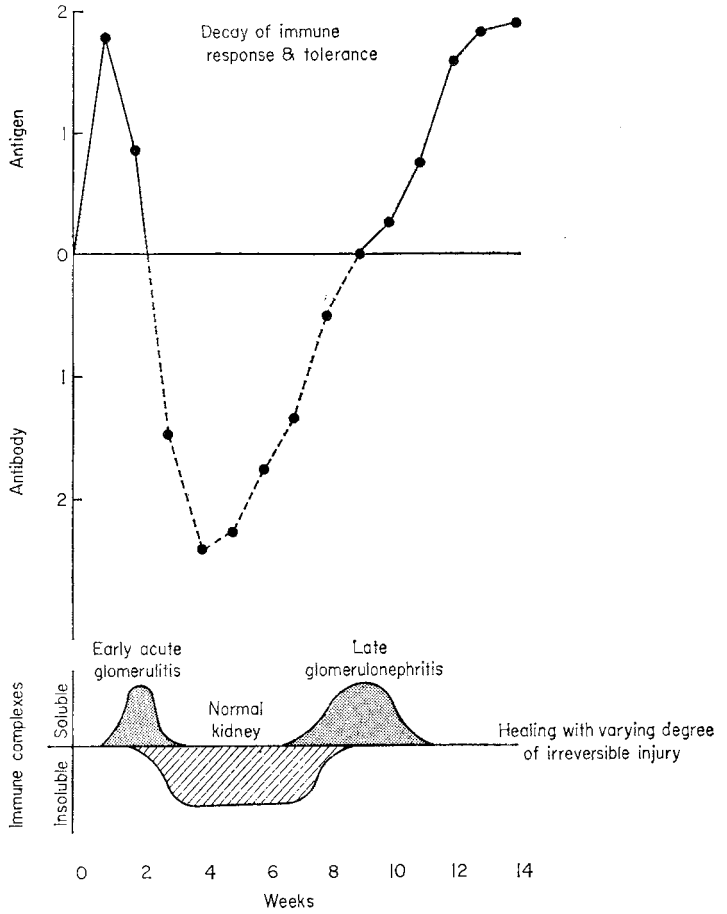


FIGURE 34.1. The sequence of events in rabbits developing late glomerulonephritis following daily intravenous injections of BSA. (From Germuth *et al.* (1967). Reproduced by kind permission.)

An initial bout of proteinuria occurs at the time at which animals first pass into antibody excess 24 hr following each intravenous dose of antigen; this is analogous to acute serum sickness following a large single dose of antigen. After a varying period in antibody excess, some animals develop increasing immunological paralysis; at about the time at which daily antibody production is only just enough to destroy the injected antigen in 24 hr, a second bout of proteinuria and glomerular damage occurs, which may become progressive.

paralysis. At about this time they again developed proteinuria, and the glomeruli showed a lesion comparable to that described by Dixon *et al* (1961). This often resolved completely in those animals remaining in gross antigen excess, but in some who stayed around equivalence for long periods chronic progres-

sive renal failure developed. At about the time of recurrence of proteinuria the Arthus reaction, previously positive, became negative (Fig. 34.1).

Boyns (1966) has given isotopically labelled BSA to rabbits, and varied the injection schedule to maintain antigen constantly present in the circulation. Estimation of complexes showed that up to 80% of antigen was bound to antibody in the circulation, and 15–20 mg of antigen were probably being destroyed in this form daily. Proteinuria in these animals was only intermittent, and most marked over the period 14–30 days. By 80 days all animals were immunologically paralysed to the antigen, and none showed proteinuria. No animals developed chronic renal lesions, and biopsies of the kidney from 50 days on showed only minor and scattered lesions of the type associated with acute serum sickness.

Dixon *et al* suggest that there is a critical size of complex, present only very close to equivalence, which can localize in the glomeruli and cause damage. If this is so then it appears that this size has only been produced experimentally in animals that have been in antibody excess for at least part of the time. Persistent antibody excess does not lead to chronic lesions. Persistent circulation of antigen, of which a high proportion is complexed to antibody, is also not associated with chronic damage (Boyns 1966). Possibly the endothelial damage induced during periods of exact antigen-antibody equivalence in the circulation potentiates the effect of circulating soluble complexes during the subsequent phase by inducing fixation within glomerular capillaries (Cochrane 1963).

This is a difficult model to understand, and merits much further investigation. So far only simple types of antigen have been investigated in this model. It remains possible that other types of antigen might be effective in much smaller doses. Also the relative importance of the different immunoglobulin types has not been studied; while IgM might be of considerable importance in the acute lesion, it seems improbable that its titre would be sufficiently high in the later chronic phases to be of significance (Wissler *et al* 1959).

III. Kidney Fixing Antibodies

In the section above the models considered concerned antibody reactions to proteins which, in the absence of antibody, show no special tendency to localize in the kidney. A number of further experiments are described in which antibody production to components of the glomeruli is produced by the injection of isologous, homologous or heterologous antigens derived from the kidney. The antibody produced in these models can be shown to fix to glomerular components, usually the basement membrane, and to induce damage.

(a) Heterologous Anti-kidney Antibody

(Masugi 1933).

Antibodies raised in a number of animals to heterologous kidney extracts will

induce renal damage when reinjected into the donor species. This damage is related to antibody against donor basement membrane. The pattern of disease is characteristically biphasic. During the initial phase the heterologous antibody is fixed to the recipient glomeruli and proteinuria ensues. This then remits wholly or partially. At 10–15 days proteinuria reappears, at this time being related to the deposition of host immunoglobulin, presumably against the donor immunoglobulin fixed to the kidney in the initial phase (Unanue & Dixon 1965). By a variety of manipulations one or other phase can be suppressed (Hammer & Dixon 1963; Halpern *et al* 1967). The chronicity of the lesion induced varies from species to species and with the dose of antibody injected, but the nephritis from a single antibody injection in low dosage will usually recover spontaneously. It has been suggested that when the lesion becomes progressive secondary mechanisms may have come into play (Pfeiffer & Federlin 1966), but this is so far by no means proved. The kinetics of this type of nephritis are complex, and are admirably reviewed by Unanue & Dixon (1967) who are themselves responsible for much of the work on the subject. Usually a fall in complement occurs at the time of maximal renal damage (Halpern *et al* 1965) and circulating polymorphs are necessary for damage to occur (Cochrane *et al* 1965). However, disease can be induced by non-complement fixing antibody in the absence of polymorphs (Hammer & Dixon 1963). Injections of heparin or warfarin concurrently with or preceding the administration of antibody will modify its action (Halpern *et al* 1965), probably by preventing progressive renal damage, rather than by inhibiting the reaction of the antiserum with the basement membrane. IgM antibody is considerably more effective, on a weight basis, than IgG (Unanue & Dixon 1965).

The antigen responsible for nephrotoxic antibody production has been shown to be a glycoprotein present in basement membrane, and constituting about 20% of its substance (Shibata *et al* 1966).

Histologically light microscopy shows varying degrees of cellular infiltration and proliferation (Reid 1956). The glomerular basement membrane is thickened, and the PA silver stain shows this to be fibrillary. Electron microscopy (Dixon 1966) shows linear deposition, subendothelially, of electron-dense material. Following repreated injections of antibody a series of such linear depositions may be built up one on the other. Immunohistological studies show linear deposition of donor immunoglobulin on the glomerular capillaries in the initial phase, and in the later phase host immunoglobulin, together with complement components and fibrinogen. Antibodies to rat collagen will also fix to the rat glomerulus (Rotbard & Watson 1961).

(B) AUTO-IMMUNE (ALLERGIC) GLOMERULONEPHRITIS

Two types of 'auto-immune' nephritis have been described. Both require the

injection of antigen in Freund's adjuvant. The first of these 'auto-immune allergic', follows the injection of heterologous kidney intramuscularly in adjuvant into sheep (Steblay 1963). At the time at which the sheep produce antibody directed against donor kidney, or shortly after, they develop a severe progressive glomerulonephritis themselves, which goes on to renal failure. Serum from these sheep will induce nephrotoxic nephritis in the donor species, but not in other sheep. Nephritis can, however, be transferred by cross-circulation experiments, or, by nephrectomizing the nephrotic sheep, harvesting the serum 8–10 days after nephrectomy and injecting this into unilaterally nephrectomized lambs, which then develop nephritis (Lerner & Dixon 1966). This shows that homologous antibody is produced but is adsorbed to the host kidney very rapidly. 160 μg of kidney fixing IgG from such nephrectomized animals will induce lesions in the unilaterally nephrectomized lambs. Similar auto-antibodies directed against basement membrane have been found in association with immunization to heterologous, or homologous (but not with autologous) preparations of glomeruli in most laboratory mammals. Most of these are less severe than in the sheep and are seldom progressive (Unanue & Dixon 1966a and b). Antibodies to renal tubular basement membrane may also be found. This model therefore appears to be the auto-allergic counterpart of nephrotoxic serum nephritis.

In the sheep the histological lesion is of a severe proliferative glomerulonephritis, with both endothelial and epithelial cell proliferation. Marked crescent formation occurs, and progresses rapidly to fibrosis (Steblay 1962). In other species histological lesions are much less severe, and often resolve spontaneously. Fluorescence microscopy shows deposition of host immunoglobulin in the glomeruli (or tubules) and on electron microscopy this is subendothelial in position.

(c) 'Auto-immune' Nephritis

The second type follows the injection of preparations of rat kidney in complete Freund's adjuvant intraperitoneally in homologous rats. The intraperitoneal route is essential, intramuscular and foot pad injection being ineffective (Heymann *et al* 1959). Production of lesions is variable between different strains of rats. Whole kidney homogenates, glomerular preparations and diazotized or unaltered mitochondrial fractions are effective (Unanue & Dixon 1967). Persistent proteinuria follows 8–10 weeks of repeated injections, but a transient proteinuria is found in many animals during the 3rd week (Barabas, personal communication).

The histological lesion closely resembles membranous glomerulonephritis in man; with thickening of the basement membrane, and disorganization by infiltration with less electron-dense material (Plate 34.5). Immunohistological studies show deposition of host immunoglobulin within the glomeruli exactly

as in the human lesion; in addition the deposits can be stained with an antiserum raised in other species to rat tubular components. This antiserum reacts with the tubular brush border in the normal rat kidney. Fluorescent IgG from affected rats stains subcellular fractions of the renal tubule, but not the affected or normal glomeruli (Barabas, personal communication). Passive transfer by serum is ineffective, but paraboisis or lymph node cells are (Hess *et al* 1962).

It is suggested (Unanue & Dixon 1967) that this model is the result of deposition of soluble antigen-antibody complexes composed of auto-antibody with a tubular antigen normally present in the circulation. There is some ultrastructural difference from the lesion of acute serum sickness. In acute serum sickness the deposits are within the epithelial cell or closely surrounded by cell cytoplasm and the basement membrane is substantially normal. In the Heymann 'auto-immune' model the deposits lie apparently between the foot processes, or within the substance of the basement membrane which is structurally grossly disorganized.

Control experiments have studied the effect of complete Freund's adjuvant alone on the rat's kidney (Watson *et al* 1965).

A similar lesion is produced by the injection intravenously of isologous kidney in pertussis vaccine (Blozis *et al* 1962).

(D) Spontaneous 'Autoimmune' Nephritis

A spontaneous nephritis occurs in the NZB/BL mice (Mellors 1965). This shows a remarkable histological similarity to disseminated lupus erythematosus, and it has recently been shown that the deposits contain DNA and IgG. Deposition may be subendothelial or subepithelial, and variable amounts of proliferation are found (Dixon 1967).

IV. Schwartzmann Phenomenon

In the generalized Schwartzmann reaction resulting from repeated injection of bacterial endotoxin preparations (see Chapter 1), bilateral renal cortical necrosis may be found as a result of the extensive vascular obstruction caused by platelet, polymorph and fibrin thrombi (McKay *et al* 1966).

V. Glomerulitis due to Physical and Chemical Agents

Primary renal injury resulting from physical or chemical agents is not truly immunological. However, it still remains possible that progressive damage, after the initial stimulus is withdrawn, may be due to secondarily excited immunological means. It therefore would seem desirable to mention such models briefly in this chapter.

(A) AMINONUCLEOSIDE NEPHROSIS

(Lannigan *et al* 1962)

Injections of the aminonucleoside from Puromycin intravenously into rats or dogs leads, after 8–10 days, to the appearance of massive proteinuria. This proteinuria resolves spontaneously after a further 10–20 days. Long-term survival in those rats who survived the acute episode is, however, reduced when compared with paired litter-mate controls (Lannigan 1963), and the injected animals develop progressive renal failure after a latent period of 6–9 months.

In the acute phase the only glomerular lesion demonstrable is a loss of foot processes of the podocytes, analogous to that seen in 'minimal change' nephrotic syndrome in man (Plate 34.7).

Immunohistological studies in this lesion show only non-specific accumulation of IgG in mesangial areas. Chemical investigation of isolated basement membrane material in treated rats shows a deficiency of hydroxylysine and hydroxyproline with an increase in lysine and proline residues (Kefalides 1966).

Studies on the general effects of aminonucleoside show excessive glycogen deposition in the liver, with a failure of normal glycogenolysis on starving (Kmetec 1966).

(B) SACCHARATED IRON OXIDE

Intravenous injection of saccharated iron oxide into rabbits has shown massive depositions of granular, iron-containing material in the glomerular capillaries. After a period of 4–8 days a proteinuria develops, at about the time at which the major part of the deposits are cleared from the capillary lumens. Repeated injections lead to azotaemia and renal failure. Electron microscopic and immunohistological studies have yet to be reported in this model (Ellis 1956).

D. CORRELATION OF HUMAN WITH EXPERIMENTAL DISEASE

From the morphological point of view it is now possible to produce in animals glomerular lesions which closely resemble those seen in the various forms of human disease.

Nevertheless, it still remains extremely doubtful as to whether any of these experimental models truly reflects the histologically similar human disease. This final section will consider some of the difficulties inherent in extrapolating too confidently from the animal to the human situation.

ACUTE STREPTOCOCCAL NEPHRITIS

Histologically this lesion shows considerable resemblance to the experimental acute serum sickness model; in addition both show an acute fall in serum complement. Endothelial cell proliferation and haematuria are both more pronounced in the human disease than in the animal model. In animals, the quantity

TABLE 34.1

	Human disease	*Histology (human)*	*Animal model*
I.	Acute streptococcal glomerulonephritis (? acute serum sickness)	Nodular subepithelial deposits. Nodular deposition of IgG and complement	Acute serum sickness *
II.	Progressive proliferative glomerulonephritis	Epithelial cell proliferation, crescent formation and scarring. Diffuse deposition of IgG and complement. Subendothelial deposits	Autoimmune allergic glomerulonephritis in sheep†
III.	Systemic lupus erythematosus	Massive deposition of immunoglobulin sub-endothelial and sub-epithelial	NZB/BL mice*
IV.	Lobular glomerulonephritis	Deposition of immuno-globulin and ? fibrinogen in mesangial zone	?
V.	Goodpasture syndrome. Systemic lupus erythematosus	Epithelial and endothelial cell proliferation. Scarring. Subendothelial deposits with basement membrane splitting. Linear intracapillary deposition of IgG and complement	Nephrotoxic antiserum nephritis†
VI.	Membranous glomerulonephritis	Thickened basement membrane with castellations. Massive deposition and vacuolation within basement membrane or subepithelially. IgG deposition in membrane	Autoimmune nephritis of rats†
VII.	Acute cortical necrosis. Haemolytic-uraemic syndrome	Endothelial cell damage with platelet and fibrin thrombi	Generalized Schwartzmann reaction
VIII.	Toxaemia of pregnancy	Fibrinogen deposition on capillary walls without immuno-globulin	?
IX.	'Minimal change' nephrotic syndrome	Loss of foot processes of epithelial cells	Aminonucleoside nephrosis of dogs and rats

* Postulated as due experimentally to soluble complex deposition (Unanue & Dixon 1967).

† Postulated as due experimentally to antibodies to basement membrane (Unanue & Dixon 1967).

of antigen destroyed as complex is more than 5 mg/kg/day, and just before the lesion in the kidney is most marked large amounts of antigen are circulating complexed with antibody. If the human disease is comparable then similar quantities of complexed antibody should be demonstrable just before or in the early stages of the disease. Much smaller amounts of complex could be the operator, if, for some reason, they fixed specifically in the glomeruli, but so far searches for streptococcal antigens with this ability have been unsuccessful. Tubular damage has been produced, and also a glomerular disease analogous to the auto-immune nephritis of rats. While this might be evidence for the antigenicity of a carbohydrate component of streptococci when injected in Freund's adjuvant, it does not correspond histologically with the findings in human disease.

Subepithelial lumpy deposits can appear following the intravenous injection of globin, and globin aggregates have been shown to pass through the basement membrane without causing marked inflammatory changes (Menefee *et al* 1964). While the deposits in acute glomerulonephritis in man may indicate antigen-antibody complex deposition, there is as yet no evidence that they cause the acute inflammatory renal lesion.

Progressive Proliferative Glomerulonephritis

There is considerable similarity in the appearances of this lesion, and of the 'auto-immune nephritis' in sheep. Patients with this type of lesion are currently being investigated for the presence of circulating antibody specific for glomerular components, in particular in patients who have had bilateral nephrectomy prior to renal transplantation. Reports of this type of work are likely to prove of the greatest value.

SLE

The lesions seen in the kidney in this condition vary widely in severity and type, from the gross 'wire-loop' lesion, with areas of glomerular necrosis, down to relatively minor proliferative changes. The histology and the pleomorphic nature of the lesions covers the whole range of experimentally induced models. Since, in SLE, a wide variety of auto-allergic phenomena are found, it seems likely that the renal lesions represent, in varying degree from case to case, a mixture of deposition of complexes involving non-renal antigen, and of specific reactions of antibody with antigen of renal origin.

Lobular Glomerulonephritis

As yet no experimental model resembling morphologically this lesion has been found. While some patients showing this lesion have a convincing past history

of acute streptococcal glomerulonephritis, in many others such a history cannot be obtained (Jennings & Earle 1961).

Haemoptysis with Renal Failure (Goodpasture's Syndrome)

The close resemblance of the lesions in this rare disease to those seen in nephrotoxic serum nephritis suggests that a similar aetiology may operate. In addition, clinical acute nephritis has been reported in association with apparently non-streptococcal pulmonary disease (Blainey 1966). Experimentally lung tissue is an effective source of potent nephrotoxic antisera (Krakower & Greenspon 1958), and therefore the circumstantial evidence suggests that primary lung damage may lead to the production of potent auto-allergic antibody cross-reacting with renal tissue in these patients. If in some of these patients a true antiglomerular antibody is demonstrated, linear deposition of host immunoglobulin on the parent membrane would be expected, and this might form a basis for definitive diagnosis (Duncan *et al* 1965).

Proliferative renal lesions are also seen in association with progressive subacute hepatitis (lupoid hepatitis), and evidence is strong that a similar cross-reacting auto-antibody is found in this condition.

Membranous Glomerulonephritis

The histological resemblance of the chronic human lesion to the Heymann model in rats is striking. In the human disease, however, the only evidence of an allergic process is the deposition of immunoglobulins in the lesion; no systemic evidence of immunological reaction has been reported in man. In addition a similar morphological lesion has been found in patients with thrombosis of the renal veins though immunoglobulin deposition has not yet been investigated. In such patients it is difficult to be sure that the lesion is truly secondary to the thrombosis, and not the primary predisposing cause; however, cases have been reported in which the thrombosis was traumatic in origin, and in whom there was no evidence for renal disease prior to its onset (Bayley *et al* 1965).

Acute Cortical Necrosis and Haemolytic Uraemic Syndrome

The resemblance histologically of these lesions to the generalized Schwartzmann reaction, and their known association with bacterial or viral infections, make it possible that they are aetiologically similar.

Toxaemia of Pregnancy

The only significant depositions seen in this lesion are of fibrinogen. Although fibrinogen deposition plays a part in the experimental lesions induced by

PLATE 34.6. Human 'minimal change' nephrotic syndrome (lipoid nephrosis).

Electron micrograph from a case of nephrotic syndrome taken during a fourth relapse. Many of the foot processes are fused (arrows).

facing p. 926

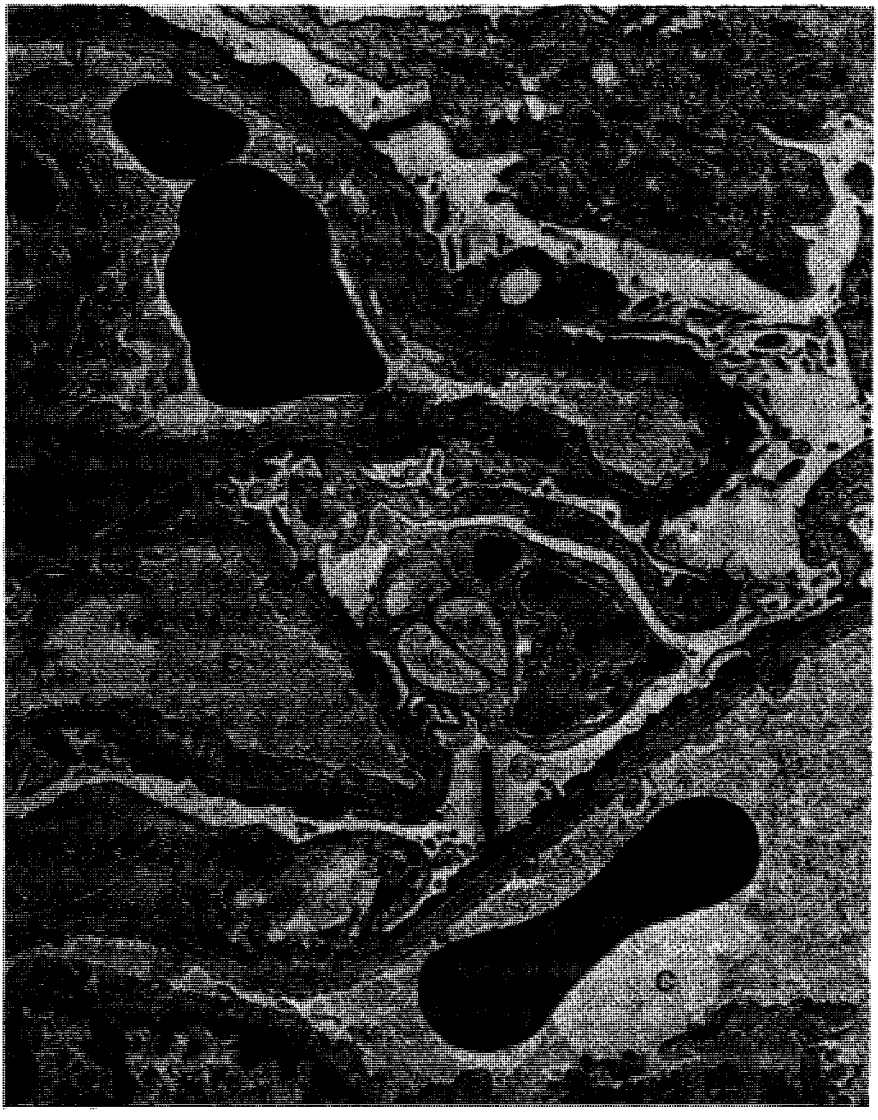


PLATE 34.7. Rat aminonucleoside nephrosis.

Electron micrograph from a rat given a single intravenous injection of mainonucleoside 5 days previously (×6000).

As in Plate 34.6 the foot processes are replaced by a continuous layer of epithelial cell cytoplasm.

soluble complexes and nephrotoxic antisera, on these occasions it is associated with marked immunoglobulin deposition. Although circulating anti-placental antibodies have been claimed to be demonstrable in some cases, the absence of immunoglobulins in the renal lesion makes it unlikely that they are of pathogenic significance.

'Minimal Change' Nephrotic Syndrome

There is as yet no clear evidence for an immunological basis for this condition. In the experimental aminonucleoside model the disease is related to the cytotoxic effects of a drug. In the human disease immunoglobulin deposition is not found. However, usually the proteinuria and the histological lesion respond rapidly to steroid therapy, and if this is ineffective, they also remit with either the azothioprine or the cyclophosphamide group of drugs. In one case in our experience (Hardwicke 1965) a series of relapses were associated on each occasion with a marked fall in serum haemolytic complement. An allergic basis to this condition therefore remains possible.

CONCLUSIONS

Very rapid advances have been made over the past few years in the study of the immunopathological mechanisms leading to glomerulonephritis in experimental animals. The various models have now been well defined, and are reproducible between laboratories. When allergic mechanisms are responsible the antigen has in many instances been characterized, whether of renal or non-renal origin. Detailed ultrastructural and immunohistological studies have been carried out on most of these models.

In human disease the technique of renal biopsy has made it possible to study glomerulonephritis in its acute stages, and to follow its subsequent progress. Many types of human disease show a remarkable morphological resemblance to one or other of the experimental models. In a limited number of investigations studies of glomerular permeability have also demonstrated similar alterations both in human disease and in its experimental morphological counterpart (Hardwicke 1965).

In spite of these similarities it would be unwise at the moment to conclude that similar immunopathological mechanisms are responsible for the apparently similar lesions. As yet, circulating complexes remain to be demonstrated preceding or in the course of human disease, no glomerular fixing antigen has been demonstrated in streptococci, and the presumed antigens responsible for the fixation of immunoglobulin and complement in glomeruli have yet to be isolated, to mention only a few of the reservations.

The experimental models have proved of the greatest value in indicating the direction in which investigations into human disease should proceed. Currently,

much work is being undertaken along these lines, and publication of the results will be awaited with interest.

ACKNOWLEDGMENTS

My thanks are due to Dr D.B.Brewer, Dr A.J.Fish, Dr R.L.Vernier and Dr. F.Germuth for permission to reproduce figures and plates. I am also grateful to Dr R.L.Lannigan for the other illustrations of glomerular morphology. I am particularly indebted to Dr E.R.Unanue for reading the manuscript, and for much helpful discussion of the section on Experimental Glomerulonephritis.

REVIEWS

MICHAEL A.F., DRUMMOND K.N., VERNIER R.L. & GOOD R.A. (1964) Immunologic basis of renal disease. *Ped. Clin. N. Amer.* **11,** 685.

MULTIPLE AUTHORS (1965) Mechanisms of injury by immunological methods. *Fed. Proc.* **24,** 93.

PETERS J.H. & FREEDMAN F. (1959) Immunological aspects of renal disease. *New Engl. J. Med.* **261,** 1166, 1225, 1281.

UHR J. (1964) *The Streptococcus, Rheumatic Fever and Glomerulonephritis.* Baltimore: Williams & Wilkins.

UNANUE E.R. & DIXON F.J. (1967) Experimental glomerulonephritis; immunological events and pathogenetic mechanisms. *Adv. in Immunol.* **6,** 1.

REFERENCES

ANAGNOSTOPOULOS T. & DE MONTERA H. (1964) Lobular glomerulonephritis. *Rev. Franc.* **9,** 934.

ANDRES G.A., SEEGAL B.C., HSU K.C., ROTHENBERG M.S. & CHAPEAU M.L. (1963) Electron microscopic studies of experimental glomerulonephritis with ferritin conjugated antibody. *J. exp. Med.* **117,** 691.

BARABAS A. (1966) Personal communication.

BAYLEY T.J., HARDWICKE J., HEATH D. & WHITFIELD A.G.W. (1965) Nephrotic syndrome due to thrombosis of the inferior vena cava and renal veins. *Postgrad. med. J.* **41,** 88.

BLAINEY J.D. (1966) Acute nephritis and pulmonary disease. *Ann. Allergy* **24,** 361.

BLOZIS G.C., SPARGO B. & ROWLEY D.A. (1962) Glomerular basement membrane changes with nephrotic syndrome produced in the rat by homologous kidney and H. pertussis vaccine. *Am. J. Path.* **40,** 153.

BOYNS A.R. (1966) Studies on soluble antigen-antibody complexes. Ph.D. thesis, Birmingham University.

BREWER D.B. (1964) Membranous glomerulonephritis. *Renal Biopsy,* p. 34. London: Edward Arnold.

CAVELTI P.A. & CAVELTI E.G. (1945) Studies on the pathogenesis of glomerulonephritis. II. Production of glomerulonephritis in rats by means of autoantibodies to kidney. *Arch. Path.* **40,** 158.

COCHRANE C.G. (1963) Studies on the localization of circulating antigen-antibody complexes and other macromolecules in vessels. I. Structural studies. II. Pathogenetic and pharmacodynamic studies. *J. exp. Med.* **118,** 489, 503.

COCHRANE C.G., UNANUE E.R. & DIXON F.J. (1965) The role of polymorphonuclear leucocytes and complement in nephrotoxic nephritis. *J. exp. Med.* **122,** 99.

DIXON F.J. (1966) Pathogenesis of immunologically induced glomerulo-nephritis. *Proc. III Int. Congr. Nephrol.*

DIXON F.J., VAZQUEZ J.J., WEIGLE W.O. & COCHRANE C.G. (1958) Pathogenesis of serum sickness. *Arch. Path.* **65,** 18.

DIXON F.J., FELDMAN J.D. & VAZQUEZ J.J. (1961) Experimental glomerulonephritis. Pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis. *J. exp. Med.* **113,** 899.

DIXON F.J. *et al* (1967) *VI Int. Symp. Immunopath.* (In press).

DRUMMOND K.N., MICHAEL A.F., GOOD R.A. & VERNIER R.L. (1966) The nephrotic syndrome in childhood: immunologic, clinical and pathologic correlations. *J. clin. Invest.* **45,** 620.

DUNCAN D.A., DRUMMOND K.N., MICHAEL A.F. & VERNIER R.L. (1965) Pulmonary haemorrhage and glomerulonephritis. *Ann. Int. Med.* **62,** 920.

ELLIS J.T. (1956) Glomerular lesions and the nephrotic syndrome in rabbits given saccharated iron oxide intravenously. *J. exp. Med.* **103,** 127.

FELDMAN J.D. (1958) Electron microscopy of serum sickness nephritis. *J. exp. Med.* **108,** 957.

FISH A.J., MICHAEL A.F., VERNIER R.L. & GOOD R.A. (1966) Acute serum sickness in the rabbit (an immune deposit disease). *Am. J. Path.* **49,** 997.

GERMUTH F.G. JR., SENTERFIT L.B. & POOLACK A.D. (1967) Immune complex disease. I. Experimental acute and chronic glomerulonephritis. *Johns Hop. Med. J.* **120,** 225.

GIANANTONIO C.A., VITACCO M. & MENDILHARZU F. (1966) The haemolytic uraemic syndrome. *Proc. III Int. Congr. Nephrol.*

GOTOFF S., FELLERS F., VAWTER G., JANEWAY C. & ROSEN F. (1966) Serum levels of β_1c-globulin, a complement component, in the nephritides, lupoid nephritis and other conditions. *New Engl. J. Med.* **273,** 524.

HABIB R., MATHIEU H. & ROYER P. (1967) Le syndrome hemolytique et uremique de l'enfant. Aspects cliniques et anatomiques dans 27 observations. *Nephron,* **4,** 139.

HALPERN B., MILLIEZ P., LAGRUE G., FRAY A. & MORARD J.C. (1965) Protective effect of heparin on immune nephritis (heretologous serum) in rabbits. *Nature,* **205,** 257.

HALPERN B., LAGRUE G., BARIETY J. & FRAY A. (1967) Recherches sur les nephrites heteroimmunes experimentales. I. Évolution spontanée de la maladie et étude de l'activité complementaire du serum. II. Prevention par les agents immunosuppressives et par certaines anticoagulants. *Path. et Biol.* **15,** 368.

HAMMER D.K. & DIXON F.J. (1963) Pathogenesis of nephrotoxic serum nephritis. *J. exp. Med.* **117,** 1019.

HARDWICKE J., SOOTHILL J.F., SQUIRE J.R. & HOLTI G. (1959) Nephrotic syndrome with pollen hypersensitivity. *Lancet* **i,** 500.

HARDWICKE J. (1965) The significance of quantitative and qualitative analyses of the proteinuria in the nephrotic syndrome. *J. clin. Path.* **18,** 559.

HAWN C.V.Z. & JANEWAY C.A. (1947) Histological and serological sequences in experimental hypersensitivity. *J. exp. Med.* **85,** 571.

HEPTINSHALL R.H. and JOEKES A, M, (1964) Focal glomerulonephritis. *Diseases of the Kidney,* ed. STRAUSS & WELT. J & A. Churchill, London.

HERDSON P.B., JENNINGS, R.B. & EARLE D.P. (1966) Glomerular fine structure in poststreptococcal acute glomerulonephritis. *Arch. Path.* **81,** 117.

HESS E.V., ASHWORTH C.T. & ZIFF M. (1962) Transfer of autoimmune nephritis in the rat by means of lymph node cells. *J. exp. Med.* **115,** 421.

HEYMANN W., HACKEL D.B., HARWOOD S., WILSON S.G.F. & HUNTER J.L.P. (1959) Production of nephrotic syndrome in rats by Freund's adjuvant and rat kidney suspensions. *Proc. Soc. exp. Biol. Med.* **100,** 660.

JENNINGS R.B. & EARLE D.P. (1961) Post streptococcal glomerulonephritis. Histopathological and clinical studies of the acute, subsiding acute and early chronic latent phases. *J. clin. Invest.* **40,** 1525.

KANTOR F.S. (1965) Fibrinogen precipitation by streptococcal M protein. I. Identity of the reactants and stoichiometry of the reactants. II. Renal lesions induced by the intravenous injection of M protein into mice and rats. *J. exp. Med.* **121,** 849, 861.

KARK R.M., MUEHRCKE R.C., PIRANI C.L. & POLLAK V.E. (1955) Clinical value of renal biopsy. *Ann. Int. Med.* **43,** 807.

KEFALIDES N.A. (1966) The chemical composition of normal and nephrotic glomerular basement membranes. *Proc. III Int. Symp. Nephrol.*

KEFALIDES N.A. & WINZLER R.J. (1966) The chemistry of glomerular basement membrane and its relation to collagen. *Biochem.* **5,** 702.

KIMMELSTIEL P. & WILSON C. (1936) Intercapillary lesions in the glomeruli of the kidney. *Am. J. Path.* **12,** 83,

KMETEC E. (1966) In vivo and in vitro effects of the nephrotoxic amino-nucleoside of Puromycin on carbohydrate metabolism in the rat. *Proc. III Int. Congr. Nephrol.*

KOFFLER D. & PARONETTO F. (1965) Immunofluorescent localization of immunoglobulin, complement and fibrinogen. I. Systemic lupus erythematosus. II. Acute, subacute and chronic glomerulonephritis. *J. clin. Invest.* **44,** 1657, 1665.

KRAKOWER C.A. & GREENSPON S.A. (1958) Localization of nephrotoxic antigen in extraglomerular tissues. *Arch. Path.* **66,** 364.

KRAMER N.C., WATT M.F., HOWE J.H. & PARRISH A.E. (1961) Circulating anti-kidney antibodies in human renal disease. *Am. J. Med.* **30,** 39.

LAGRUE G., BARIETY J., HALPERN B. & MILLIEZ P. (1966) Le complement serique dans les nephropathies. *J. Urol. et Nephrol.* **72,** 909.

LANGE K., SLOBODY L. & STRANG R. (1955) Prolonged intermittent ACTH and cortisone therapy in the nephrotic syndrome. Immunological basis and results. *Pediatrics* **15,** 156.

LANGE K., TRESER G., SAGEL I., TY A. & WASSERMAN E. (1966) Routine immunohistology in renal diseases. *Ann. int. Med.* **64,** 25.

LANNIGAN R., KARK R. & POLLAK V.E. (1962) The effect of a single intravenous injection of aminonucleoside of Puromycin on the rat kidney. A light and electron microscope study. *J. Path Bact.* **83,** 357.

LANNIGAN R. (1963) The production of chronic renal disease in rats by a single intravenous injection of aminonucleoside of Puromycin and the effect of low dosage continuous hydrocortisone. *Brit. J. exp. Path.* **44,** 326.

LANNIGAN R., BLAINEY J.D. & BREWER D.B. (1964) Electron microscopy of the diffuse glomerular lesion in diabetes mellitus with special reference to early lesions. *J. Path. Bact.* **88,** 255.

LANNIGAN R. & INSLEY J. (1965) Light and electron microscopic appearances in renal biopsy material from cases of recurrent haematuria in children. *J. clin. Path.* **18,** 178.

LERNER R.A. & DIXON F.J. (1966) Transfer of experimental allergic glomerulonephritis by serum in sheep. *J. exp. Med.* **124,** 431.

LONGCOPE W.T. (1913) The production of experimental nephritis by repeated proteid intoxication. *J. exp. Med.* **18,** 678.

MCCLEAN C.R., FITZGERALD J.D.L., YOUNGHUSBAND O.Z. & HAMILTON J.D. (1951) Diffuse glomerulonephritis induced in rabbits by small intravenous injections of horse serum. *Arch. Path.* **51,** 1.

McCLUSKEY R.T., BENACERRAF B. & MILLER F. (1962) Passive acute glomerulonephritis induced by antigen-antibody complexes solubilized in hapten excess. *Proc. Soc. exp. Biol. Med.* **111,** 764.

McCLUSKEY R.T., VASSALI P., GALLO G. & BALDWIN D.S. (1966) An immunofluorescent study of the pathogenic mechanisms in glomerular diseases. *New Eng. J. Med.* **274,** 695.

McKAY D.G., MARGERETTEN W. & CSAVOSSY I. (1966) An electron microscopic study of the effects of bacterial endotoxin on the blood vascular system. *Lab. Invest.* **15,** 1815.

MASUGI M. (1933) Uber das wesen der spezifischen veranderungen der Niere und der Leber durch das nephrotoxin bzw. das hepatotoxin. *Beitr. Path. Anat.* **91,** 82.

MELLORS R.C. (1965) Autoimmune disease in NZB/BL mice. *J. exp. Med.* **122,** 25.

MENEFEE M.G., MUELLER C.B., BELL A.L. & MYERS J.K. (1964) Transport of globin by the rat glomerulus. *J. exp. Med.* **120,** 129.

MICHAEL A.F., DRUMMOND K.N., GOOD R.A. & VERNIER R.L. (1966) Acute post-streptococcal glomerulonephritis: immune deposit disease. *J. clin. Invest.* **35,** 237.

MOVAT H.Z. & McGREGOR D.D. (1959) The fine structure of the glomerulus in membranous glomerulonephritis in adults. *Am. J. clin. Path.* **32,** 109.

PFEIFFER E.F. & FEDERLIN K. (1966) Experimental glomerulonephritis. *Proc. III Int. Congr. Nephrol.*

PIRANI C.L., POLLAK V.E., LANNIGAN R. & FOLLI G. (1963) The renal glomerular lesions of pre-eclampsia: electron microscopic studies. *Am. J. Obst. Gyn.* **87,** 1047.

POLLAK V.E., FOLLI G., PIRANI C., REID R.T.W. & MUERHCKE R.C. (1958) An electron microscopic recognition and clinical cure of lipoid nephrosis in adults. *J. clin. Invest.* **37,** 922.

RAMMELKAMP C.H. (1964) Concepts of pathogenesis of glomerulonephritis derived from studies in man. *The Streptococcus, Rheumatic Fever and Glomerulonephritis*, p. 288, ed. UHR. J. Baltimore: Williams & Wilkins.

REID R.T.W. (1956) Electron microscopy of glomerulus in nephrotoxic nephritis. *Austr. J. exp. Biol.* **34,** 143.

RHODIN J. (1962) Electron microscopy of the kidney. *Renal Disease*, ed. BLACK D.A.K. Oxford: Blackwell.

ROTBARD S. & WATSON R.F. (1961) Antigenicity of rat collagen. *J. exp. Med.* **113,** 1041.

STEBLAY R.W. (1962) Glomerulonephritis induced in sheep by injections of heterologous basement membrane and Freund's complete adjuvant. *J. exp. Med.* **116,** 253.

SHIBATA S., NAGASAWA T., TAKUMA T., NARUSE T. & MIYAKAWA Y. (1966) Isolation and properties of the soluble antigen specific for the production of nephrotoxic glomerulonephritis. II. Purification of the active principle from the soluble antigen. *Jap. J. exp. Med.* **36,** 143.

TALMAGE D.W., DIXON F.J., BUKANTZ S.C. & DAMMIN G.T. (1951) Antigen elimination from the blood as an early indication of the immune response. *J. Immunol.* **67,** 243.

TAN E.M., HACKEL D.B. & KAPLAN M.H. (1961) Renal tubular lesions in mice produced by group A streptococci grown in intraperitoneal diffusion chambers. *J. Infect. Dis.* **108,** 107.

UNANUE E.R. & DIXON F.J. (1965) The importance of autogenic antibody to the injected γ-globulin in nephrotoxic nephritis in rats. *J. exp. Med.* **121,** 715.

UNANUE E.R. & DIXON F.J. (1966a) Experimental allergic glomerulonephritis induced in the rabbit with heterologous renal antigens. *J. exp. Med.* **125,** 149.

UNANUE E.R. & DIXON F.J. (1966b) Experimental allergic glomerulonephritis induced in the rabbit with homologous renal antigens. *J. exp. Med.* **125,** 163.

VASSALI P., MORRIS R.H. & McCLUSKEY R.T. (1963) The pathogenic role of fibrin deposition in the glomerular lesions of toxaemia of pregnancy. *J. exp. Med.* **118,** 467.

VAZQUEZ J.J. & DIXON F.J. (1956) Immunohistochemical analysis of amyloid by the fluorescence technique. *J. exp. Med.* **104,** 727.

VERNIER R.L., FARQUHAR M.G. BRUNSON J.G. & GOOD R.A. (1958) Chronic renal disease in childhood. Correlation of clinical findings with morphological characteristics seen by light and electron microscopy. *Am. J. dis. Child.* **96,** 306.

WATSON R.F., DIXON F.J. & FELDMAN J.D. (1965) Effect of complete Freund's adjuvant on rat's kidneys. *Lab. Invest.* **14,** 1559.

WISSLER R., SMALL K. & LESH J.B. (1959) The effects of various horse serum fractions in producing cardiovascular and renal lesions in rabbits. *J. exp. Med.* **90,** 577.

CHAPTER 35

THYROID AUTO-ALLERGIC DISEASE*

DEBORAH DONIACH

& I.M.ROITT

AUTOANTIBODIES IN THYROID DISEASE

Autoantibodies to several normal thyroid constituents are found in the serum of patients with a variety of thyroid diseases and this is related to the presence of varying degrees of lymphadenoid change in the thyroid gland. A similar lesion can be produced in experimental animals by immunization with homologous thyroid antigens in Freund's adjuvant (Rose & Witebsky 1956; Rose *et al* 1966)

*In spite of some editorial pressure (cf. Introductory chapter) the authors are unwilling to accept the terminology proposed by the editors for auto-allergic disease.

and lends support to the view that the aetiology of the human disease is closely connected with the autoimmunity (Roitt & Doniach 1965; Rose, Witebsky & Beierwaltes 1965).

In the most severe forms of autoimmunizing thyroiditis the destructive lymphadenoid invasion involves the entire thyroid gland and ultimately leads to myxoedema. In Hashimoto's disease the gland appears to compensate for the destructive effect by formation of new thyroid acini. This presumably leads to an increased immunological stimulus and to an ever-increasing proliferation of lymphoid cells as evidenced by the formation of germinal centres throughout the thyroid and a marked reactive hyperplasia in the regional lymph nodes surrounding the gland. The lymphoid overgrowth and the regeneration of thyroid tissue lead to thyroid enlargement usually accompanied by high levels of circulating antibodies. The size of Hashimoto goitres varies widely and may reach over 350 g, i.e. fifteen times the normal thyroid size. However, the average weight of Hashimoto goitres is 40–70 g and it is probable that many small lymphadenoid goitres escape attention unless the neck is carefully palpated. Adult primary myxoedema is closely related to Hashimoto's disease both in the histological appearance of the basic lesion, and the antibody response. In this instance, however, the gland fails to regenerate and the final result is complete fibrous replacement with persistence of small lymphoid foci and low titres of circulating antibodies. There is clinical evidence to show that small Hashimoto goitres may regress spontaneously and lead to thyroid atrophy indistinguishable from primary myxoedema. This is also the end result in over 50% of Hashimoto patients with large goitres who are given thyroxine replacement therapy for many years (Buchanan 1965). Histological studies carried out in cases with repeated biopsies or thyroidectomies suggest that progression to increased fibrosis and epithelial atrophy occurs in about 50% of Hashimoto glands; in the remainder the processes of destruction and regeneration are balanced in a state of dynamic equilibrium and the degree of lymphadenoid infiltration remains of the same order for up to 15 or 20 years (Vickery & Hamlin 1961). There are many milder forms of lymphadenoid goitre which do not readily fit into the clinical group of Hashimoto's disease and may not lead to myxoedema (Buchanan, Harden & Clark 1965). All degrees of lymphadenoid replacement from minute scattered foci of lymphocytes to almost complete invasion may be encountered in various types of underlying goitres and in 'normal' glands at post-mortem (Williams & Doniach 1962). All these forms of thyroiditis appear to be associated with immunization to the same thyroid antigens as in Hashimoto's disease proper.

The reason for the difference in cell regeneration between the goitrous and atrophic variants of autoimmune thyroiditis is of interest. The sex ratio is approximately five females to one male in the atrophic variant while it rises to 12F/1M in the goitrous cases (Fig. 35.1). The incidence of atrophic thyroiditis

appears to increase progressively with age while Hashimoto goitres reach a peak in the sixth decade and decrease thereafter. Cell regeneration in thyroiditis is certainly not dependent upon TSH since the highest levels of this hormone are found in primary myxoedema and its pituitary output is normal in Hashimoto

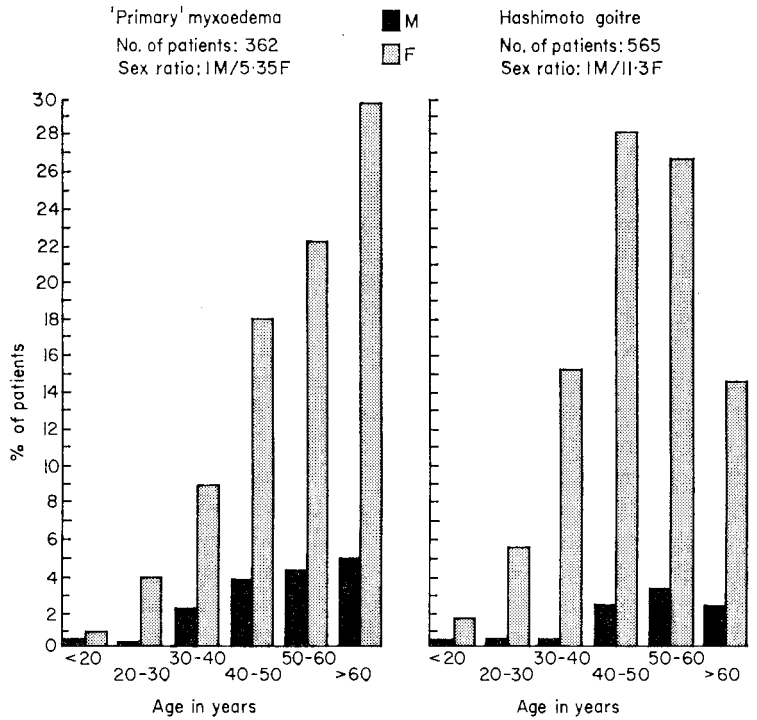


FIG. 35.1. The frequency of atrophic thyroiditis increases steadily with age whereas the goitrous variants reach a peak at the menopause and show a greater preponderance of female patients.

goitre unless hypothyroidism has developed. In view of the cell-stimulating effects described for certain antibodies, the possibility cannot be dismissed that some such mechanism might be discovered to account for the acinar regeneration in Hashimoto glands and possibly for the active proliferation of mitochondria in Askenazy cells, another unexplained phenomenon.

Clinically, there is a continuous gradation in the intensity and the progressiveness of the disease and many cases remain entirely symptomless unless the goitre is removed when there is an appreciable incidence of post-operative myxoedema. The majority of Hashimoto goitres seem to arise in apparently normal

thyroid glands, but autoimmunizing thyroiditis of all degrees of intensity is particularly common in Graves' disease, and even in Hashimoto patients without past or present evidence of thyrotoxicosis there is a high familial incidence of hyperthyroidism suggesting a basic relationship between the two diseases. A small proportion of Hashimoto patients give a history of thyroid swelling since adolescence with an increase in the size of the goitre and onset of hypothyroidism around the menopause. The thyroiditis can develop in a colloid goitre, when histological examination shows remaining areas of distended acini compatible with this diagnosis. In other cases a mild juvenile thyroiditis could either progress slowly or become more active at the time of the menopause.

Tests for the detection of thyroid antibodies have proved clinically useful and their routine application in the study of goitres and the follow up of thyroid patients undoubtedly helps the clinician to select cases for thyroidectomy on a rational basis and to decide on the choice of definitive treatment in Hashimoto's disease and in thyrotoxicosis. The main diagnostic uses of thyroid antibody tests are the differential diagnosis of Hashimoto's disease from non-toxic nodular goitres and from thyroid tumours, its distinction from virus thyroiditis (de Quervain's disease), and Riedel's disease, and the detection of clinically significant thyroiditis in thyrotoxic patients. Furthermore, thyroid antibody tests have proved of value in distinguishing mild thyrotoxicosis from anxiety states and for the correct assessment of unilateral occular proptosis in the absence of thyroid overactivity. Serological investigations are of undoubted value for the diagnosis of myxoedema, particularly in cases of partial deficiency associated with a low thyroid reserve. The tests are of interest in the investigation of autoimmune disease affecting other organs, since an unduly high incidence of low titre thyroid antibodies may be an indication of a more general immunological disturbance (Hijmans *et al* 1961). Finally they have provided important information in familial studies of thyroid disease.

THYROID ANTIGENS AND ANTIBODY TESTS

Three distinct organ-specific antigen-antibody systems were identified in human thyroiditis between 1956 and 1961 (Table 35.1) and no additional systems have been reported since. The thyroid constituents acting as auto-antigens are all found in the normal gland and none of the methods used so far have proved sufficiently sensitive to detect any subtle chemical abnormalities if these exist. The first antigen to be identified was thyroglobulin (Roitt *et al* 1956; Witebsky *et al* 1957), then followed the microsomal antigen (Belyavin & Trotter 1959; Anderson, Goudie & Gray 1959; Roitt & Doniach 1958) and last, the second antigen of the acinar colloid (Balfour *et al* 1961). Antibodies to the same three antigens are found in patients with mild non-progressive thyroiditis, but in

Hashimoto's disease the titres are usually higher and the three types of antibody are present together whereas in focal thyroiditis only one or other may be detected in titres which require the most sensitive methods.

TABLE 35.1

The thyroid autoimmune systems

Antigen	Properties of antigen	Antibody tests
1. Microsomal antigen	Localized in thyroid epithelial cytoplasm. Intimately associated with lipoprotein membrane of microsomal vesicles. Abundant in thyrotoxic and dyshormonogenetic glands. ? Precursor of thyroglobulin	Complement fixation test (CFT). Immunofluorescence. Tissue culture cytotoxic test
2. Thyroglobulin	Molecular weight 650,000. Storage form of thyroid hormones. Constitutes 75% of protein in thyroid colloid. Contains nearly all the iodine in the gland	Precipitin. Tanned red cell agglutination (TRC). Latex particle agglutination. Immunofluorescence (floccular staining pattern in colloid). Passive cutaneous anaphylaxis. Co-precipitation with ^{131}I-thyroglobulin
3. Second colloid antigen	Constitutes less than 1% of proteins in thyroid colloid. Present in all glands. Contains no iodine	Immunofluorescence (uniform staining pattern in colloid)

THYROGLOBULIN ANTIBODIES

Thyroglobulin, the main hormone storage protein of the thyroid acini constitutes over 75% of the total protein content of the colloid. Its molecular weight is of the order of 650,000 and it contains nearly all the iodine trapped in the gland. Thyroglobulin can no longer be regarded as a secluded antigen since it is detected in trace amounts in up to 60% of normal individuals, in all pregnant women and in cord blood (Hjort 1963a; Assem 1964; Torrigiani 1965; Roitt *et al* 1967). When thyroid hormone is required, thyroglobulin stored in the colloid is phagocytosed back into the thyroid cell forming microdroplets. These later merge with lysosomal particles leading to proteolytic degradation and the release of thyroxine and tri-iodothyronine. These small molecules are liberated at the base of the cell directly into the capillaries and thyroglobulin molecules

which have escaped proteolysis find their way into the lymphatics. Avoiding artefacts due to radiation or manipulation of the gland, Daniel *et al* (1967a and b) have conclusively demonstrated the presence of thyroglobulin in the cervical lymph of monkeys and rats by a sensitive radioimmunoassay.

Thyroglobulin antibodies can at present be detected by three simple methods all suitable for the routine clinical laboratory.

1. *The precipitin test.* This can be done in agar gel with crude saline extracts of post-mortem thyroid glands or with thyroglobulin purified by salt fractionation, provided the antibody content is high (i.e. over 0.2 mg/ml of serum). If a Hashimoto serum and thyroglobulin are placed in adjacent wells of an Ouchterlony plate an opaque line appears after 24–48 hr. When the antibodies are 'non-precipitating', a 'clear line' may be seen where small antigen-antibody complexes have clarified the agar (Goudie, Anderson & Gray 1959). The precipitin test is the most reliable index of widespread and intense thyroiditis but it is negative with about 55% of Hashimoto sera. In some Hashimoto patients precipitin titres may reach high values and classical precipitin curves can be made by the Heidelberger method, some cases giving 'rabbit-type' and others 'horse-flocculation-type' curves. Thyroglobulin precipitins are mostly IgG, but IgA and IgM thyroglobulin antibodies have been described.

2. *The latex agglutination test* using thyroglobulin coated particles (Hyland TA test) is slightly more sensitive than the precipitin and it is convenient as a quick screening test. However, like the precipitin test it will miss all the Hashimoto cases with low titre thyroglobulin antibodies. The test should be done as recommended by the manufacturers, using a 1/20 dilution of the patient's serum. With undiluted serum false positives can occur if rheumatoid factors are present, presumably owing to traces of human immunoglobulins present in the thyroglobulin coating the particles.

3. *The tanned red cell agglutination test* (TRC) is about 1000 times more sensitive than the precipitin reaction. The test is extremely specific immunologically, and should be carried out with a stable preparation of formalinized cells (Fulthorpe *et al* 1961). It is simple to perform and gives valuable results in thyroid diseases. Serial dilutions of serum are set up in a Takatsy microtitration apparatus using calibrated loops for semi-automatic titration. Hashimoto patients with precipitins usually have titres from 1/5000 upwards. The test can be conveniently run together with the complement fixation test (CFT), and if these two tests are combined, about 90% of Hashimoto patients can be diagnosed correctly. In primary myxoedema the TRC may be the only test giving positive results as anti-thyroglobulin is the most persistent of the thyroid antibodies when hormonal failure becomes evident. For instance, high titre TRC may be found for many years after subtotal thyroidectomy in Hashimoto patients. The extreme sensitivity of the test has certain disadvantages since high titres are sometimes obtained with minute quantities of antibodies. TRC titres of 1/2500 to 1/40,000

do not necessarily indicate widespread thyroiditis unless the precipitin test is also positive, or if there are associated strong positive complement fixing antibodies.

The TRC test has proved extremely useful in the familial investigation of thyroid autoimmunity and in studies of theoretical interest such as the incidence of thyroid antibodies in other disorders.

The Microsomal Antibody System

In human thyroiditis this may prove the most significant pathogenetically, since at least one variety of microsomal antibodies is cytotoxic to thyroid cells in culture (Forbes *et al* 1962; Irvine 1962; Kite *et al* 1965). Hashimoto sera have by far the highest concentrations of cytotoxic factor when studied quantitatively but a high proportion of thyrotoxic sera also have weak cytopathic activity. The microsomal antigen is present in low concentrations in the normal thyroid and is up to ten times more abundant in glands from patients with Graves' disease which makes these glands particularly suitable for complement fixation and tissue culture studies. The antigen is intimately associated with the lipoproteins in the microsomal fraction of thyroid extracts and it has not yet proved possible to separate it from the membranous part of smooth surfaced vesicles. It is destroyed by surface-active substances such as deoxycholate or the non-ionic detergent Lubrol and by fixatives such as alcohol and formalin, but is unaffected by substances which inactivate RNA or remove ribosomes. The most sensitive and specific test for microsomal antibodies is the Coons immunofluorescent test done on unfixed thyroid sections, in which positive sera give rise to a bright specific staining of the cytoplasm in thyroid epithelial cells (Plate 35.1). Cytoplasmic fluorescence correlates well with cytotoxicity titres (Fig. 35.2) and it is likely that these two methods measure the same antibody. The complement fixation test is less sensitive and Kite *et al* (1965) have found occasional sera showing thyroid specific CFT but lacking cytotoxic properties, suggesting some heterogeneity in the biological properties of antibodies directed against the microsomes. In clinical practice, the CFT using two minimum haemolytic doses of complement, is most useful for the titration of microsomal antibodies and gives useful diagnostic information as it is rarely positive except in active thyroiditis. The Takatsy apparatus is used routinely and all sera giving cytoplasmic fluorescence are titrated from 1/2 to 1/256. It is also possible to use the autoanalyser for thyroid and gastric CFT (Irvine 1966a) but the instrument does not perform titrations and is uneconomical in the use of complement and antigen. It is important to establish the organ specificity of the thyroid reactions by including a human kidney section in the fluorescent test and fresh rat kidney homogenate in the CFT. This applies particularly to studies of the serological overlap between thyroiditis, the connective tissue disorders and liver diseases, since patients with these conditions are prone to have non-organ-specific antibodies which react

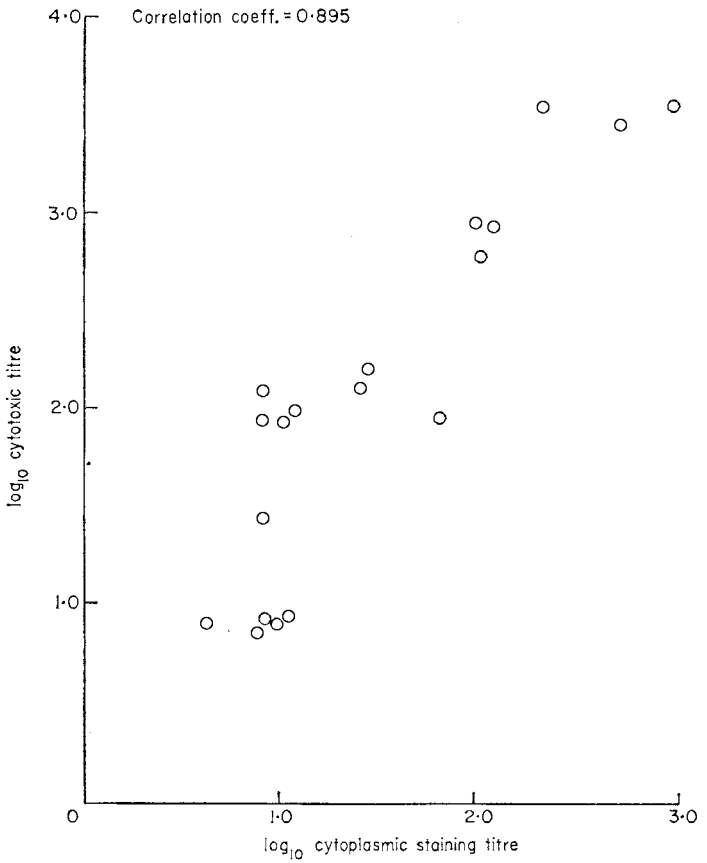


FIG. 35.2. The titres of thyroid cytoplasmic immunofluorescence obtained with thyroiditis sera correlate closely with those of cytotoxic effects on trypsinized thyroid cell cultures. (Reproduced from Forbes *et al* (1962). *J. Clin. Invest.* **41**, 996.)

with thyroid cytoplasm in the immunofluorescent test, and fix complement with thyroid and other organ homogenates. A striking example are the mitochondrial antibodies found in almost all patients with primary biliary cirrhosis and used clinically for the differential diagnosis of this chronic disease from jaundice due to extrahepatic obstructions (Doniach *et al* 1966). These antibodies give a granular fluorescence on thyroid cells and a preferential staining of mitochondria-rich cells such as Askenazy, gastric parietal and distal renal tubule cells. In active chronic hepatitis and in some patients with SLE, rheumatoid arthritis and other collagenoses, non-organ-specific cytoplasmic reactions have been reported due to ribosomal, lysosomal and occasionally mitochondrial antibodies. Specific thyroid microsomal antibodies of moderate to high titre are

only found in Hashimoto's disease, myxoedema and Graves' disease, and are very uncommon in nodular colloid goitres, cancer of thyroid and granulomatous thyroiditis. A high titre CFT (1/64 or greater) indicates Hashimoto's disease in a euthyroid or hypothyroid patient, but in hyperthyroidism high titres are occasionally found in patients with multifocal thyroiditis affecting only parts of the gland and does not always imply progression to diffuse thyroiditis since the titres may fall with clinical improvement.

The Second Antigen of the Acinar Colloid

The raised γ-globulin values found in a few Hashimoto patients who failed to react both with tanned cells and in the complement fixation test (Doniach, Hudson & Roitt 1960) prompted the search for antibodies to a further thyroid antigen. It was found that if these sera were applied to fixed thyroid sections using the indirect fluorescent technique, a uniform bright specific staining was produced in the acinar colloid when the fluorescein-conjugated anti-human immunoglobulin serum is added (Plate 35.2) and this contrasted with the floccular pattern obtained with anti-thyroglobulin sera (Plate 35.3) (Balfour *et al* 1961; Hjort 1963b). Further studies were carried out using column chromatography and it has been possible to separate the second antigen from thyroglobulin. It constitutes only a small percentage of the proteins in the colloid and appears to contain no iodine. This was shown by the fact that no radioactivity was trapped in coprecipitates prepared with radioiodine labelled thyroid extract, Hashimoto sera containing the antibody and rabbit anti-human immunoglobulin serum. When this method is applied to sera with thyroglobulin precipitins, virtually all of the radioactivity comes down in the coprecipitate. The second antigen is distinct from the thyroid protease having optimal activity at pH 3.6. The antibody to this antigen is frequently demonstrable in thyroid diseases although with Hashimoto sera the staining is much brighter. Over 50% of thyrotoxic sera react with colloid in the fluorescent tests. The colloid stains up in about half the cases of de Quervain's disease in its active stage and with a third of the sera from patients with cancer of the thyroid. The test is positive in many cases of focal thyroiditis and in 6% of blood donors. Many symptomless relatives of Hashimoto patients give positive reactions. Patients with collagen diseases react only rarely in the fluorescent thyroid antibody tests.

Very occasionally Hashimoto sera fail to agglutinate tanned cells yet give a positive precipitin reaction with both purified thyroglobulin and crude thyroid extracts. When tested by the fluorescent test these sera produce a colloid staining with a floccular pattern similar to that obtained with thyroglobulin precipitins. These findings suggest the existence of a fourth thyroid antigen. We have tested two myxoedema patients who had raised immunoglobulins yet

gave negative results by all the thyroid antibody tests known to date suggesting that further antigen-antibody systems may be revealed in the future.

It is now possible to combine on one slide the immunofluorescent test for thyroid with the tests for anti-nuclear factors (ANF), gastric parietal cell, mitochondrial, and smooth muscle antibodies by using a frozen section cut from a composite block containing human thyroid, stomach and renal cortex tissues. This combined fluorescent test can be used as a screening procedure for thyroid cases and patients with other autoimmune conditions. If the fluorescent thyroid tests are negative Hashimoto's disease is excluded, and the absence of nuclear staining practically excludes systemic lupus erythematosus. The cases giving positive reactions can then be further tested by TRC and CFT in serial dilutions, or have their ANF titres determined.

DIAGNOSTIC APPLICATIONS

Antibodies in Hashimoto's Disease

Virtually all Hashimoto patients have some circulating antibodies (Table 35.2). In our series of over 600 cases, only three gave negative results by all available tests; two of the patients were myxoedematous and in the third the histological picture was so atypical that the diagnosis of lymphosarcoma could not be ruled out. The particular types of antibodies and their titres differ considerably according to the histological variant of the disease. In the 'fibrous variant' of Hashimoto's disease (Hazard 1955; Senhauser 1964) which is characterized by active fibroplasia with some architectural disruption of the gland and a cellular infiltrate predominantly composed of plasma cells, the antibody titres are high as a rule and the precipitin test is positive. These cases are also positive by the Hyland TA test, and they give tanned cell titres of 1/5000 to several million. The complement fixation titre is frequently in the range of 1/128 to over 1/512 provided the patient is tested before operation or before prolonged thyroxine treatment has reduced the antibody level. Antibodies to the second antigen of the colloid are also present but in precipitin positive cases the serum has to be absorbed with thyroglobulin before these can be demonstrated. Clinically this variety of Hashimoto goitre is very firm and is liable to be confused with thyroid cancer, particularly when the goitre enlarges rapidly due to vigorous overgrowth of fibrous tissue. Not infrequently, patients with the fibrous variants are clinically hypothyroid when they are first seen. This severe form of autoimmune thyroiditis is sometimes seen in men and the sex ratio is about 1M/5F whereas in the less destructive oxyphil variant this is nearer 1M/20F. Patients with the 'oxyphil' variant of Hashimoto's disease are usually euthyroid or may even have 'pseudothyrotoxic' symptoms at the onset. They complain of palpitations and shortness of breath and feel generally nervous; their gland is less firm and can be easily confused with colloid goitre. The radioiodine uptake may be normal or high

TABLE 35.2

Incidence of thyroid auto-antibodies in thyroid disorders

Diagnosis histological or clinical	Number patients tested	% Positive all tests	Precipitin test +ve	TRC and CFT high	CFT high TRC low or −ve	TRC high CFT low or −ve	CFT and/or TRC low	Only fluores. tests+ve	Negative by all tests
Hashimoto's disease hypothyroid cases	234	99.6	52%	42.8%	21.3%	18.8%	10.7%	6%	0.4%
Lymphadenoid goitre euthyroid cases	287	99.7	40.5%	32.7%	28.6%	17.1%	18.5%	2.8%	?0.3% Histology ? lympho-sarcoma
Primary myxoedema	320	93.4	12.2%	11.2%	8.3%	19.7%	40.7%	13.5%	6.6%
Graves' disease	500	85	1.6%	3.6%	9.7%	4.5%	41.2%	26%	15%
Non-toxic nodular goitre	335	48.6	0	0	1.1%	1.1%	25%	21.4%	51.4%
Thyroid cancer	85	44.8	2.4%	0	2.4%	8.3%	27%	7.1%	55.2%
De Quervain subacute thyroiditis	68	53	1.7%	0	0	3%	13%	37%	47%

Diagnosis: 217 cases of Hashimoto's disease were proved by histology. The other patients were diagnosed clinically and with radioiodine and serum protein tests. The cases of Graves' disease include treated and untreated patients with and without exophthalmos. Cancer cases were all proved by histology.

Precipitin test: Double diffusion in agar (Ouchterlony) purified thyroglobulin as antigen. Patients with positive precipitins are also included in the two other columns with high TRC titres.

TRC: Tanned red cell agglutination test for thyroglobulin antibodies.
High = titre $\geqslant$ 1/25,000 (dilution with single pipette; equivalent to 1/2500 using Takatsy loops).
Low = titre $\leqslant$ 1/2500 (dilution with single pipette; equivalent to 1/1280 using Takatsy loops).

CFT: Complement fixation test with thyrotoxic thyroid extract, for the detection of the thyroid microsomal antibody.
High = titre $\geqslant$ 1/64; Low = titre $\leqslant$ 1/32.

Fluorescent tests: Coons's method, sandwich technique, using rabbit anti-human immunoglobulin conjugated with fluorescein isothiocyanate, and frozen sections of human thyroid gland. Tests include cytoplasmic staining for microsomal CF thyroid antibodies and colloid staining for anti-thyroglobulin and/or for antibodies to the second antigen of the thyroid colloid.

but is invariably suppressible with triiodothyronine. Histologically, the thyroid shows marked eosinophilic metaplasia of the epithelial cells (Askenazy cell change), there are numerous lymphoid germ centres and the infiltrate is composed of small lymphocytes with a low percentage of plasma cells. Antibody tests usually show strong microsomal complement fixation with titres frequently up to 1/512 but the precipitin is rarely positive and tanned cell agglutination is either negative or of low titre. The fluorescent tests show bright staining of the colloid due to antibodies reacting with its second antigen, and cytoplasmic staining is obtained in unfixed sections. Some Hashimoto glands show a mixture of the oxyphilic and fibrous variants histologically and in these cases the antibody levels have been high. In some of the patients the goitre was tender and of recent onset, thus simulating de Quervain's thyroiditis.

There is a small proportion of Hashimoto cases with advanced and diffuse thyroid destruction who have persistently low antibody titres, i.e. TRC of less than 1/5000 and CFT below 1/64; about 5% of Hashimoto patients have antibodies detectable only by the fluorescent tests so that they cannot be distinguished serologically from focal non-progressive thyroiditis occurring in a colloid goitre. If the clinical evidence is suggestive of Hashimoto's disease and the patient gives abnormal results with radioiodine (i.e. a normal or raised 24 hr neck uptake and a high plasma protein-bound radioactivity at 48 hr in a patient who is euthyroid or mildly hypothyroid), it is advisable to supplement the immunological tests with quantitative determinations of the immunoglobulins since these are frequently elevated in Hashimoto's disease, This unusual discrepancy between low antibody titres and raised serum immunoglobulin levels is not found in focal non-progressive thyroiditis. Thyroid needle biopsies are of great value in difficult cases.

Thyroid Antibody Tests in Primary Myxoedema

Primary hypothyroidism has an insidious onset more often than is realized and many patients have vague ill health for several years before the deficiency becomes severe enough to give abnormal results in the PBI and radioiodine tests. Since it is now thought that most adult cases result from autoimmune destruction of the gland, it would be of great clinical value if the thyroid antibody tests could give a definite answer in incipient hypothyroidism. Although antibodies are found in over 90% of myxoedema patients and nearly 40% have titres in the 'Hashimoto' range (Owen & Smart 1958; Skanse & Nilsson 1961), the majority of cases give low results in all available tests (Table 35.2). In young subjects low titre antibodies are highly significant, especially if several different types are present together or if the CFT is positive. In the higher age groups of 50–60, however, symptomless non-progressive thyroiditis and low titre antibodies are

present in 20% of women (Serafini, Torrigiani & Masala 1964; Dingle *et al* 1966) so that the tests do not have the same high diagnostic index; none the less a complete absence of antibodies is strong evidence against the diagnosis of hypothyroidism. Here again, if the complement fixation test is carried out with two minimum haemolytic doses of complement no positive results are found in control groups though there are traces of microsomal antibodies detectable with the fluorescent method in 8% of middle-aged female subjects without overt thyroid disease. If symptoms suggest mild hypothyroidism and antibodies are found in the absence of a goitre, it is likely that the patient has a 'low-reserve-thyroid' (Bastenie *et al* 1965). In this premyxoedematous stage the gland is under increased TSH stimulation and produces just sufficient hormone to maintain the PBI and radioiodine uptake within the normal range under resting conditions but the patient has symptoms when the demand for thyroxine increases. The diagnosis of this condition depends upon the failure of injected TSH to increase the thyroid uptake of radioiodine.

Myxoedematous patients giving negative results by all available antibody tests are usually long-standing cases tested after many years of replacement therapy but some are of recent onset and it may well be that circulating antibodies are never formed in sufficient quantities to be detectable. On the other hand thyroid antibodies of fairly high titre are found in myxoedema coma so that advanced hormonal failure is compatible with the persistence of high antibody levels.

Differential Diagnosis between Hashimoto's Disease and Colloid Goitre

Classical cases of Hashimoto's disease can be distinguished from non-toxic colloid goitre on clinical grounds when the patient is a middle-aged woman, if the goitre is diffuse, firm and symmetrical, and there are signs of thyroid deficiency. Such cases are rather uncommon and in many milder cases the goitre is only moderately firm, it may be larger on one side or there are marked interlobar grooves giving an impression of vague nodularity on palpation; the patient may be euthyroid or may even have symptoms reminiscent of thyrotoxicosis. For all these reasons, many diagnostic mistakes are made unless thyroid antibody tests are performed in every patient before thyroidectomy.

In typical cases of colloid goitre substantial antibody titres are found in 2.2% (Table 35.2) though traces of antibody are detectable by the tanned cell and fluorescent methods in about 46% of the patients. Precipitin tests are always negative and complement fixation titres of over 1/4 occurred in 3.5% of a large series. In the majority of the cases showing antibodies, a focal thyroiditis or even more extensive lymphadenoid changes superimposed on the colloid goitre can be demonstrated on histological examination. Complement fixation provides the most useful test for distinguishing Hashimoto's disease from colloid goitre

owing to the very high titres found in Hashimoto cases and the rarity of positive results in colloid goitre.

When thyroid antibody tests are done routinely on all goitre patients, it is possible to detect mild cases of thyroiditis which do not fall into any of the clinically recognized categories and yet present interesting clinical problems (El Kabir, Doniach & Turner-Warwick 1963). Antibody titres are low as a rule and needle biopsy studies reveal a 'half-way house' between focal thyroiditis and Hashimoto's disease proper. The great majority of the patients are young women who remain euthyroid for many years unless the goitre is removed, when many of them become myxoedematous. Serum antibody tests followed by needle biopsy in the positive cases provides the best management of this problem.

Distinction of Hashimoto's Disease from Thyroid Malignancy

This is the most serious challenge to any diagnostic methods and in this context the thyroid antibody tests can only be used in conjunction with careful clinical assessment of each individual patient and of radio-iodine studies including scintigrams. There is no doubt that some thyroid cancers are surrounded by areas of thyroiditis and therefore one would expect to find thyroid antibodies in the serum of some cancer patients (Goudie 1966). Of eighty-five cases examined, fifty-two were negative by TRC and CFT and of these the majority were also negative by the fluorescent antibody method. In seven cases the TRC titre was 1/25,000 or greater with positive precipitins in two instances; CFT was positive in twelve cases of which nine were low and three had titres of 1/32, 1/64 and 1/512 respectively but in no instance were high titres found in both CFT and TRC tests. Taking the two tests together, ten cancer patients (12%) had one or more antibodies in titres which might have suggested the wrong diagnosis. In the remaining cases antibodies were only present in trace amounts. This shows the importance of combining antibody tests with needle biopsy or open operation in all suspicious cases. Immunoglobulins are rarely raised in thyroid cancer.

In clinical practice the differential diagnosis of cancer is made easier by the fact that in many instances there is lymph node involvement when the patient is first seen or the tumour is clearly unilateral and takes up no iodine. Thus suggestive clinical evidence of thyroid malignancy was obtained in seven of the ten patients in whom we found antibody titres which might have confused the issue. Antibody studies have not yet been correlated systematically with the different types of thyroid tumours and with the extent of thyroiditis in the surrounding tissue. Histologically the closest association is with papillary carcinoma (Hirabayashi & Lindsay 1966) and in view of the higher cumulative survival rate of patients showing an associated thyroiditis, it is suggested that this repre-

sents a defensive immune mechanism to protect the normal tissues from tumour invasion. Malignant lymphomas of the thyroid are sometimes found in association with chronic thyroiditis and this is of interest in view of the increasing link-up between thymic tumours or maturation arrests, dysglobulinaemias, autoimmune phenomena and the malignant lymphomas (Good, Peterson & Gabrielsen 1965).

Distinction of Hashimoto's Disease, de Quervain's and Riedel's Thyroiditis

Subacute thyroiditis is a self-limiting disease and the mumps virus has been proved responsible in one epidemic and in several isolated cases, while other viruses may be implicated. Thyroid autoantibodies are detected in trace amounts by the fluorescent tests in over 50% of the cases between 6 and 12 weeks after the onset of the infection but the tests become negative again on recovery and there are indications that virus thyroiditis rarely initiates a progressive autoimmunization. Table 35.2 shows the results obtained with thyroid autoantibody tests in sixty-eight cases studied. The highest titres were found in two patients who may well have had autoantibodies before the onset of the mumps thyroiditis. One was a thyrotoxic patient (Felix-Davies 1958) who subsequently had a permanent remission of the hyperthyroidism. In the other there was a history suggestive of mumps thyroiditis with definite mumps contact 6 months before the onset of myxoedema but the histological picture of a thyroid needle biopsy was indistinguishable from lymphadenoid goitre.

Hashimoto's disease may be confused with virus thyroiditis when the goitre develops fairly rapidly and the thyroid is tender. As mentioned previously, the cases of autoimmune thyroiditis with a rapidly developing tender goitre have been associated with high antibody levels, and CFT titres of 1/256 together with positive precipitins are not unusual. The distinction between the two diseases may be difficult if low antibody titres are obtained; however, in granulomatous thyroiditis, the radioiodine uptake is low or absent whereas it is normal or raised in all euthyroid cases of autoimmune thyroiditis.

Classical Hashimoto patients occasionally give a past history which suggests that an upper respiratory virus infection might have initiated the autoimmunity or stimulated a clinically latent process to greater activity, but until viruses can be grown from thyroid biopsies obtained in the acute phase it is impossible to evaluate their role. Certainly, the great majority of cases of de Quervain's disease recover full thyroid function and have no evidence of residual lesions.

Riedel's invasive thyroiditis is extremely rare and quite distinct from Hashimoto's disease. Antibody tests are negative. The condition is sometimes associated with retroperitoneal or mediastinal fibrosis and it is fundamentally a vascular or perivascular inflammatory process which spreads to the thyroid (Turner-Warwick, Nabarro & Doniach 1966).

Thyroiditis and Graves' Disease

The close relationship which exists between primary thyrotoxicosis and autoimmune thyroiditis has proved a fruitful approach to the study of the aetiopathology of both disorders. Using the sensitive fluorescent antibody method and the TRC, 85% of patients with Graves' disease can be shown to have circulating thyroid antibodies (Table 35.2) and the majority of thyrotoxic thyroid glands removed at operation contain small areas of lymphoid infiltration. Whereas Hashimoto's disease and myxoedema are much more common in women, particularly in later life, the thyroiditis associated with thyrotoxicosis affects men and women equally and antibodies are found almost as frequently in young as in elderly thyrotoxic patients. In the great majority of cases the thyroiditis is minimal and non-progressive so that it does not affect the clinical course. However, in about 18% of thyrotoxic patients, fairly high antibody titres are found. Occasionally the lymphoid invasion undoubtedly progresses to a more widespread thyroiditis and final hypothyroidism, and for these cases, treatment with drugs would be suitable since these patients so frequently become myxoedematous after subtotal thyroidectomy (Buchanan *et al* 1962) or ^{131}I therapy, while the spontaneous progression is slow and may never lead to complete loss of thyroid function. However, it has become increasingly evident that in some thyrotoxic patients with high titres, the hyperthyroidism is of a severe and persistent nature. The patient may be poorly controlled with drugs, requiring high maintenance doses, the goitre and exophthalmos showing no decrease, so that post-operative myxoedema and life-long thyroxine replacement may be a preferable choice. During anti-thyroid drug treatment or in natural remissions, the CFT titres frequently decrease, only to return to a high level with exacerbations of the thyrotoxic process. Thus a surprising number of recurrent thyrotoxics, operated on several years previously, are found to have high CFT titres. The CFT under these circumstances is not likely to represent a complement fixing type of long-acting thyroid stimulator (LATS) since thyrotoxic sera reacting by CFT usually give cytoplasmic fluorescence of comparable titre whereas many patients with high LATS levels do not react with any structure in thyroid tissue by immunofluorescence. Occasionally patients with low initial antibody levels have shown a gradual increase in the complement fixation titre during the course of drug treatment and this may indicate a more progressive type of thyroiditis though proof of this assumption has not yet been obtained in a sufficient number of cases to allow a final evaluation.

The tanned red cell test is positive in 46% of thyrotoxic patients and is usually of low titre. Very few thyrotoxic patients have precipitins (1.6%) and when this test is positive there is usually a diffuse thyroiditis though the patient may remain hyperthyroid. The widespread lymphadenoid involvement of true Hashimoto's disease with rapid thyroid enlargement occurs very occasionally in

an actively thyrotoxic patient; more frequently high antibody titres are detected in patients who become myxoedematous some years after a course of anti-thyroid drugs given for well authenticated Graves' disease.

Antibody titres tend to be higher in patients with progressive endocrine exophthalmos. In some cases ophthalmoplegia develops in patients without a history of clinically overt hyperthyroidism and occasionally these patients present with spontaneous myxoedema. It is in this group of cases that antibodies have been particularly high, the precipitin test being positive in many instances. In view of the lymphoid infiltration found in the ocular muscles in some cases of malignant exophthalmos, and the resemblance of this lesion to that of lupus myositis and dermatomyositis, an autoimmune aetiology might be sought for the ocular myositis, though the proptosis and mucopolysaccharide infiltration of the retro-orbital tissues may be related to the presence of LATS or exophthalmos-producing substance (EPS).

The effect of radioiodine therapy on thyrotoxic patients with high thyroid antibody titres is less clear cut than that of surgical removal of the goitre. The incidence of myxoedema after ^{131}I therapy for Graves' disease increases proportionally with the period of follow-up and autoimmunity may not be involved to any extent in its causation. We have found that of the cases tested at intervals before and after therapy, two-thirds showed moderate increases in complement fixation titres but this appeared to be transient in many instances. The formation of microsomal antibodies was not initiated by the treatment since all the patients with positive CFT results after ^{131}I already showed cytoplasmic antibodies by immunofluorescence or CFT before the therapy dose was given. Thyroglobulin antibodies showed even less variation as a result of isotope administration. The effect of β- and γ-radiation is of a complex nature and interferes with the future ability of epithelial cells to divide, thus leading to a gradual depletion of cells in the gland. The known long survival of thyroid cells and their infrequent mitosis may contribute to the delay in onset of hypothyroidism observed after ^{131}I therapy. Furthermore, since actively dividing lymphoid cells are particularly sensitive to radiation effects, it is probable that some of the immunocytes are destroyed together with the thyroid tissue and this would lead to an inhibition of the inflammatory reaction.

MECHANISMS OF TISSUE DESTRUCTION IN AUTOIMMUNE THYROIDITIS

The presence of autoantibodies in virtually every Hashimoto patient studied and the comparable thyroiditis produced in animals experimentally following autoimmunization make it seem most probable that the lesions in the Hashimoto gland result from an autoimmune response to the patient's own thyroid

constituents (Plates 35.4 and 35.5). However, the precise mechanisms operating in this immunologically activated tissue damage are not yet clearly defined (Roitt & Doniach 1967). Much evidence argues against the circulating antibodies alone being responsible for the thyroid lesions. Injection of autologous antithyroid antibodies into normal animals has consistently failed to evoke inflammatory changes. Furthermore, although autoantibodies in high titre can cross the placenta, thyroid abnormalities are rarely seen in the offspring of mothers with thyroiditis. The notion that athyreotic cretinism is due to placental transfer of cytotoxic antibodies from the mother has not been substantiated. On the other hand the unequivocal demonstration of the cytotoxic effect of Hashimoto serum on trypsinized thyroid cells in culture suggests that slight surface injury to these cells may allow access of the complement-fixing antibodies to their antigenic substrate and that this results in cell death.

Experimentally, it has been shown that in rats mild trauma by radioiodine or X-rays potentiates an inflammatory reaction in the gland by injection of heterologous antithyroglobulin antibodies (Roitt, Jones & Doniach 1962). In human thyroiditis there is a possibility that sensitized lymphocytes produce this initial damage to the cell surface. In some cultures derived from Hashimoto glands, the lymphocytes were seen to move over the surface of the thyroid cells in close contact with the cell membrane and several lymphocytes appeared to move freely within the confines of the cytoplasm (emperipolesis) (Ling *et al* 1964). Penetration of thyroid cells by lymphocytes has also been observed in experimental autoimmune thyroiditis (Jones & Roitt 1961). Further evidence that lymphocytes play an important part in autoimmune tissue damage is provided by passive transfer of allergic encephalomyelitis by sensitized lymphoid cells (Paterson 1966). Perlmann & Broberger (1963) have been able to show that autosensitized lymphocytes from patients with ulcerative colitis cause a release of radioactivity from labelled colon cells in tissue culture, suggesting that lymphoid cells can disturb the structural integrity of the surface of epithelial cells to which they have acquired sensitivity. Studies on the homograft reaction also underline the role of lymphocytes in effecting tissue damage. In thyroiditis unequivocal evidence for a direct cytotoxic effect is still lacking.

The factors initiating lymphadenoid thyroiditis are not yet understood. Bacterial or viral infections of the thyroid do not lead to Hashimoto's disease in most cases studied and in the few instances where an upper respiratory infection occurred before the lymphadenoid goitre became clinically evident, a pre-existing autoimmunization could not be excluded. The high incidence of focal thyroiditis and of thyroid antibodies in apparently normal individuals, particularly women, indicates that this defect must be widespread in later life, but that lesions become progressively destructive only in a small proportion of cases. Additional, as yet unknown factors must operate in patients developing primary myxoedema or Hashimoto's disease (Irvine 1964).

PATHOGENESIS OF GRAVES' DISEASE

The most notable advance in the study of thyroid autoimmune disease is the characterization of the long-acting thyroid stimulator (LATS), first discovered by Adams & Purves (1956), as an autoantibody, possibly reacting with a TSH-sensitive site in the thyroid cell (Adams 1965). This has led to the inclusion of Graves' disease as a thyroid autoimmune disorder (Anderson *et al* 1964; McKenzie 1965) along with Hashimoto's thyroiditis and primary myxoedema, a concept which helps to explain many features of the disease.

Indirect evidence for thyrotoxicosis as a primary autoimmune disease. Apart from the associations with thyroiditis already described, the familial incidence, and the occurrence of Graves' disease in the same families as cases of Hashimoto goitre and myxoedema, the female preponderance and the clinical course with exacerbations and remissions are well known. The association of thyrotoxicosis with other autoimmune disorders such as pernicious anaemia (Irvine 1964), non-tuberculous Addison's disease (Irvine *et al* 1965), myasthenia gravis, thrombocytopoenic purpura, 'lupoid hepatitis', systemic lupus, rheumatoid arthritis, Sjogren's syndrome, other collagenoses, and acquired haemolytic anaemia, has received increasing attention. Stigmata such as vitiligo occur in Graves' with the same increased frequency as in Hashimoto families. Thymic hyperplasia with germinal centre formation which is a notable feature of myasthenia gravis, is seen to a more pronounced degree in Graves' disease than in Hashimoto's thyroiditis, rheumatoid arthritis or lupus erythematosus, particularly in cases showing extensive focal lymphoid infiltrates of the thyroid gland (Irvine 1966). Furthermore, the incidence of gastric antibodies is identical with that of thyroiditis patients (30%).

Direct evidence. LATS can be detected by McKenzie's mouse assay in up to 80% of Graves' patients (Carneiro, Dorrington & Munro 1966a), when IgG concentrates are tested. The use of a phylogenetically distant animal for the assay may be responsible for the low values found in the majority of sera tested. LATS mimics pituitary TSH in its biological effects, the main difference lying in the longer time course of its action which probably derives from its greater persistence in the circulation. LATS cannot be extracted from pituitary glands or other human organs except possibly from lymphoid cells. On gel filtration LATS activity is recovered in the 7S peak and TSH in a 4S peak (McKenzie 1965). Anti-TSH serum inactivates TSH and leaves LATS intact while anti-human globulin removes the latter. LATS has been identified with IgG immunochemically (Kriss *et al* 1965) and thyroid-stimulating activity is recovered in Fab fragments obtained by papain digestion of thyrotoxic IgG. The activity is absorbed out preferentially with thyroid microsomes (Beall & Solomon 1966) and can be eluted again under acid conditions, a characteristic of antigen-antibody reactions (El Kabir *et al* 1966). Administration of steroids depresses the level of LATS,

which returns to initial values on withdrawal of therapy (Kriss *et al* 1965).

It is likely that LATS is responsible for the continued overactivity of the thyroid gland. Not only does it stimulate the animal thyroid in the bioassay but experimental infusion of LATS serum into adult human volunteers also produced release of iodinated proteins (Arnaud *et al* 1965). Furthermore, it is likely that the transient neonatal thyrotoxicosis seen in babies of patients with high levels of LATS is due to the placental transmission of maternal IgG (McKenzie 1964). The disease lasts for 4–8 weeks and subsides with the known disappearance rate of maternal antibody. The condition is rare since high LATS values are exceptional in young women, and severe thyrotoxicosis often leads to impaired fertility. By far the highest LATS levels are found in middle-aged patients with pretibial myxoedema (Hoffmann & Hetzel 1966; Carneiro *et al* 1966b), sometimes with progressive exophthalmos. These patients have frequently been treated with ^{131}I or operation and are not necessarily hyperthyroid. Owing to the occurrence of fairly severe thyroiditis, some cases with exophthalmos and LATS never show any signs of thyroid overactivity and may even present with myxoedema. It is likely that in the presence of a small iodine pool, the few remaining thyroid cells are incapable of giving rise to clinical thyroid overactivity in spite of being fully stimulated by LATS. This accounts for ^{131}I uptake values in the euthyroid range which cannot be suppressed with triiodothyronine. The mode of action of LATS and pituitary TSH on the thyroid cell is still unknown. It is unlikely that LATS stimulates the cells by combining with a hypothetical TSH inhibitor since thyrotoxicosis can develop after total hypophysectomy and the thyrotrophs in the pituitary glands of thyrotoxic patients appear inactive on histological examination. Indeed, patients with Graves' disease have a functioning pituitary-thyroid axis as shown by their ability to develop a normal goitrogenic response to overdosage with anti-thyroid drugs. The concept that antibodies may sometimes stimulate cells rather than damage them is supported by the finding of blast-cell transformation of small lymphocytes by anti-lymphocyte and anti-IgG allotype sera (see Chapter 20).

POPULATION STUDIES

In view of the known high frequency of focal thyroiditis in post-mortem series (Williams & Doniach 1962), studies have been made on the incidence of thyroid antibodies in hospital patients without overt thyroid disease and in healthy individuals. Most authors measured anti-thyroglobulin by the TRC. Although this reflects only part of the true incidence since cytoplasmic and other colloid antibodies are ignored, it is none the less useful for comparison between different populations. The incidence in mixed hospital patients varies from 3.7% in a study partly derived from a pediatric practice (Blizzard *et al* 1959) to 18% in a hospital series biased towards connective tissue disorders (Hackett, Beech &

Forbes 1960). The mean for some 5000 hospital patients reported up to 1965 was 8.7%. It is of interest that of 3000 normal individuals similarly tested in several countries, 7.6% gave positive results. In both groups there is a steady increase with age, and the incidence in females is about three times that in males. This sex differential disappears after the age of 70 when thyroglobulin antibodies are found in over 20% of normal individuals of both sexes. In most cases the titres are low, usually not exceeding 1/250. When all three types of thyroid antibodies are included (Serafini *et al* 1964), the total incidence goes up by less than one-third due to the fact that focal thyroiditis, even in its non-progressive form, is often associated with detectable levels of more than one, and sometimes all three types of thyroid antibodies (Senhauser 1964).

FAMILIAL STUDIES

Hashimoto's disease and primary myxoedema can occur in more than one member of a family and several reports describe the occurrence of lymphadenoid

TABLE 35.3

Family studies in thyroid autoimmunity

Proband sole member with clinically overt disease

Healthy relatives	Number tested	Thyroid antibodies Positive	Statistical significance
Males	60	32%	$0.01<P<0.02$
Controls	60	12%	
Females	76	45%	$P<0.001$
Controls	76	14%	

goitres in mono-ovular twins (Irvine *et al* 1961). Twin studies in Graves' disease (Harvald & Hauge 1956; Hassan *et al* 1966) have shown a high concordance rate of 60% in monozygotic pairs compared with only 9% in dizygotic pairs of the same sex and this suggests a strong genetic predisposition. Following the early example of Hall, Owen & Smart (1960) family studies have been carried out on the relatives of Hashimoto patients (Doniach, Roitt & Taylor 1965) and patients with Graves' disease (Saxena 1965). In all these, a high incidence of clinical and subclinical thyroiditis was found, with corresponding serum antibodies. The methods of ascertainment of probands in these studies have been criticized (Masi, Hartman & Shulaman 1965) but recalculation of our own results after exclusion of clinically affected relatives and rejection of all families with more than one overtly affected member, showed that the incidence of thyroid antibodies was

still significantly higher in relatives than in matched controls (Table 35.3) (Doniach & Roitt 1967). Parent studies (Hall, Saxena & Owen 1962; Doniach, Nilsson & Roitt 1965) in cases of juvenile thyroiditis suggest that in children the tendency to autoimmunity is frequently transmitted from both sides of the family but can also come entirely from the father (Hall, Owen & Smart 1964), excluding the intrauterine environment as a significant factor.

OVERLAP OF THYROIDITIS WITH OTHER DISEASES

This has already been mentioned in connection with thyrotoxicosis; a similar spectrum of conditions has been reported in association with primary myxoedema and Hashimoto's thyroiditis (Roitt & Doniach 1965). Clinically and serologically the most significant association is with atrophic gastritis and pernicious anaemia (Irvine *et al* 1965; Ardemann *et al* 1966) 30% of patients with thyroid autoimmune disorders have gastric parietal cell antibodies although only 1–3% have intrinsic factor antibodies and these are the patients who are liable to progress from simple atrophic gastritis to megaloblastic anaemia. Conversely over 50% of patients with pernicious anaemia have detectable thyroid antibodies, with titres indicative of an active thyroiditis in about 25%. A significant overlap with thyroiditis is also found in Sjögren's syndrome and myasthenia gravis (Downes, Greenwood & Wray 1966). In rheumatoid arthritis and sarcoidosis there is no increase in the incidence of thyroid antibodies, yet the clinical impression is that thyroid disorders are more frequent. However, epidemiological studies indicate that minor clinical thyroid abnormalities are found when especially looked for in 12% of normal women (Dingle *et al* 1966). In 4% of healthy women over 30, high antibody titres can be detected and these often coincide with a palpable firm thyroid gland. Retrospective histological studies have shown a 2% incidence of diffuse autoimmune thyroiditis (Masi 1965). In view of these findings, claims of significant aetiological connections between autoimmune diseases and other disorders have to be carefully evaluated (Mulhern, Masi & Shulman 1966).

ACKNOWLEDGMENTS

We gratefully acknowledge support by the Medical Research Council, the Arthritis and Rheumatism Council, the Nuffield Foundation and the World Health Organization.

REFERENCES

ADAMS D.D. (1965) Pathogenesis of the hyperthyroidism of Graves' disease. *Brit. med. J.* **i,** 1015.

ADAMS D.D. & PURVES H.D. (1956) Abnormal responses in the assay of thyrotrophin. *Proc. Univ. Otago Med. School* **34,** 11.

ANDERSON J.R., GOUDIE R.B. & GRAY K.G. (1959) The 'thyrotoxic' complement-fixation reaction. *Scot. med. J.* **4,** 64.

ANDERSON J.K., GRAY K.G., MIDDLETON D.G. & YOUNG J.A. (1964) Autoimmunity and thyrotoxicosis. *Brit. med. J.* **ii,** 1630.

ARDEMAN S., CHANARIN I., KRAFCHIK B. & SINGER W. (1966) Addisonian pernicious anaemia and intrinsic factor antibodies in thyroid disorders. *Quart. J. Med.* **35,** 421.

ARNAUD C.D., KNEUBUHLE H.A., SEILING V.L., WIGHTMAN B.K. & ENGBRING N.H. (1965 Responses of normal human to infusions of plasma from thyrotoxics. *J. clin. Invest.* **44,** 1287.

ASSEM E.S.K. (1964) Thyroglobulin in the serum of parturient women and newborn infants. *Lancet* **i,** 139.

BALFOUR B.M., DONIACH D., ROITT I.M. & COUCHMAN K.G. (1961) Fluorescent antibody studies in human thyroiditis: autoantibodies to an antigen of the thyroid colloid distinct from thyroglobulin. *Brit. J. exp. Path.* **42,** 307.

BASTENIE P.A., NEVE P., BONNYNS M., ERMANS A.M. & MALAISSE-LAGAE F. (1965) Thyroiditе chronique latente et myxoedeme spontane de l'adulte. *Bull. Acad. Roy. Med. Belg.* **5,** 137.

BEALL G.N. & SOLOMON D.H. (1966) Inhibition of LATS by thyroid particulate fractions. *J. clin. Invest.* **45,** 552.

BELYAVIN G. & TROTTER W.R. (1959) Investigations of thyroid antigens reacting with Hashimoto sera. *Lancet* **i,** 648.

BLIZZARD R.M., HAMWI G.J., SKILLMAN T.G. & WHEELER W.E. (1959) Thyroglobulin antibodies in multiple thyroid diseases. *N. Engl. J. Med.* **250,** 112.

BUCHANAN W.W. (1965) Primary hypothyroidism and Hashimoto's thyroiditis a continuing spectrum. *Arch. Int. Med.* **115,** 411.

BUCHANAN W.W., HARDEN R.McG. & CLARK D.H. (1965) Hashimoto's thyroiditis. *Brit. J. Surg.* **52,** 430.

BUCHANAN W.W., KOUTRAS D.A., CROOKS J., ALEXANDER W.D., BRASS W., ANDERSON J.R., GOUDIE R.B. & GRAY K.G. (1962) The clinical significance of the complement fixation test in thyrotoxicosis. *J. Endocrinol.* **24,** 115.

CARNEIRO L., DORRINGTON K.J. & MUNRO D.S. (1966a) Recovery of the long acting thyroid stimulator from serum of patients with thyrotoxicosis by concentration of immunoglobulin. *Clin. Sci.* **31,** 215.

CARNEIRO L., DORRINGTON K.J. & MUNRO D.S. (1966b) Relation between LATS and thyroid function in thyrotoxicosis. *Lancet* **ii,** 878.

DANIEL P.M., PRATT O., ROITT I.M. & TORRIGIANI G. (1967a) The release of thyroglobulin from the thyroid gland into thyroid lymphatics; the identification of thyroglobulin in thyroid lymph and in the blood of monkeys by physical and immunological methods and its estimation by radioimmunoassay. *Immunology.* **12,** 489,

DANIEL P.M., PRATT O., ROITT I.M. & TORRIGIANI G. (1967b) Thyroglobulin in the lymph draining from the thyroid gland and in the peripheral blood of rats. *Quart. J. exp. Physiol.* **52,** 184,

DINGLE P.R., FERGUSON A., HORN D.B., TUBMAN J. & HALL R. (1966) The incidence of thyroglobulin antibodies and thyroid enlargement in a general practice in North East England. *Clin. exp. Immunol.* **1,** 277.

DONIACH D., HUDSON R.V. & ROITT I.M. (1960) Human autoimmune thyroiditis: clinical studies. *Brit. med. J.*, **i,** 365.

DONIACH D., NILSSON L.R. & ROITT I.M. (1965) Autoimmune thyroiditis in children and adolescents. Immunological correlations and parent study. *Acta pediatr.* **54,** 260.

DONIACH D., ROITT I.M. & TAYLOR K.B. (1965) Autoimmunity in pernicious anaemia and thyroiditis: a family study. *Ann. N.Y. Acad. Sci.* **124,** 605.

DONIACH D., ROITT I.M., WALKER J.G. & SHERLOCK S. (1966) Tissue antiboides in primary biliary cirrhosis, active chronic hepatitis and other liver diseases. *Clin. exp. Immunol.* **1,** 237.

DOWNES J.M., GREENWOOD B.M. & WRAY S.H. (1966) Autoimmune aspects of myasthenia gravis. *Quart. J. Med.* **35,** 85.

EL KABIR D.J., BENHAMOU-GLYNN N., DONIACH D. & ROITT I.M. (1966) Absorption of thyroid-stimulating globulin from thyrotoxic sera by organ homogenates. *Nature,* **210,** 319.

EL KABIR D.J., DONIACH D. & TURNER-WARWICK R. (1963) Serum content of TSH in human thyroiditis. Low TSH levels with high radioiodine uptake in mild autoimmune thyroiditis. *J. clin. Endocr.* **23,** 510.

FELIX-DAVIES D. (1958) Autoimmunization in subacute thyroiditis associated with evidence of infection by mumps virus. *Lancet* **i,** 880.

FORBES I.J., ROITT I.M., DONIACH D. & SOLOMON I.L. (1962) The thyroid cytotoxic autoantibody. *J. clin. Invest.* **41,** 996.

FULTHORPE A.J., ROITT I.M., DONIACH D. & COUCHMAN K.G. (1961) A stable sheep cell preparation for detecting thyroglobulin autoantibodies and its clinical applications. *J. clin. Path.* **14,** 654.

GOOD R.A., PETERSON R.D.A. & GABRIELSON A.E. (1965) The thymus and immunological deficiency disease. In *Immunological Diseases,* p. 344, ed. SAMTER *et al.* London: Churchill.

GOUDIE R.B. (1966) Thyroiditis and thyroid carcinoma. *Int. Colloquium Tumours of the Thyroid Gland, Marseilles,* 1964, p. 292. Basel: Karger.

GOUDIE R.B., ANDERSON J.R. & GRAY K.G. (1959) Non-precipitating antithyroglobulin studied by the Ouchterlony technique. *Immunology* **4,** 309.

HACKETT E., BEECH M. & FORBES I.J. (1960) Thyroglobulin antibodies in patients without clinical disease of the thyroid gland. *Lancet* **ii,** 402.

HALL R., OWEN S.G. & SMART G.A. (1960) Evidence for genetic predisposition to formation of thyroid autoantibodies. *Lancet* **ii,** 187.

HALL R., OWEN S.G. & SMART G.A. (1964) Paternal transmission of thyroid autoimmunity. *Lancet* **ii,** 115.

HALL R., SAXENA K.M. & OWEN S.G. (1962) A study of the parents of patients with Hashimoto's disease. *Lancet* **ii,** 1291.

HARVALD B. & HAUGE M. (1956) A catamnestic investigation of Danish twins. *Dan. Med. Bull.* **3,** 150.

HASSAN T.H.A., GREIG W.R., BOYLE J.A., BOYLE I.T. & WALLACE T.J. (1966) Toxic diffuse goitre in monozygotic twins. *Lancet* **ii,** 306.

HAZARD J.B. (1955) Thyroiditis: a review. *Amer. J. clin. Path.* **25,** 399.

HIJMANS W., DONIACH D., ROITT I.M. & HOLBOROW E.J. (1961) Serological overlap between lupus erythematosus, rheumatoid arthritis, and thyroid autoimmune disease. *Brit. med. J.* **ii,** 909.

HIRABAYASHI R.N. & LINDSAY S. (1966) Thyroid carcinoma and chronic thyroiditis of the Hashimoto type; a statistical study of their relationship. *Int. Colloquium Tumours of the Thyroid Gland, Marseilles,* p. 272. Basel: Karger.

HJORT T (1963a) Thyroglobulin in the serum. *Acta Med. Scand.* **174,** 137.

HJORT T. (1963b) The occurrence of antibody against 'second colloid antigen' in patients with and without thyroid disease. *Acta Med. Scand.* **174,** 147.

HOFFMANN M.J. & HETZEL B.S. (1966) The clinical significance of plasma thyroid stimulating activity in hyperthyroidism. *Australas. Ann. Med.* **15,** 204.

IRVINE W.J. (1962) Studies on the cytotoxic factor in thyroid diseases. *Brit. med. J.* **i,** 1444.

IRVINE W.J. (1964) Thyroid autoimmunity as a disorder of immunological tolerance. *Quart. J. exp. Physiol.* **49,** 324.

IRVINE W.J. (1966a) Automated determinations of thyroid and gastric CF antibodies. *Clin. exp. Immunol.* **1,** 341.

IRVINE W.J. (1966b) Discussion on histopathology of the thymus. In *The Thymus*, p. 465. Ciba Symposium. London: Churchill.

IRVINE W.J., DAVIES S.H. & SUMERLING M.D. (1965) The immunopathology of thyroid diseases. In *Current Topics in Thyroid Research*, p. 773. New York: Academic Press.

IRVINE W.J., MACGREGOR A.G., STUART A.E. & HALL G.H. (1961) Hashimoto's disease in uniovular twins. *Lancet* **ii,** 850.

JONES H.E.H. & ROITT I.M. (1961) Experimental autoimmune thyroiditis in the rat. *Brit. J. exp. Path.* **42,** 546.

KITE J.H., ROSE N.R., KANE K. & WITEBSKY E. (1965) Cytotoxicity of human thyroid autoantibodies. *Ann. N.Y. Acad. Sci.* **124,** 626.

KRISS J.P., PLESHAKOV V. & CHIEN J.R. (1964) Isolation and identification of long acting thyroid stimulator and its relation to hyperthyroidism and circumscribed pretibial myxoedema. *J. clin. Endocr.* **24,** 1005.

LING N.R., ACTON A.B., ROITT I.M. & DONIACH D. (1964) Interaction of lymphocytes from immunized hosts with thyroid and other cells in culture. *Brit. J. exp. Path.* **46,** 348.

MCKENZIE J.M. (1964) Neonatal Graves' disease. *J. clin. Endocr.* **24,** 660.

MCKENZIE J.M. (1965) Etiology of Graves' disease. *J. clin. Endocr.* **25,** 424.

MASI A.T. (1965) Hashimoto's disease. An epidemiological study based on a community-wide hospital survey. *J. chron. Dis.* **18,** 35.

MASI A.T., HARTMAN W.H. & SHULMAN L.E. (1965) Hashimoto's disease, an epidemiological critique. *J. chron. Dis.* **18,** 1.

MULHERN L.M., MASI A.T. & SHULMAN L.E. (1966) Hashimoto's disease. A search for associated disorders in 170 clinically detected cases. *Lancet* **ii,** 508.

OWEN S.G. & SMART G.A. (1958) Thyroid antibodies in myxoedema. *Lancet* **ii,** 1034.

PATERSON P.Y. (1966) Experimental allergic encephalomyelitis. An autoimmune disease. In *Advances in Immunology*, vol. 5, p. 131.

PERLMANN P. & BROBERGER O. (1963) In vitro studies of ulcerative colitis. II. Cytotoxic action of white blood cells from patients on human fetal colon cells. *J. exp. Med.* **117,** 717.

ROITT I.M., DONIACH D., CAMPBELL P.N. & VAUGHAN HUDSON R. (1956) Autoantibodies in Hashimoto's disease (lymphadenoid goitre). *Lancet* **ii,** 820.

ROITT I.M. & DONIACH D. (1958) Human autoimmune thyroiditis: serological studies. *Lancet* **ii,** 1027.

ROITT I.M. & DONIACH D. (1965) Recent advances in autoimmunity. In *The Scientific Basis of Medicine*, Annual Reviews p. 110. Athlone Press, London.

ROITT I.M. & DONIACH D. (1967) Delayed hypersensitivity in autoimmune diseases. *Brit. med. Bull.* **23,** 66.

ROITT I.M., JONES H.E.H. & DONIACH D. (1962) Mechanisms of tissue damage in experimental and human autoimmune thyroiditis. In *2nd Internat. Symp. on Immunopathology, Michigan, 1962, p.* 174.

ROITT I.M., LING N.R., DONIACH D. & COUCHMAN K.G. (1964) The cytoplasmic autoantigen of the human thyroid. *Immunol.* **7,** 375.

ROITT, I.M. & DONIACH, D. (1967) A reassessment of studies on the aggregation of thyroid autoimmunity in families of thyroiditis patients. Clin. exp. Immunol. **2,** 727.

ROITT I.M., TORRIGIANI G. & DONIACH D. (1967) Serum thyroglobulin in thyroid disorders. To be published.

ROSE N.R., SKELTON F.R., KITE J.H. & WITEBSKY E. (1966) Experimental thyroiditis in rhesus monkey. *Clin. exp. Immunol.* **i,** 171.

ROSE N.R. & WITEBSKY E. (1956) Changes in the thyroid glands of rabbits following active immunization with rabbit thyroid extracts. *J. Immunol.* **76,** 417.

ROSE N.R., WITEBSKY E. & BEIERWALTES W.H. (1965) Thyroiditis. In *Immunological Diseases*, p. 821, ed. SAMTER M. *et al.* Boston: Little Brown & Co.

SAXENA K.M. (1965) Inheritance of thyroglobulin antibodies in thryotoxic children. *Lancet* **i,** 583, 1965.

SENHAUSER D. (1964) Immunopathologic correlations in thyroiditis. In *The Thyroid*, ed. HAZARD J.B. & SMITH D.E.

SERAFINI U., TORRIGIANI G. & MASALA C. (1964) The incidence of autoantibodies in the normal population. *Reports of the 5th Int. Congress of Allergology, Madrid.*

SKANSE B. & NILSSON S.B. (1961) Thyroid antibodies in hypothyroidism. *Acta Med. Scand.* **170,** 461.

TORRIGIANI G. (1965) Ph.D. thesis, London University.

TURNER-WARWICK R., NABARRO J.D.N. & DONIACH D. (1966) Riedel's thyroiditis and retroperitoneal fibrosis. *Proc. Roy. Soc. Med.* **59,** 595.

VICKERY A.L. & HAMLIN E. (1961) Struma lymphomatosa (Hashimoto's thyroiditis). Observations on repeated biopsies in sixteen patients. *New Engl. J. Med.* **264,** 226.

WILLIAMS E.D. & DONIACH D. (1962) The post-mortem incidence of focal thyroiditis. *J. Path. Bact.* **83,** 255.

WITEBSKY E., ROSE N.R., TERPLAN K., PAINE J.R. & EGAN R.W. (1957) Chronic thyroiditis and autoimmunization. *J. Amer. med. Assoc.* **164,** 1439.

CHAPTER 36

THE LUNG IN ALLERGIC DISEASE

W.E.Parish & J.Pepys

INTRODUCTION

The lung and the skin are the two organs most frequently observed to undergo diverse and dramatic allergic reactions. The sudden onset and possibly fatal result of some reactions in the lungs demands a quick and accurate diagnosis, whilst other more chronic conditions may resemble infectious disease. Accurate diagnosis requires a clear understanding of the nature of the allergic reactions taking place.

This chapter is confined to a consideration of the changes occurring in allergic reactions in the lung, and the mechanisms by which these changes are induced. The properties of the antibodies mediating them have been described elsewhere in this book. No rigid classification has been adopted, and the allergic conditions of the lung are considered and compared according to the type of allergic reaction mediating the response. However, it is important to realize that in any one disease more than one type of allergic response may be present, and also in any one type of allergic response there may be more than one form of antibody, e.g. tissue-sensitizing and precipitating antibodies.

THE SHOCK ORGAN

The presence of antibodies capable of sensitizing tissue to elicit a Type I response on challenge does not mean that reactions can be produced in all the tissues of an individual. It is not known whether this variation is due to the organ or the nature of the antibody, or both. Halpern, Biozzi & Benacerraf (1955) have provided one explanation for a particular organ becoming a shock organ. In the sensitized lung or skin, exposure to small amounts of antigen as well as other

inflammatory stimuli initiates vasodilatation and exudation of serum which results in more antibody entering the organ and therefore further sensitization of the tissues and an increase in the susceptibility of the organ. In some sensitized individuals only the alimentary tract, or the skin, or the lungs appear susceptible to Type I reactions to a particular antigen. Evidence of bronchial sensitivity in the absence of skin sensitivity has been found by Tiffenau (1958). Schwartz (1952) has suggested that the tendency of the lung to react as the shock organ is heritable.

THE TYPE I ALLERGIC RESPONSE

Anaphylaxis

Anaphylaxis in man, fatal or otherwise, is rare despite the frequency with which individuals are sensitized to antigens of their environment or to drugs. Sudden death has occurred following the injection of antiserum, toxoid or pollen extracts (Lamson 1924; Vaughan & Pipes 1936; Vance & Strassman 1942), penicillin (Mayer *et al* 1953; Chao-Ling & Chih-Tze 1956), bee or wasp stings (Wegelin 1948; Schenken, Tamisiea & Winter 1953) or contrast medium in intravenous urography (Pendergrass *et al* 1958).

The sensitivity of the lungs to anaphylaxis, as in some patients with asthma, cannot always be demonstrated by skin testing. Preliminary testing of the patient by eye, mouth or skin may not provide evidence of sensitivity to material capable of causing a fatal reaction on entering the blood stream (Lamson 1924; Pendergrass *et al* 1958).

Signs of Anaphylaxis in Man

Anaphylaxis in man may involve the skin resulting in urticaria and intense itching, the gastro-intestinal tract resulting in vomiting and diarrhoea, or the respiratory system resulting in severe dyspnoea. A patient may develop signs in any one or more organs, together with vascular reactions, a fall in blood pressure, tachycardia, vasodilatation and cyanosis. Anaphylaxis need not be accompanied by respiratory distress, as in the single non-fatal case reported by Parish & Oakley (1940), the fatal case of Dean (1922) and one fatal case of James & Austen (1964).

The onset of anaphylaxis occurs within minutes of the antigen entering the body. The patient may complain of discomfort, giddiness, a feeling of tight bands being drawn about the head and chest, a desire to urinate or defaecate and a burning sensation of the skin. The progression of the symptoms to signs of respiratory distress and collapse will depend upon the degree of sensitivity, the organs most susceptible in that patient, the nature and concentration of the antigen and the route by which it enters the body.

The Post-mortem Changes of Anaphylaxis

The main post-mortem changes in individuals dying of acute anaphylaxis with severe respiratory distress are distension of the lungs, oedema of the trachea and bronchioles, and a varying degree of congestion of other organs.

There is little doubt that death is due to asphyxia resulting from spasm of the smooth muscle of the bronchioles, to which the oedema of the mucosa and the larynx may be an additional factor.

Asthma

Asthma, rhinitis and conjunctivitis in atopic subjects are classical examples of Type I allergic reactions. In spite of the abundant evidence of the role of reaginic antibody there is still considerable difficulty with regard to the causation and mechanism of asthma in those subjects in whom allergens cannot be found. It is not always recognized that the only clearly demonstrable mechanism with measurable evidence for the consistent production of asthmatic reactions is the Type I allergic reaction, and any analysis of the causes of asthma must be influenced by the presence or absence of its characteristic features. The increase in capillary permeability and the influence upon it of increased arteriolar blood flow is the focal point of the Type I reactions and it is here that other factors can readily exert their effect by modifying the peripheral blood flow (see Chapter 6).

The emphasis upon Type I allergy does not exclude the participation of other factors, either proven or suspected, or even their predominant influence in the established state. It is most important, however, from the practical aspect, to assess the order of appearance and relative importance of the different factors in the development of the asthmatic state.

Until recent years the mechanism of allergic asthma in man was deduced from the pulmonary manifestations of anaphylaxis or experimental asthma in the guinea-pig. Such comparisons have much to teach, and *in vivo* and *in vitro* studies in man in recent years have confirmed the conclusions already drawn.

Experimental Asthma in Animals

Experimental asthmatic reactions have been produced in animals sensitized by injection and challenged by injection or inhalation of antigen solutions (Meltzer 1910; Busson 1911). A picture more closely resembling that in man has been produced in guinea-pigs by the inhalation of dry allergenic dusts, which produce a 'regional' sensitization of the respiratory tract, an exposure of 5–7 hr being optimal for sensitization. No classical anti-anaphylaxis or refractory period appears and repeated exposures lead to repeated and prolonged attacks of asthma, resembling status asthmaticus in man, though as in man, some animals had periods during which they did not react. Bronchial reactions to pharmacological agents such as histamine or acetylcholine were shown to be more

rapidly reversible than allergic reactions (Ratner, Jackson & Gruehl 1927; Ratner 1953; Kallos & Pagel 1937). In an elaborate study, Noellp-Eschenhagen & Noellp (1954) showed in guinea-pigs that a conditioning stimulus at the time of challenge with an allergen aerosol, could lead to a partial asthmatic reaction on subsequent exposure to the conditioning stimulus without the presence of the allergen aerosol. This conditioned state was more readily produced in the allergically induced asthma than in that produced by histamine. These findings also resemble the provocation of asthma in man by what appear to be conditioned stimuli.

The typical histological changes in experimental asthma in animals resemble the findings in asthma in man and consist of oedema of the bronchial mucosa, hyperplasia and hypersecretion of goblet cells with an increase in mucus and the presence of mucus plugs, together with eosinophil cell infiltration of the bronchial and other tissues.

Experimental Asthma in Man

There is now a well-documented literature on the deliberate production of typical asthmatic reactions in man by the inhalation of allergen solutions (Colldahl 1952; Herxheimer & Prior 1952; Tiffenau 1958; ten Cate 1961; and others). The relationship between the degree of allergic sensitivity to the allergen and the reactivity of the bronchi to histamine and acetylcholine has been shown by Tiffenau (1958). Increased sensitivity to the pharmacological agents appears in patients with prolonged, chronic asthma.

Direct observations of the reaction to inhaled allergens have been made under bronchoscopy by Dubois de Montreynaud (1950). An explosive oedema of the bronchial mucosa and narrowing of the lumen was found in allergic subjects in whom asthmatic reactions were provoked by inhalation of specific allergens. In asthmatic subjects in whom allergens could not be found the bronchial lumen was also decreased during attacks. The mucosa was dark and congested but not oedematous, and hypersecretion was marked. The picture resembled that of asthmatic reactions due to pharmacological agents. The sputum in both types contained many eosinophil cells which were thought to come from the smaller bronchioles.

In vitro studies on bronchial muscle removed at operation from asthmatic patients (Schild, Hawkins, Mongar & Herxheimer 1951; Rosa & McDowall 1951) have given results like those in Schultz-Dale tests on bronchial muscle from sensitized guinea-pigs. The bronchial muscle contracted and could be desensitized after addition of a specific allergen. Addition of other specific allergens provoked further contractions, thereby demonstrating multiple specific sensitivity. The allergic reaction was accompanied by the release of histamine and other active substances, thus providing in an *in vitro* test, with a purely immunological mechanism, the factors required for production of asthma *in vivo*.

Pathology of Asthma

The pathology of asthma has been studied in biopsy specimens taken *in vivo* and in post-mortem material. The presence of oedema, eosinophil cell infiltration and hypertrophy of bronchial muscle are strongly suggestive of Type I allergy. Comparison of biopsy specimens shows an eosinophil infiltration in asthma but not in bronchitis (Glynn & Michaels 1960). Other differences are described in post-mortem material by Gough (1961). Whilst the cellular infiltration in asthma did not extend to the terminal bronchioles, these showed hypertrophy of the muscle. In chronic bronchitis the inflammation extended to the terminal bronchioles and was accompanied by destructive emphysema which was absent in asthma. Shedding of sheets of ciliated epithelium with eosinophil cell infiltration in the tissues and in the viscid mucous plugs has been found in fatal cases of asthma (Houston, de Navasquez & Trounce 1953; Cardell & Pearson 1959). The presence of shed columnar epithelial cells in small or large aggregates has been found by Naylor (1962) to occur almost exclusively in asthma and in particular during attacks. Great amounts of epithelium and of ciliated cells are probably shed. A similar shedding of epithelium with eosinophil infiltration has been found in respiratory-type epithelium in an ovarian teratoma of a patient dying of asthma (Thomson 1945).

Classification of Asthma

Asthma and rhinitis may be classified into those in whom allergens are demonstrable, the so-called extrinsic group, and those in whom no allergens are found, the intrinsic group. Whilst these terms are in common usage, 'intrinsic' does not mean that an endogenous cause is known or suspected, nor that the condition is non-allergic, but rather that the cause has not been found. Broadly speaking, the extrinsic group are atopic subjects who become readily sensitized to common allergens and the intrinsic group are non-atopic. The late onset of symptoms in the intrinsic group suggests that they are less easily sensitized and that there are as yet obscure immunological differences between the two groups, although evidence of Type I allergic reactions are present in both. In both the extrinsic and intrinsic groups, eosinophil cells in the tissues and the blood are a feature and both respond in varying degree to adrenergic agents and to corticosteroids.

Whilst the term 'intrinsic' does not mean that auto-allergy is responsible, Hall, Turner-Warwick & Doniach (1966) report that either non-organ-specific antibodies, or thyroid or gastric antibodies, but seldom both together, are present more frequently in intrinsic than in extrinsic asthma. The majority of the patients with goitre and thyroid antibodies and classifiable chest disease were found to be suffering from intrinsic asthma. It is important to exclude the presence of thyroid antibodies before giving such patients iodides in treatment for the asthma. Serafini, Torrigiani & Masala (1965), however, found a comparable raised

incidence of thyroid and gastric antibodies in both extrinsic and intrinsic asthma. An auto-allergic mechanism is suggested by the report of antibodies specifically against bronchial mucosa in patients with asthma but not in those with bronchitis (Wagner, Tomsikova, Sach, Jankova & Novackova 1965).

It is often difficult to distinguish between 'intrinsic' asthma and chronic bronchitis. Some light may be thrown on this by the finding of species-specific precipitins against *H. influenzae* in 69% of patients with chronic bronchitis, who had mucopurulent sputum and airways obstruction; in 25% of patients with simple chronic bronchitis; in 35% of patients with asthma and chronic bronchitis; in 8% with asthma and in 6% of controls (Burns & May 1967). In addition to variable airway obstruction the asthma group had an eosinophilia, an important point of distinction from chronic bronchitis.

The distinguishing features of the two groups are as follows:

	Extrinsic	Intrinsic
Age of onset	Infancy to young adult life	Mainly in adult life and particularly in older subjects
Clinical picture	Dermal-respiratory syndrome, infantile eczema, rhinitis, asthma, long history of allergic manifestations	Asthma and rhinitis. No previous history of allergic manifestations
Other features	Blood eosinophilia up to 10–15%	Blood eosinophilia frequently high, 15% or more
Causal agents identified	Foods in infancy, followed by inhalant allergens	Aspirin sensitivity in a small proportion
Response to palliatives	Good on the whole	Become refractory to palliatives
Prognosis	Good for life and for control of symptoms	Poor. High proportion of deaths due to asthma occur in this group, within a few years of onset in many cases

Allergic management is indicated in the extrinsic group, whereas it has little place in the intrinsic group, in whom corticosteroid treatment is indicated when usual palliatives fail.

SUDDEN DEATH IN INFANCY (COT DEATH AS A CONSEQUENCE OF AN ALLERGIC REACTION IN THE LUNG)

A Type I allergic response appears to be responsible for sudden death in infancy which is not uncommon, there being about 1090 cases in England and Wales in

1960 (Ministry of Health Report 1965). The cause of many of these deaths remains undiagnosed, though a sufficiently large number die with a very similar history and post-mortem changes for the condition to be recognized as a common entity, conveniently known as cot death. Attempts to define this condition and the possible contributory factors have been made recently in two public health reports (Ministry of Health Report 1965; Wedgwood & Benditt 1965).

It occurs in infants 2 weeks to 2 years of age with a peak incidence at 2–3 months. The usual history is that an apparently normal child, or one that has had a slight cold for the previous 2 or 3 days, is fed, put to bed, and during the next 3–4 hr dies without making a noise or attempting to struggle.

Post-mortem Findings in Cot Death

Some degree of cyanosis is usually observed on post-mortem examination, with generalized congestion and petechial haemorrhages on the pericardium and the lungs. In the lungs the main histological changes are diffuse severe congestion with scattered haemorrhages, oedema infiltrated by a variable number of macrophages, and a few areas of partial collapse. The walls of the bronchi are infiltrated by lymphocytes and plasma cells and occasionally a slight increase in the number of polymorphonuclear leucocytes (Barrett 1954; Stewart 1957). The lumen of the bronchi and bronchioles may contain increased amounts of single intact desquamated epithelial cells from the mucosa, sometimes in such profusion as to completely fill the lumen of the smaller bronchioles (Bodian & Heslop 1956; Stewart 1957).

Inability to Isolate an Infective Agent

Careful investigations for several years failed to reveal the presence of any pathogenic bacteria or viruses in the great majority of these cases (Ministry of Health Report 1965) and though potential pathogenic bacteria have been isolated from some infants dying suddenly (Ministry of Health Report 1965; Johnstone & Lawy 1966) there is little evidence that they caused death. In a careful review of the problem Barrett (1954) suggested that such deaths may follow the inhalation of food or vomit, not in such amounts as to cause mechanical asphyxia, but enough to initiate shock in the lungs of specifically sensitized individuals.

Investigations were then made to test the possibility that such deaths may be due to a Type I allergic response following the inhalation of milk into the lungs of a specifically sensitized child.

Experimental Evidence that Cot Death may be due to Hypersensitivity to Cow's Milk

Cow's milk, either in its natural form or in proprietary preparations, is the commonest foreign protein to which infants are exposed. Antibodies to milk

proteins are stimulated soon after milk is first fed (Gunther, Cheek, Matthews & Coombs 1962) and antibodies to milk can cause disease of allergic origin in infants (Anderson & Schloss 1923; Schloss 1924–25; Tallerman 1934; Clein 1958; Heiner & Sears 1960; Peterson & Good 1963). Nearly all infants have agglutinating antibody to milk proteins as measured by the tanned cell test, but sera of cot-death cases tend to have higher titres (Gunther, Aschaffenburg, Matthews, Parish & Coombs 1960; Parish, Barrett, Coombs, Gunther & Camps 1960; Parish, Richards, France & Coombs 1964). However, these agglutination tests only demonstrate that antibody has been stimulated by milk in the diet: they do not detect antibodies that sensitize tissues passively and predispose them to anaphylaxis.

Experimental Reproduction of the Changes in Cot Death Cases in Guinea-pigs Sensitized to Cow's Milk

It has been demonstrated that guinea-pigs sensitized to milk proteins to produce a comparable titre of circulating antibody to that in babies died quickly and quietly with post-mortem changes in the lungs closely resembling those of cot death when they were lightly anaesthetized to simulate sleep and were given inhalations of a small amount of milk, or stomach contents of cot-death cases containing milk (Parish, Barrett & Coombs 1960; Parish *et al* 1960). Death in these guinea-pigs was associated with bronchial constriction as in anaphylaxis in conscious animals (Parish *et al* 1964; Parish, Akester & Gregg 1964).

One Probable Cause of Cot Death

Milk proteins have been found in the lungs of cot-death infants (Parish *et al* 1964b) and a case of sudden death has been observed after inhalation of a small amount of milk by a sensitized child (Kaufmann, Lantz & Burgin-Wolff 1963), providing further evidence that it is possible for an infant sensitized to milk, during sleep to regurgitate and inhale a small amount of its last meal containing milk protein in some form, resulting in a fatal Type I reaction in the lungs. In experimental studies the clinical manifestations and histological changes, including eosinophil infiltration, of the reaction are modified in the sleeping state when compared with anaphylaxis in the conscious subject (Parish *et al* 1960a; Parish, Hall & Coombs 1963).

It is possible that death in adults following inhalation of stomach contents when under anaesthesia may be due to a similar Type I allergic reaction in individuals sensitized to other food proteins.

LOBAR PNEUMONIA

A Type I allergic sensitivity may be responsible for the lobar pneumonia in adults whose tissues have been sensitized by previous infection with pneumococci,

in contrast to infants in whom this condition is uncommon (Lauche 1928). This is an example in which a state of hypersensitivity is not the same as immunity, for the sera of adults has a bactericidal action on pneumococci and they develop a Type I allergic sensitivity as shown by immediate reaction on skin testing (Sutliff & Finland 1932). Yet the oedema and mucus secretion resulting from the Type I allergic reaction potentiates the pneumococcal infection. Similar extensive oedema and leucocyte infiltration may follow the intratracheal injection of extracts of pneumococci in sensitized animals (Sharp & Blake 1930; Lindau 1933).

THE TYPE III ALLERGIC RESPONSE

Serum sickness (see Chapter 24) is the classical example of the Type III, precipitin-mediated, tissue-damaging allergic reaction. It may be associated with, and complicated by, Types I and IV reactions. There are similarities between serum sickness and certain pulmonary hypersensitivity diseases caused by organic antigens reaching the lungs by inhalation and perhaps also through the circulation after injection or ingestion. More is known about the effects of inhaled organic dusts as a result of investigations into pulmonary aspergillosis in its various forms and into farmer's lung and related diseases.

Effects of Inhaled Organic Antigens

There are at present three main factors which have an obvious influence upon the effects of inhaled organic dusts:

1. The immunological reactivity of the subject.
2. The nature of the organic dust.
3. The nature of the exposure.

These factors determine the appearance of different types of pulmonary hypersensitivity disease in populations exposed to the same dusts.

Immunological Reactivity

In this respect people may be divided into two groups, atopic and non-atopic. Atopic subjects are constitutionally predisposed to develop Type I allergy in response to limited ordinary, everyday, contact with antigens, resulting in bronchial, asthmatic reactions, mediated by non-precipitating skin-sensitizing, reaginic antibody. However, both non-atopic and atopic subjects may produce precipitating antibody in response to intensive antigenic exposure likely to occur at work or under special circumstances. These precipitins may be associated with Type III reactions in the tissues in and around the bronchi, and in the peripheral alveolar tissues.

Nature of the Organic Dusts

The capacity of the microflora of the dusts to grow in the lungs and the particle sizes of the inhaled dusts influence the nature and situation of the pulmonary reactions produced. Such antigens may be derived from vegetable, porcine, bovine, avian, insect or other sources, and from their varied micro-organismal flora. Certain spores such as those of the *Aspergillus* genus, and in particular of *A.*

Table 36.1

Nature and sources of organic dust antigens in farmer's lung type disease

Disease	Dust exposure	Source of antigen and precipitins against
Farmer's lung	Mouldy overheated hay, etc.	Thermophilic actinomycetes *Micropolyspora* sp. *Thermoactinomyces vulgaris*
Bagassosis	Mouldy overheated sugar-cane bagasse	Mouldy bagasse ?Thermophilic actinomycetes
Mushroom-picker's lung	Mushroom dust	?Thermophilic actinomycetes
Fog-fever in cattle	Mouldy hay, etc.	*Micropolyspora* sp.
Maple-bark pneumonitis	Maple bark	*Cryptostroma (Coniosporium) corticale*
Wheat weevil disease	Wheat flour	*Sitophilus granarius*
Bird breeder's (fancier's) lung	Pigeon and budgerigar droppings	Serum protein and droppings antigen
Pituitary snuff-taker's lung	Porcine and bovine posterior pituitary powder	Serum protein and pituitary antigens
Suberosis	Oak-bark, cork-dust	Not known
Smallpox-handler's lung	Smallpox scabs	Not known

fumigatus, can grow in the lungs, thus providing local sources of increased amounts of antigen. The spores, which are 2.5 to 3 μ in diameter, are also present in the air in chains longer than 8–10 μ, and in hypersensitive subjects tend to be retained in the medium-sized bronchi, where their growth results in reactions affecting the peribronchial tissues. The fungus may grow saprophytically in damaged lung producing the aspergillus mycetoma or aspergilloma.

The spores of the thermophilic actinomycete *Micropolyspora* sp. (*Thermopolyspora polyspora*), the main source of the antigens in mouldy hay responsible for farmer's lung, can grow at 37°C but there is no evidence of their growth in the lungs. They are inhaled in large numbers and, being 1 μ in diameter, easily penetrate to the alveoli where the tissue reactions of farmer's lung occur (Pepys, Jenkins, Festenstein, Gregory, Lacey & Skinner 1963). The spores of *Cryptostroma corticale*, which are responsible for maple-bark disease, do not grow at 37°C. They are 4–6 μ in diameter (Emanuel, Wenzel & Lawton 1966); recent evidence (Johnston 1966) suggests that particles of this size may penetrate into the alveoli, where the main reaction of this disease also occurs.

Nature of Exposure to the Dusts

This may influence the response in various ways. Intensive exposure may lead to the production of allergic sensitivity and, in already sensitive subjects suffering, for example, from farmer's lung, heavy exposure may provoke severe disease in weakly allergic subjects and vice versa (Pepys & Jenkins 1965). The frequency of exposure is also important, since in farmer's lung (Pepys & Jenkins 1965) and in bird breeder's (fancier's) lung (Hargreave, Pepys, Longbottom & Wraith 1966) intermittent exposure is associated with attacks of acute onset coming on usually 5–6 hr or so after inhalation of the relevant dust. Regular, frequent, exposure is associated with an insidious development of disease, the more dangerous because the relationship to the causal exposure is not obvious.

Broncho-pulmonary Aspergillosis

In atopic subjects the *Aspergillus* genus, and *A. fumigatus* in particular, may cause asthma, which may become complicated by a form of pulmonary eosinophilia. In both non-atopic and atopic subjects, growth of the fungus may result in an aspergilloma. The fungal growth is confined to the lungs, with limited, if any, local invasion. Invasion of the tissues of the body does occur, however, in patients with reticuloendothelial disturbances.

Three main patterns of immunological response to the fungus are observed in three groups of patients.

Group (*a*). Patients with uncomplicated asthma of whom 38% may give immediate Type I reactions to prick tests with crude extracts of *A. fumigatus* (Longbottom & Pepys 1964). Precipitins against *A. fumigatus* may be found in about 9% of cases, almost all of these giving Type I skin-test reactions.

Group (*b*). Patients with asthma complicated by pulmonary eosinophilia, consisting of transitory, recurrent and usually isolated infiltrations of the collapse-consolidation type, together with blood eosinophilia. This is a serious and not uncommon complication of asthma in atopic subjects in the U.K. and

needs to be sought elsewhere. The patients usually have a long history of extrinsic asthma, which becomes more severe when they develop febrile episodes, accompanied by the appearance of pulmonary infiltrations and the coughing up of sputum plugs containing fungal mycelium and of hard brownish fungal particles of varying sizes.

A characteristic cylindrical bronchiectasis with normal peripheral filling develops in the medium-sized bronchi at the sites of the peribronchial infiltrations. Positive sputum cultures are given with greater (statistically significant) frequency in asthmatic than in non-asthmatic subjects (Pepys, Riddell, Citron, Clayton & Short 1959). But, since positive cultures may be obtained from anyone inhaling and expectorating these ubiquitous spores, and since negative cultures may be obtained in proven cases, sputum culture is not sufficient for diagnostic purposes.

Immunological tests provide the best supporting evidence for diagnosis. In the survey of Longbottom & Pepys (1964) most of the patients with asthma and pulmonary eosinophilia gave positive Type I reactions to prick tests with *A. fumigatus* and about two-thirds also had precipitins. The greater incidence of precipitins in the patients with asthma and pulmonary eosinophilia as compared with those with uncomplicated asthma is statistically highly significant. These findings have been supported by intensive studies at the Brompton Hospital on forty-two patients with asthma and pulmonary eosinophilia. Precipitins were found in thirty-nine cases, the serum of one-quarter of the patients having to be concentrated to show them. Agar-gel tests showed limited numbers of arcs in contrast to the abundant arcs obtained with the sera of patients with aspergilloma. Intracutaneous tests with the protein antigens of *A. fumigatus* gave vigorous dual, Type I and Type III reactions in thirty-six cases, in only one of whom were precipitins not demonstrated, and a further four patients gave Type I reactions only.

In patients allergic to *A. fumigatus* and with uncomplicated asthma and without precipitins, skin tests give only Type I reactions. In patients with aspergilloma and abundant precipitins Type I skin test reactions are obtained in less than one-quarter, and in these subjects a Type III reaction may follow. In the majority, however, neither Type I nor Type III reactions are elicited in spite of the abundant precipitins. Thus, it seems that antibodies capable of giving Type I reactions have to be associated, whatever the mechanisms concerned, with the precipitins, if the Type III component of the dual reaction is to be elicited.

The fact that a vigorous Type III component of the dual reaction is produced in patients with weak precipitins is of interest, since it may be that it is in such cases that, with the limited test doses acceptable in man, moderate antigen excess may be achieved. Ishizaka (1963) has shown that it is the antigen-antibody complexes formed in moderate antigen excess which are the most active in causing tissue damage. A further possibility to be considered is that the precipitins in atopic

and non-atopic subjects and the individual precipitins in any one subject, may differ in their biological activity.

Histological examination of the dual reactions has shown polymorphonuclear neutrophil infiltration and debris, with lymphoid cells and eosinophil cells, the latter being attributable to the preceding Type I reaction. Perivascular granulomata of Type IV reactions were not seen.

These changes are like those of Type III, Arthus, reactions in experimental animals, except for the absence of fibrinoid necrosis of vessels and haemorrhage. This may be due to the limited test doses used in man. Fibrinoid necrosis of tissue collagen has, however, been found in two out of sixteen cases and it seems likely that it is responsible for the bronchiectasis.

There are analogies between the Type III component of the skin-test reaction and the pulmonary infiltrations in the patients with asthma and pulmonary eosinophilia. Both occur in patients with reagins and precipitins, and both the pulmonary infiltration and the Type III component of the skin-test reaction are inhibited by corticosteroid drugs.

Group (*c*). Patients with aspergilloma of the lungs. The *Aspergillus* genus, and especially *A. fumigatus*, colonizes damaged areas of lung such as the open-healed cavities of treated pulmonary tuberculosis, emphysematous bullae and bronchiectasis. The mycelial growth gives rise to the 'fungal-ball' typically surrounded by an air-halo. Sputum cultures of the infecting species are obtained only if the aspergilloma communicates with a bronchus. Intense, specific precipitation reactions consisting of large numbers of precipitin arcs in agar-gel are given by the sera of most of these patients, and specific reactions may be obtained where the aspergilloma is due to other species than *A. fumigatus*, such as *A. nidulans*, *A. flavus* and *A. niger* (Longbottom, Pepys & Temple-Clive 1964). Less than one-quarter of these patients give Type I reactions to prick tests and it is in only these subjects in whom asthma and pulmonary eosinophilia may also be present that Type III reactions have been elicited on intracutaneous testing.

There appears to be a place for precipitin tests in the diagnosis of the invasive form of aspergillosis, since a positive reaction to *A. nidulans* has been obtained in a patient infected with it (Redmond, Carré, Biggart & Mackenzie 1965).

C. albicans in Asthma and Pulmonary Eosinophilia

The possibility that precipitins against *C. albicans* may be related to the pulmonary infiltrations in some patients with asthma, and especially in those who have no precipitins to *A. fumigatus*, is suggested by recent investigations of Pepys, Faux, Longbottom, McCarthy & Hargreave (1967). Whereas *A. fumigatus* is chiefly a respiratory pathogen, *Candida albicans* infects many other parts of the body. This makes it more difficult to establish the relationship of precipitins to *C. albicans* to respiratory disease, though there is no doubt that the subjects

with precipitins are capable of responding to the antigens of *C. albicans* by skin, systemic and pulmonary reactions. These reactions are very similar to those reported in patients with pulmonary aspergillosis, farmer's lung, bird breeder's lung and related diseases.

Farmer's Lung and Related Diseases

Farmer's lung is defined for National Insurance purposes as 'pulmonary disease due to the inhalation of the dust of mouldy hay or of other vegetable produce, and characterized by symptoms and signs attributable to a reaction in the peripheral part of the broncho-pulmonary system and giving rise to a defect in gas exchange' (Farmer's Lung 1964). Except for the nature of the offending dust, this definition applies to a group of closely similar and important diseases, due to the inhalation of an ever-increasing number of inhaled organic dusts, against which precipitins have been found in most instances (Table 36.1). Only a small proportion of affected subjects have a history or show evidence of atopic sensitivity, and the pulmonary reaction takes place in the peripheral alveolar tissues, whereas in atopic subjects exposed to the same dusts reactions occur in the bronchi with the production of asthma. The alveolar reaction is accompanied by impairment of gas-exchange and decrease in compliance or elasticity of the lungs.

Diseases of this type have been termed 'hypersensitivity granulomatous interstitial diseases' in the U.S.A. This term is not altogether appropriate since the reaction takes place in the walls of the alveoli which enclose capillaries only, so that the use of the term interstitial is not apt. An alternative, and perhaps better, title for these diseases would be 'extrinsic allergic alveolitis'.

The attacks may be acute, developing 5–6 hr or so after the inhalation of the offending dust, particularly where there is an interval of several days between the exposures, or they may develop insidiously in constantly exposed subjects; in a small number of cases there may be a rapidly developing asthmatic reaction followed some hours later by the farmer's lung type of reaction. Systemic manifestations are prominent, consisting of fever, chills, rigors, muscle aches and malaise, accompanied by a notable loss of weight. Respiratory manifestations follow consisting of cough and severe dyspnoea together with fine crepitant rales indicative of an alveolar reaction. A widespread fine miliary infiltration may be seen on X-ray and in chronic cases the appearances are those of diffuse pulmonary fibrosis which may follow repeated or even isolated attacks.

a Farmer's Lung

The thermophilic actinomycetes *Micropolyspora* (*Thermopolyspora*) *faeni* and to a lesser extent *Thermoactinomyces* (*Micromonospora*) *vulgaris* have been found to be the main sources of farmer's lung hay, FLH, antigens in mouldy hay responsible for farmer's lung. Precipitins are present in about 90% of affected subjects (see

Chapter 3), though weaker positive reactions may also be obtained in sera from some exposed but apparently unaffected subjects (Pepys, Riddell, Citron & Clayton 1961, 1962; Pepys *et al* 1963; Pepys & Jenkins 1965; Kobayashi, Stahmann, Rankin & Dickie 1963; Bishop, Melnick & Raine 1963; Symposium on farmer's lung 1965). These organisms flourish in damp hay and other vegetable produce which becomes overheated due to bacterial and fungal growth. The discovery of their role in farmer's lung confirms the original description by Ramazzini of Diseases of Sifters and Measurers of Grain. He observed that the severe pulmonary disease with which they were affected was due to the inhalation of the dust which crumbled from grain which had been stored damp and had subsequently become overheated (Ramazzini 1713).

The precipitins against the FLH antigens of *Micropolyspora faeni* are evidence of exposure to mouldy hay and appear to be pathogenetically related to the disease, since there is a statistically significant correlation between the grade of FLH precipitin reaction and the frequency and severity of attacks and the degree of clinical sensitivity to the dust (Pepys & Jenkins 1965; Davies & Yull 1966). This is supported by the provocation of typical attacks of farmer's lung in affected subjects coming on 5–6 hours or so after the inhalation of extracts of mouldy hay (Williams 1963) and of extracts containing the 'protein' and 'glycopeptide' FLH antigens obtained from cultures on ordinary media of *Micropolyspora faeni* (Pepys & Jenkins 1965). This interval between exposure and reaction is the same as that observed in patients presenting with attacks of acute onset, and the same reaction is produced by inhalation tests in patients whose disease has developed insidiously, thus showing that both forms of onset of attacks are features of the same disease, their differences being related to the circumstances of exposure to the dust. Furthermore, the interval is consistent with the time required for a precipitin-mediated reaction.

The spores of *M. faeni* are 1 μ in size, small enough to penetrate and be retained in the alveoli. Vast numbers are present in mouldy hay and it has been calculated that 750,000 are retained in the lungs per minute by farmers working in a moderately dusty atmosphere (Lacey & Lacey 1964) thus explaining the widespread alveolar reactions observed. The organisms have been cultured from the lungs of affected subjects (Wenzel, Emanuel, Lawton & Magnin 1964). Histological examination has shown infiltration of the alveolar walls with lymphoid, plasma and neutrophil cells (Emanuel, Wenzel, Bowerman & Lawton 1964), and in well-developed cases epithelioid cell granulomata are a feature (Dickie & Rankin 1958). Skin testing has not so far been helpful in farmer's lung, as prick tests with extracts of good and mouldy hays of *M. faeni* give negative reactions and intracutaneous tests give non-specific irritant reactions.

Cattle fed on mouldy hay develop a respiratory disease included in the 'fog-fever' group, and precipitins against the FLH antigens of are present in the majority of cases (Jenkins & Pepys 1965). Parish (1961, 1963) found that

experimental animals reared on mouldy corn and hay developed precipitins and that inhalation of extracts of mouldy hay provoked dyspnoea appearing after 6–9 hr, thus resembling the reactions to inhalation tests in patients with farmer's lung.

Diseases Resembling Farmer's Lung

Bagassosis

The inhalation of the dust of mouldy overheated sugar cane bagasse causes a farmer's lung type of disease. Precipitins have been found against certain extracts of mouldy bagasse in affected subjects (Weill, Buechner, Gonzalez, Herbert, Aucoin & Ziskind 1966; Salvaggio, Buechner, Seabury & Arquembourg 1966). Whilst it seems likely, by analogy with mouldy hay in farmer's lung, that the source of the relevant antigens may be from thermophilic organisms developing in the mouldy overheated bagasse, none have so far been identified.

Mushroom picker's lung may also belong in this group. Thermophilic actinomycetes can grow in mushroom houses, and a farmer's lung disease has been described in workers handling the compost (Bringhurst, Byrne & Gershon-Cohen 1959). Precipitins against *Thermoactinomyces vulgaris* and *M. faeni* have been found in cases in the U.K. (Sakula 1967).

Maple bark disease is due to the inhalation of the spores of *Cryptostroam* (*Coniosporium*) *corticale* which infects the bark of the maple tree. Precipitins have been found, and immediate and more slowly developing skin-test reactions have been obtained against its extracts (Emanuel, Wenzel & Lawton 1966).

Mill-worker's Asthma and Lung Disease due to the Wheat Weevil

The antigens of *Sitophilus granarius* have been found to cause asthma and to give Type I skin and inhalation test reactions, and to excite the production of precipitins (Jiminez-Diaz, Lahoz & Canto 1947; Frankland & Lunn 1965; Lunn 1966; Lunn & Hughes 1966). In patients with precipitins dual, Type I and Type III, reactions were obtained on skin testing (see Chapter 7) and asthma followed by farmer's lung type reactions occurred on inhalation test. It is clear that insect antigens merit study.

Bird Breeder's (fancier's) Lung

Inhalation of antigens in the dry dust of the droppings of pigeons and budgerigars stimulates formation of precipitins and leads to a farmer's lung type of reaction (Barboriak, Sosman & Reed 1965; Reed, Sosman & Barbee 1965; Hargreave, Pepys, Longbottom & Wraith 1966). Pigeon breeders who are exposed intermittently tend to have attacks of acute onset, whereas the more frequently exposed budgerigar fanciers tend to develop the disease insidiously.

As in farmer's lung, precipitins are present in affected subjects but may also be present in a small proportion of exposed though apparently unaffected subjects and not in non-exposed subjects. In affected subjects skin tests give dual reactions, and inhalation tests provoke typical attacks of disease coming on after 5–6 hr. The precipitins react with antigens present in the bird sera, extracts of their droppings and of egg yolk and white, and of skin. Feather extracts give only weak reactions, if at all.

Respiratory Disease Due to Pituitary Snuff

The nasal insufflation of porcine and bovine pituitary snuff for the treatment of diabetes insipidus may cause Type I asthmatic reactions and also Type III alveolar reactions like those of farmer's lung. This is not surprising in view of the repeated inhalation each day over long periods of heterologous tissue and serum proteins. Precipitins are produced against the heterologous serum and pituitary antigens and also against homologous human pituitary antigens, the latter providing an example of iatrogenic hetero-stimulated production of auto-reactive antibodies (see Chapter 21) (Pepys, Jenkins, Lachmann & Mahon 1966; Mahon, Scott, Ansell, Manson & Fraser 1967).

Dual reactions are obtained to skin tests with the heterologous serum proteins. Tests with the pituitary snuffs themselves are modified by the vasoconstrictor action of the vasopressin, which probably mitigates the severity of clinical reactions to this allergenic material. The inhalation of synthetic lysine vasopressin (Syntopressin, Sandoz) is effective for the treatment of the diabetes insipidus and for the relief of symptoms due to the pituitary snuffs.

General

Other pulmonary diseases probably belonging in this group are suberosis due to cork dust, and small-pox handler's lung in nurses treating cases of this disorder (Morris-Evans & Foreman 1963). Variations in this type of disease produced by vegetable dusts may perhaps include byssinosis in which Massoud & Taylor (1964) have found precipitins against extracts of the cotton plant receptacle particularly in affected subjects. Workers with sisal, coffee, hemp and other dusts develop precipitins against them (Pepys, Longbottom & Jenkins 1964) and the possibility that these may mediate disease needs to be investigated. The inhalation of any organic dust should be suspect in subjects with pulmonary disease, particularly of the alveolar tissues.

Type III Pulmonary Reactions to Injected Antigens

The injection of foreign serum with the production of serum sickness and polyarteritis nodosa-like lesions attributable to a Type III reaction, is a familiar

finding in experimental animals, and in man may be associated, as may certain drug reactions, with pulmonary changes (see Longcope 1943). Thus, where polyarteritis nodosa of unknown aetiology is found, Type III allergy is postulated, and will be dealt with briefly here, though this does not imply that injected antigens are necessarily responsible. Rose & Spencer (1957) divide their series into those with and without lung involvement, such as bronchitis, pneumonia and asthma which appears to be of the 'intrinsic' type, and they suggest that polyarteritis nodosa should be considered in atypical lung diseases with these manifestations.

A large number of disorders are thought, though the validity of this has yet to be determined, to be part of this group, having features in common: asthma with polyarteritis nodosa (Herrman 1933; Rackeman & Greene 1939; Trasoff & Scarff 1940; Wilson & Alexander 1945), pulmonary granulomatosis with polyarteritis nodosa (Wegener's granulomatosis) (Wegener 1939; Fienberg 1953; Fahey, Leonard, Churg & Godman 1954; Godman & Churg 1954; Walton 1958), granulomatous ulceration of the upper respiratory tract without involvement of the lungs (Singh, Stokes, Drury & Walshe 1958), and Löffler's syndrome or severe pulmonary eosinophilia (Fienberg 1955; Rose & Spencer 1957).

Type III Pulmonary Reactions Due to Ingested Antigens

The possibility must be considered that ingested food antigens may enter the circulation and combine with antibodies to form aggregates capable of producing lung damage, though the possibility that the offending food, particularly milk, has been inhaled must be kept in mind. Milk and precipitins against it figure prominently in reports on infants and children, in whom are found respiratory diseases such as recurrent or chronic pneumonia, pulmonary haemosiderosis and bronchiectasis, together with failure to thrive, anaemia due to gastro-intestinal bleeding and hepatosplenomegaly (Diner, Kniker & Heiner 1961; Heiner, Sears & Kniker 1962; Holland, Hong, Davis & West 1962; Peterson & Good 1963; Wilson, Heiner & Lacey 1964; Collins-Williams 1962).

The inhalation of milk in patients with oesophageal lesions, and probably in mongols, is associated with the presence of milk precipitins and chronic recurrent lung disease (Peterson & Good 1963; Nelson 1964; Handelman & Nelson 1964). A raised incidence of precipitins was not found in patients with milk allergy and suffering from eczema, asthma and gastro-intestinal symptoms (Luz & Todd 1964; Saperstein, Anderson, Goldman & Kniker 1963) whereas the precipitins were present in patients with recurrent pneumonia and pulmonary haemosiderosis (Luz & Todd 1964). The different clinical picture in the atopic and non-atopic groups of subjects resembles the Type I asthmatic reactions to inhaled organic antigens in atopic subjects and the Type III alveolar reactions in non-atopic subjects.

THE TYPE IV ALLERGIC RESPONSE

The classical example of a Type IV reaction is the hypersensitivity resulting from infection by the tubercle bacillus. The basic histological feature following infection is the formation of tubercles comprising epithelioid cells and occasional giant cells surrounded by fibrous tissue and lymphocytes. The nature of the stimulus causing these changes is unknown (Rich 1951) but they may be induced by living or dead tubercle bacilli or the phosphatide or wax fractions of the bacilli (Raffel 1946, 1948, 1950).

The association between hypersensitivity of the delayed type and the presence of lesions containing epithelioid cells and lymphocytes, or well-defined tubercles as found in tuberculosis, has given rise to the general belief that these histological changes are the result of a delayed-type allergic response, or develop concomitantly with it. This does occur as a result of infection by *Coccidioides immitis* (Cox & Smith 1939; Aronson, Saylor & Parr 1942; Alznauer, Rolle & Pierce 1955; Hyde 1958) and by *Histoplasma capsulatum* (Parsons & Zarafonetis 1945; Puckett 1953; Straub & Schwarz 1955). Skin tests with broth cultures filtered after supporting growth of these organisms causes a delayed indurated skin reaction in individuals sensitive to coccidioidin (Aronson, Saylor & Parr 1942) and histoplasmin (Palmer 1945; Tucker 1951) and some cross-reaction may be present.

The evidence that a state of delayed-type sensitivity is induced by infection with these organisms is as conclusive as that following infection with *Mycobacterium tuberculosis*. However, circulating antibodies are also formed in the same patients as those who give a delayed skin reaction on testing, and these may be used as an aid to diagnosis by precipitation, complement fixation and collodion particle agglutination tests (Salvin & Furcolow 1954; Campbell 1958). Similar tests may reveal the presence of circulating antibodies in patients with tuberculosis, but they are unreliable as evidence of infection (Rich 1951; Boyden 1958).

However, it is by no means certain that tubercle formation is solely the result of a delayed hypersensitivity response. It is possible that the tubercles, or diffuse granulomatous lesions which have resulted from infection by *Aspergillus* species or *Candida albicans* in man (Renon 1897; Ikeda 1937; Hetherington 1943; Smith 1947; Hertzog, Smith & Goblin 1949) and in experimental animals (Castellani 1927; Schattenberg & Flinn 1939; Cooper 1946; Zettergren 1950; Parish 1961), and those following inhalation of silica or beryllium may result from precipitates formed in a Type III reaction or even stimuli without any allergic basis. The significance of these changes resembling tuberculosis is yet to be determined, particularly in sarcoidosis and silicosis.

Decreased Delayed Hypersensitivity in Sarcoidosis

There is much evidence of altered immunological responses in sarcoidosis, such as increased production of circulating antibody (Sands, Palmer, Maycock &

Greger 1955), and decreased delayed hypersensitivity, although the incidence of the disease is not related to the degree of tuberculin sensitivity present previously (Sutherland, Mitchell & Hart 1965). Failure to develop delayed sensitivity to tuberculin may persist after recovery from sarcoidosis, vaccination with BCG either failing to sensitize or producing weak transient sensitivity (Israel & Sones 1965), yet patients with sarcoidosis can be sensitized passively with leucocytes taken from tuberculin-positive individuals (Urbach, Sones & Israel 1952) indicating that the tissues are able to participate in delayed sensitivity reactions, but there is a defect in the reticuloendothelial system resulting in an inability to develop delayed sensitivity. It has been suggested that this immunological defect is prerequisite to the disease (Israel & Sones 1965). BCG vaccination repeated on two occasions was associated with positive Kveim tests in clinically normal young adults who failed to become sensitive to tuberculin (Hart, Mitchell & Sutherland 1964) though there was no evidence of any association of sarcoidosis with BCG vaccination (Sutherland *et al* 1965). The possibilities of occult sarcoidosis or some immuno-chemical change not directly related to sarcoidosis have been invoked to explain this finding. The Kveim test reaction (see Chapter 7) is not sufficient evidence, in itself, to establish that this disorder is due to immunological factors.

The Changes in the Lungs following Inhalation of Silica or Beryllium and Evidence that Hypersensitivity may be Induced

Silica may produce pulmonary lesions identical with those produced by *Mycobacterium tuberculosis* (see Kettle 1930; Gardner 1932, 1937) though the same type of change is not found in all affected individuals. Silica may become closely bound with body proteins including plasma globulins which have been isolated from silcotic nodules (Ceppellini & Pernis 1958; Vigliani & Pernis 1958) and the resulting complex could become antigenic (Powell & Gough 1959).

Inhalation of beryllium may result in an acute pneumonitis in which there is a diffuse infiltration by lymphocytes, plasma cells and large mononuclear cells, and in some patients well-defined granulomata are found (Van Ordstrand, Hughes, DeNardi & Carmody 1945; Dutra 1948). In the chronic condition there is a focal and diffuse fibrosis and scattered granulomata whose features include lymphocytes, fibroblasts and Langhans-type giant cells, though epithelioid cells are relatively infrequent. The condition resembles sarcoid from which it may be differentiated by the lower number of epithelioid cells on histological examination and the detection of beryllium in the tissues (Dutra 1948; Hardy 1956). Beryllium is known to be a skin-sensitizing agent, and patch tests have caused a delayed-type response in affected individuals indicating that dermatitis and

probably the pneumonitis results from hypersensitivity to the beryllium ion (Curtis 1951).

The stimulus to produce this type of lesion in these conditions is still unknown, but there is some evidence that a delayed hypersensitivity may be induced in both.

TYPE OF ALLERGY UNKNOWN

In many pulmonary conditions an allergic mechanism is suspected on indirect evidence. The lesions may resemble those of many other conditions, in particular polyarteritis nodosa, for which there is evidence of an allergic origin. These are usually accompanied by an increase in circulating serum globulins and the conditions are generally alleviated by drugs that are effective in treating known allergic inflammatory reactions.

Lung involvement in the so-called 'collagen disease complex' or 'connective tissue disorders' is concomitant with the generalized disease of the body, and may not occur at all. The nature of the allergic reaction, if any, in this group is not known.

Pneumonia in rheumatic fever and rheumatoid arthritis

Rheumatic fever is a condition chiefly affecting the cardiovascular and connective tissue system but which may cause severe changes in many organs and the joints. Severe reactions in the lungs may be a major contributing cause of death in the acute diseases, and chronic inflammatory changes may cause death by right-sided heart failure, independent of any lesions of the heart valves (Gouley 1938). The possible importance of streptococcal infection and the nature of the allergic response is reviewed in Chapter 30.

The relationship of rheumatoid disease to acute rheumatic fever is unknown. In rheumatoid disease joint and lung lesions may occur together (Caplan 1953; Ellman & Cudkowicz 1954), though it is not known whether the pulmonary lesions are due to an allergic response affecting blood vessels or are an inflammatory reaction to inhaled irritant or perhaps antigenic dusts in individuals whose tissues react abnormally due to the co-existing rheumatoid disease.

Changes in the lungs associated with diseases affecting connective tissue

There are several diseases usually considered as a group under the title of 'collagen diseases' or 'connective tissue disorders' in which the lungs may be affected as part of the process affecting the whole body. It is believed that they are of allergic origin but there is no conclusive evidence that this is the cause or in what manner the changes may be initiated.

In scleroderma and in fibrosing alveolitis (Hamman-Rich syndrome) a diffuse fibrosis of the alveolar walls gives rise to the 'honeycomb' lung. Some of the features of this syndrome are claimed by Read (1958) to have been induced in

rats by the intratracheal injection of rabbit anti-rat lung serum. Whilst no specific auto-antibodies for lung were found in patients with interstitial pulmonary fibrosis, non-organ-specific antibodies of different types, particularly rheumatoid factor and anti-nuclear antibodies, were present in two-thirds of the cases by Turner-Warwick & Doniach (1965). It should be noted that chronic inflammatory changes may follow inhalation of organic antigens.

Changes in the lungs resembling rheumatic pneumonia and polyarteritis nodosa may occur in disseminated lupus erythematosus as part of the systemic manifestations (Ellman 1956). Typical LE cells may be found in the pleural effusions. No agreement has been reached whether the pulmonary lesions are specific for this condition (Ellman & Cudkowicz 1954; Ellman 1956) or whether they are the result of secondary tissue degeneration or bacterial infection.

Pulmonary Eosinophilic Infiltrations and Possible Types of Allergy

Possible Functions of the Eosinophil Cell

Little is known of the functions of the eosinophil polymorphonuclear leucocyte. It is often found in increased numbers in the blood stream, and sometimes in focal lesions in individuals during or following an allergic reaction or parasitic infestation, and the association with these states is so constant that it is often accepted as diagnostic evidence. Increased numbers are found after Types I and III reactions. They have also been found by Bassett (1962) in increased numbers in relation to fibroblasts in oestrous and pregnant animals. It is suggested that they may be concerned with regulation of extracellular protein contributed to collagen and ground substance by the enlarged fibroblasts, which showed an increase in the RNA content and the Golgi apparatus.

Eosinophils have been found to have antihistamine activity and the intact cells or their extracts can prevent much of the effect of a local injection of histamine or 5-hydroxytryptamine (Archer 1963). Eosinophils may ingest antigen (Speirs & Speirs 1963; Roberts 1966) or antigen-antibody complexes (Sabesin 1963; Litt 1964a) though in these respects they behave like other phagocytic leucocytes (Archer & Hirsch 1963). Both histamine and antigen-antibody complexes can stimulate increased numbers of eosinophils in the peritoneal fluid or peripheral blood of guinea-pigs, though histamine is the less potent (Litt 1964b; Parish & Coombs 1968).

A Classification of Pulmonary Eosinophilic Infiltrations

A classification of infiltration of the lungs associated with eosinophilia is presented in Table 32.2 which is based on the review by Crofton, Livingstone, Oswald & Roberts (1952).

TABLE 36.2

Pulmonary eosinophilia and presumed types of allergy

Nature of eosinophilia	Presumed type of allergy and antibody demonstrated					Etiology
	* I (a) reagin	* I (b) ppt.	II	III	IV	
(1) Simple pulmonary eosinophilia or Löffler's syndrome	Presumed	Yes	No	Yes	No	Parasites chiefly
(2) Prolonged or recurrent pulmonary eosinophilia without asthma	?	?	No	?	?	
(3) Pulmonary eosinophilia with asthma (long history of asthma)	Presumed	Yes	No	Yes	No	*Aspergillus fumigatus* about 90%
(4) Tropical eosinophilia usually with asthma of recent onset	No	?	No	?	No	? parasites
(5) Polyarteritis nodosa. If asthma occurs it is of recent onset (e.g. serum sickness)	No	Yes	No	Yes	No	

* The type of allergy is a tentative suggestion where no known data exists. The Type I allergic response has been subdivided into (a) responses due to the reagin antibody sensitizing the tissues, and (b) responses due to that part of the precipitating antibody also capable of predisposing tissues to a Type I allergic response.

In some tests human heat stable precipitating-type antibody has been claimed to passively sensitize human skin for short periods following intradermal injection.

Their definition of pulmonary eosinophilia as 'pulmonary infiltration with blood eosinophilia (over 6% total white cells)' included a wide range of different diseases which extended from the mild and transient changes of true Löffler's syndrome to the severe manifestations of polyarteritis with lung involvement. They stressed that the groups were a continuous series with overlapping features in each group, but that it was convenient to subdivide them. 'Tropical eosinophilia' or 'tropical pulmonary eosinophilia' was a particularly well-defined group and its relationship to the rest of the series was uncertain.

This classification brings together those conditions in which eosinophilia occurs, but in which eosinophilia is not necessarily the main sign of disease.

Possible Causes of the Allergic Responses, and Types of Allergy Associated with Pulmonary Eosinophilia

Many agents other than fungi and especially *A. fumigatus*, already discussed, have been incriminated as the cause of transient eosinophilia. These include pollens, vegetable dusts, helminth larvae, including ascaris species and schistosomes, and amoebae in pneumonitis associated with amoebic dysentery. Some of the evidence is reviewed by Crofton *et al* (1952).

The true Löffler's condition consists of a simple transient pulmonary eosinophilia possibly associated with fever, but without asthma (Löffler 1932, 1936) due to a mild allergic reaction to a very large number of antigens which are inhaled or distributed through the body from other sites. On the available evidence this could be a Type I or a Type III reaction. The absence of associated disease of the respiratory system, alimentary tract or skin, argues against the participation of reagins or skin-sensitizing antibody, although they may be demonstrated by skin tests as in hypersensitivity to extracts of Ascaris, an important cause of Löffler's syndrome.

Whether the second group of patients presenting with prolonged or recurrent eosinophilia without asthma are an extension of Löffler's syndrome due to repeated exposure to the antigen, or whether there is a difference in the nature of the antigen or type of allergic response is yet to be discovered. In the following discussion of the possible antigens it will be seen that antibody responses capable of mediating both Types I and III reactions may be found in some patients.

Possible Agents Causing the Eosinophilic Infiltrations, the Role of the Normal Helminth Parasites of Man, and Visceral Larva Migrans

There is no doubt that helminth infestation is the cause of the transient eosinophilia of Löffler's syndrome in many patients. The larvae that penetrate the tissues in the normal life cycle in man are probably the cause, and *Ascaris lumbricoides* seems the commonest. The ingestion of embryonated eggs under experimental conditions in man has resulted in a transient eosinophilia (see Crofton *et al* 1952).

Ascaris is known to contain potent antigens and laboratory workers not infrequently develop signs of a Type I allergic response after working with this parasite, hay fever, asthma and urticaria. Some insight into the immunology of Löffler's syndrome could be obtained by comparing the clinical picture in patients with Type I reactions, i.e. asthma, and those with pulmonary infiltrations without asthma. The question is whether skin-sensitizing or precipitating antibodies are, or are not, present in patients with Löffler's syndrome and whether, if present, pulmonary infiltrations occur as well as asthma. A similar

situation is discussed in relation to pulmonary eosinophilia due to *Aspergillus fumigatus*.

Purified antigens of Ascaris have been prepared and used to detect agglutinating antibody (Kagan 1958; Oliver-Gonzalez 1960) and precipitating antibody (Kagan, Jeska & Gentskow 1958; Kent 1960) and skin tests of infected individuals resulted in an immediate response (Kent 1960). These, among many other uncited experiments, conclusively demonstrate the presence of a Type I allergic response to Ascaris infection, which could result in a transient pulmonary eosinophilia during the period of migration of the larvae.

Visceral larva migrans

This condition is caused by the larval stages of the helminths which have infected a species other than their true host. The larvae do not mature but wander into many organs of the body before dying and being destroyed. They may cause disease by trauma and allergic reactions with the sensitized tissues. The chief culprit recognized in man is the larval stage of *Toxocara canis*.

The chief signs are pulmonary infiltration resulting in respiratory distress, usually wheezing, which may be a prominent feature and result in death, eosinophilia which may be transient or persist for months, hyperglobulinaemia chiefly γ-globulin, enlargement of the liver and occasionally skin rashes (Beaver, Snyder, Carrera, Dent & Lafferty 1952; Heiner & Kevy 1956; Beaver 1957; Shrand 1964).

Despite the detection of circulating antibodies in these individuals (Heiner & Kevy 1956; Sadum, Norman & Allain 1957; Jung & Pacheco 1960), skin testing with *T. canis* antigen extracts by Duguid (1961) in twelve patients with ocular lesions due to this parasite resulted in a delayed response in all of them, and eight control patients had no response. Type I reactions have been reported by Shrand (1964) and by Woodruff, Bisseru & Bowe (1966) to extracts of *T. canis* prepared according to Fairley (1931), in cases of epilepsy. There is further evidence of a Type IV or possibly a mixed Type IV and Type III allergic response from the examination of the lesions of tissues obtained from cases proven to be infected (Churg & Strauss 1951; Brill, Churg & Beaver 1953; Dent, Nichols, Beaver, Carrera & Staggers 1956).

Tropical eosinophilia, usually with asthma of recent onset

Tropical eosinophilia, or tropical pulmonary eosinophilia as described by Ball (1950) has four main features:

1. History of persistent cough with or without asthma.
2. Eosinophilia high enough to produce leucocytosis.
3. Miliary mottling of chest X-ray in about 50% of cases.
4. Therapeutic response to arsenic. In more recent times a response to diethylcarbamazine would probably be taken as supporting evidence.

When these features are compared with other similar conditions of man in which more is known of the nature of the response, tropical pulmonary eosinophilia would appear to result from a Type I or Type III allergic response. However, the nature of the antigen is unknown and therefore there is no reliable evidence even that it is allergic in origin.

CONCLUSIONS

The lung is continuously exposed to antigenic stimulation. Inspired air contains 'dusts' that are foreign antigens, and agents entering the venous system from any part of the body will pass through the vascular network of the lung and its reticuloendothelial cells before being disseminated through the body by the arterial system. This may result in the lung becoming the particular shock organ as in asthma, or being associated with disease affecting the whole body as in polyarteritis nodosa or tuberculosis. In some conditions, particularly those associated with the Type I allergic response, many specific antigens are known as well as the nature of the physiological responses which initiate the histological changes. The smooth muscle of the bronchioles and the large number of capillaries make the lung a predilection site in several species for asthmatic responses of a Type I reaction.

In other conditions such as farmer's lung, bird fancier's lung and related diseases, and in certain forms of pulmonary aspergillosis, the evidence favours the participation of Type III reactions. Precipitins are demonstrable and the responses to skin and inhalation tests have the features of Type III reactions, though in certain instances there is a close association and dependence upon coexisting and preceding Type I reactions. In addition to the accepted role of Type IV reactions in, for example, tuberculosis, its participation in other diseases described above cannot be excluded. Some allergic reactions may consist of two or three types of allergic response superimposed, and the individual contribution of each type to the general reaction will depend upon their different speeds of development and their influence upon each other. Recognition of this may help in the understanding of obscure diseases, in which hypersensitivity is suspect but unproven.

Table 36.3 summarizes and classifies under types of allergic response most of the conditions dealt with in this chapter.

TABLE 36.3

(*a*) TYPE I

Evidence of allergic state	Clinical manifestations	Histology of affected tissues	Aetiology
Type Ia or Ib Allergic basis established	Anaphylaxis	Smooth muscle spasm Bronchial mucosal oedema Hypersecretion Eosinophil cell infiltration	Extraneous allergens, e.g. pollens, fungi, bee stings, vegetable dusts Drugs and foreign sera
Type Ia Allergic basis established	Rhinitis and nasal polyposis	Nasal mucosal oedema Hypersecretion Eosinophil cell infiltration Plasma cells	Extrinsic—extraneous allergens Idiopathic (intrinsic)—not known
Type Ia	Asthma	Bronchial mucosal oedema Hypertrophy of smooth muscle Hypersecretion and increase of mucous glands Eosinophil cell infiltration	As for rhinitis
Type Ib Presumed allergic	Cot death	Bronchial mucosal oedema Hypersecretion without increase in mucous glands Shedding of cells of mucosa	Inhalation of milk in sleeping child
Type I Allergic component	Lobar pneumonia	Oedema, mucus secretion, extensive leucocyte infiltration	Streptococcus pneumoniae

(*b*) TYPE III AND COMBINED TYPES

Evidence of allergic state	Clinical manifestations	Histology of affected tissues	Aetiology
Type III only			
Allergic	Serum sickness, limited pulmonary changes as part of system reaction	Fibrinoid necrosis of arterioles, increased capillary permeability with oedema, neutrophil or eosinophil cell infiltration, plasma cells Can be haemorrhagic	Heterologous serum. Similar picture due to drugs or antibiotics
Type III			
Presumed allergic	Polyarteritis nodosa. Chiefly experimental evidence	Like that of serum sickness above	As above
Type III predominant plus Type I			
Allergic	Pulmonary aspergillosis with pulmonary eosinophilia in asthmatic subjects	Bronchial mucosal oedema, eosinophil cell infiltration, may be bronchiectasis Parenchymal eosinophil cell infiltration	*A. fumigatus* ? other aspergillus species
Type III (? plus Type IV)			
Allergic	Farmer's lung Bagassosis and pneumoconiosis due to other vegetable dusts	Epithelioid cell infiltration in interstitial tissues leading to interstitial fibrosis	Vegetable dusts, in particular mouldy dust

(*c*) TYPE IV

Evidence of allergic state	Clinical manifestations	Histology of affected tissues	Aetiology
Allergic	Allergy of infection, e.g. tuberculosis Histoplasmosis Coccidioidomycosis	Granuloma with epithelioid, lymphoid and giant cells—caseation	Infective agents
Allergic	Beryllium	Epithelioid cell granuloma—no caseation	Beryllium compounds
Possibly allergic component	Silicosis	As above	Certain silica containing compounds

(*d*) TYPE AND/OR ROLE OF ALLERGY NOT ESTABLISHED

Evidence of allergic state	Clinical manifestations	Histology of affected tissues	Aetiology
Presumed allergic	Pneumonia in rheumatic fever, lung changes in rheumatoid arthritis, the so-called 'collagen diseases', vascular 'allergic' disorders, including DLE, scleroderma, dermatomyositis, fibrosing alveolitis	May be fibrinoid necrosis with variable cellular infiltration	Unknown
Presumed allergic	Sarcoidosis	Epithelioid cell granuloma—no caseation Fibrosis	Unknown

REFERENCES

ALZNAUER R.L., ROLLE C. & PIERCE W.F. (1955) Analysis of focalized pulmonary granulomas due to *Coccidioides immitis*. *Arch. Path.* **59,** 641.

ANDERSON A.F. & SCHLOSS O.M. (1923) Allergy to cow's milk in infants with nutritional disorders. *Amer. J Dis. Child.* **26,** 451.

ARCHER G.T. & HIRSCH J.G. (1963) Motion picture studies on degranulation of horse eosinophils during phagocytosis. *J. exp. Med.* **118,** 287.

ARCHER R.K. (1963) *The Eosinophil Leucocytes.* Oxford: Blackwell.

ARONSON J.D., SAYLOR R.M. & PARR ERMA I. (1942) Relationship of coccidioidomycosis to calcified pulmonary nodules. *Arch. Path.* **34,** 31.

BALL J.D. (1950) Tropical pulmonary eosinophilia. *Trans. R. Soc. trop. Med. Hyg.* **44,** 237, 258.

BARBORIAK J.J., SOSMAN A.J. & REED C.E. (1965) Serological studies in pigeon breeder's disease. *J. Lab. Clin. Med.* **65,** 600.

BARRETT A.M. (1954) Sudden death in infancy. In *Recent Advances in Paediatrics*, p. 301, ed. GAIRDNER D. London: J. & A. Churchill.

BASSETT E.G. (1962) Infiltration of eosinophils into the modified connective tissue of oestrous and pregnant animals. *Nature* **194,** 1259.

BEAVER P., SNYDER H., CARRERA G., DENT J. & LAFFERTY J. (1952) Chronic eosinophilia due to visceral larva migrans. Report of three cases. *Paediatrics* **9,** 7.

BEAVER P.C. (1957) Wandering nematodes as a cause of disability and disease. *Amer. J. trop. Med. Hyg.* **6,** 433.

BISHOP J.M., MELNICK S.C. & RAINE J.L. (1963) Farmer's lung: studies of pulmonary function and aetiology. *Quart. J. Med.* **32,** 257.

BODIAN M. & HESLOP, BARBARA (1956) Sudden death in infancy. *Abstracts 8th Int. Congr. Paediatrics*, p. 91.

BOYDEN S.V. (1958) The immunological response to antigen of the tubercle bacillus. *Progr. Allergy* **5,** 149.

BRILL R., CHURG J. & BEAVER P.C. (1953) Allergic granulomatosis associated with visceral larva migrans. *Amer. J. clin. Path.* **23,** 1208.

BRINGHURST L.S., BYRNE R.N. & GERSHON-COHEN J. (1959) Respiratory disease of mushroom workers. *J.A.M.A.* **171,** 15.

BURNS M.W. & MAY J.R. (1967) *Haemophilus influenzae* precipitins in the serum of patients with chronic bronchial disorders. *Lancet* **i,** 354.

BUSSON B. (1911) Über Eiveissanaphylaxie von den huftinegen ans. *Wien. Klin. Wschr.* **24,** 1492.

CAMPBELL, CHARLOTTE C. (1958) Serological diagnosis and epidemiological aspects of histoplasmosis. In *Fungus Diseases and Their Treatment*, p. 142, ed. RIDDLE R.W. & STEWART G.T. London: Butterworth.

CAPLAN A. (1953) Certain unusual radiological appearances in the chest of coal-miners suffering from rheumatoid arthritis. *Thorax* **8,** 29.

CARDELL B.J. & PEARSON R.S.B. (1959) Death in asthmatics. *Thorax* **14,** 341.

CASTELLANI A. (1927) Notes on certain bronchomycoses which may simulate pulmonary tuberculosis. *Amer. Rev. Tuberc.* **16,** 541.

CEPPELLINI R. & PERNIS B. (1958) Presence of plasma globulins in the hyaline tissue in cases of silicosis. *Nature (Lond.)* **181,** 55.

CHAO-LING C. & CHIH-TZE C. (1956) Penicillin hypersensitivity and anaphylaxis. A review with report of ten cases including five autopsies. *Chinese Med. J.* **74,** 513.

CHURG J. & STRAUSS L. (1951) Allergic granulomatosis, allergic angiitis and periarteritis nodosa. *Amer. J. Path.* **27,** 277.

CLEIN N.W. (1958) Cow's milk allergy in infants and children. *Int. Arch. Allergy* **13,** 245.

COLLDAHL H. (1952) Study of provocation tests on patients with bronchial asthma. *Acta allerg. (Kbh.)* **5,** 133, 143, 154.

COLLINS-WILLIAMS C. (1962) Cow's milk allergy in infants and children. *Int. Arch. Allergy* **20,** 38.

COOPER N.S. (1946) Acute bronchopneumonia due to *Aspergillus fumigatus* Fresenius. *Arch. Path.* **42,** 644.

COX A.J. & SMITH C.E. (1939) Arrested pulmonary coccidioidal granuloma. *Arch. Path.* **27,** 717.

CROFTON J.W., LIVINGSTONE J.L., OSWALD N.C. & ROBERTS A.T.M. (1952) Pulmonary eosinophilia. *Thorax* **7,** 1.

CURTIS G.H. (1951) Cutaneous hypersensitivity due to beryllium. A study of thirteen cases. *Arch. Derm. Syph. (N.Y.)* **64,** 470.

DAVIES D.G. & YULL A.J. (1966) A comparison of the precipitin test in asymptomatic and symptomatic cases of farmer's lung. *Mon. Bull. Minist. Health* **25,** 75.

DEAN H.R. (1922) The histology of a case of anaphylactic shock occurring in a man. *J. Path. Bact.* **25,** 305.

DENT J.H., NICHOLS R.L., BEAVER P.C., CARRERA G.M. & STAGGERS R.J. (1956) Visceral larva migrans. With a case report. *Amer. J. Path.* **32,** 777.

DICKIE H.A. & RANKIN J. (1958) Farmer's lung: an acute granulomatous interstitial pneumonitis occurring in agricultural workers. *J.A.M.A.* **167,** 1069.

DINER W.C., KNIKER W.T. & HEINER D.C. (1961) Roentgenologic manifestations in the lungs in milk allergy. *Radiology* **77,** 564.

DUBOIS DE MONTREYNAUD J.M. (1950) Étude experimentale, bronchoscopique et anatomique des bronches au cours de la crise d'asthme. *Ann. med.* **51,** 712.

DUGUID I.M. (1961) Features of ocular infestation by *Toxocara*. *Brit. J. Ophthal.* **45,** 789.

DUTRA F.R. (1948) The pneumonitis and granulomatosis peculiar to beryllium workers. *Amer. J. Path.* **24,** 1137.

ELLMAN P. (1956) Pulmonary manifestations in the systemic collagen diseases. *Post. Grad. med. J.* **32,** 370.

ELLMAN P. & CUDKOWICZ L. (1954) Pulmonary manifestations in the diffuse collagen diseases. *Thorax* **9**, 46.

EMANUEL D.A., WENZEL F.J., BOWERMAN C.I. & LAWTON B.R. (1964) Farmer's lung. Clinical, pathologic, and immunologic study of twenty-four patients. *Amer. J. Med.* **37**, 392.

EMANUEL D.A., WENZEL F.J. & LAWTON B.R. (1966) Pneumonitis due to *Cryptostroma corticale* (maple-bark disease). *New Engl. J. Med.* **274**, 1413.

FAHEY J.L., LEONARD E., CHURG J. & GODMAN G.C. (1954) Wegener's granulomatosis. *Amer. J. Med.* **17**, 168.

FAIRLEY H.N. (1931) Serological and intradermal tests in filariasis. *Trans. R. Soc. trop. Med. Hyg.* **24**, 635.

FARMER'S LUNG (1964) Report by the Industrial Injuries Advisory Council. H.M.S.O. Cmnd. 2403.

FIENBERG R. (1953) Necrotizing granulomatosis and angiitis of the lungs. *Amer. J. clin. Path.* **23**, 413.

FIENBERG R. (1955) Pathogenic granulomatosis (editorial). *Amer. J. Med.* **19**, 829.

FRANKLAND A.W. & LUNN J.A. (1965) Asthma caused by the grain weevil. *Brit. J. industr. Med.* **22**, 157.

GARDNER L.U. (1932) Studies on experimental pneumokoniosis. VIII. Inhalation of quartz dust. *J. Indust. Hyg.* **14**, 18.

GARDNER L.U. (1937) The similarity of the lesions produced by silica and by the tubercle bacillus. *Amer. J. Path.* **13**, 13.

GLYNN A.A. & MICHAELS L. (1960) Bronchial biopsy in chronic bronchitis and asthma. *Thorax* **15**, 142.

GODMAN G.C. & CHURG J. (1954) Wegener's granulomatosis. Pathology and review of the literature. *Arch. Path.* **58**, 533.

GOUGH J. (1961) Post mortem differences in 'asthma' and in chronic bronchitis. *Acta allerg. (Kbh.)* **16**, 391.

GOULEY B.A. (1938) The role of mitral stenosis and of post-rheumatic pulmonary fibrosis in the evolution of chronic rheumatic heart disease. *Amer. J. med. Sci.* **196**, 11.

GUNTHER, MAVIS, ASCHAFFENBURG R., MATTHEWS R.H., PARISH W.E. & COOMBS R.R.A. (1960) The level of antibodies to the proteins of cow's milk in the serum of normal human infants. *Immunology* **3**, 296.

GUNTHER, MAVIS, CHEEK E., MATTHEWS R.H. & COOMBS, R.R.A. (1962) Immune responses in infants to cow's milk proteins taken by mouth. *Int. Arch. Allergy* **21**, 257.

HALL R., TURNER-WARWICK M. & DONIACH D. (1966) Auto-antibodies in iodide goitre and asthma. *Clin. exp. Immunol.* **1**, 285.

HALPERN B.R., BIOZZI G. & BENACERRAF B. (1955) Mise en evidence du rôle des facteurs irritatifs et des traumatismes dans la localisation éléctive des anticorps dans les organes de choc. *Sem. Hôp. Paris* **31**, 1955.

HANDELMAN N.I. & NELSON T.L. (1964) Milk precipitins associated with aspiration. *Pediatrics* **34**, 699.

HARDY, HARRIET L. (1956) Differential diagnosis between beryllium poisoning and sarcoidosis. *Amer. Rev. Tuberc.* **74**, 885.

HARGREAVE F.E., PEPYS J., LONGBOTTOM J.L. & WRAITH D.G. (1966) Bird breeder's (fancier's) lung. *Lancet* **i**, 445.

HART P.D'ARCY, MITCHELL D.N. & SUTHERLAND I. (1964) Associations between Kveim test results, previous B.C.G. vaccination and tuberculin sensitivity in healthy young adults. *Brit. Med. J.* **i**, 795.

HEINER D.C. & KEVY S.V. (1956) Visceral larva migrans. Report of the syndrome in three siblings. *New Engl. J. Med.* **254,** 629.

HEINER D.C. & SEARS J.W. (1960) Chronic respiratory disease associated with multiple circulating precipitins to cow's milk. *Amer. J. Dis. Child* **100,** 500.

HEINER D.C., SEARS J.W. & KNIKER W.T. (1962) Multiple precipitins to cow's milk in chronic respiratory disease. *Amer. J. Dis. Child* **103,** 634.

HERRMAN W.G. (1933) Pulmonary changes in a case of periarteritis nodosa. *Amer. J. Roentgenol.* **29,** 607.

HERTZOG A.J., SMITH T.S. & GOBLIN M. (1949) Acute pulmonary aspergillosis. Report of a case. *Pediatrics* **4,** 331.

HERXHEIMER H. & PRIOR F.N. (1952) Further observations on induced asthma and bronchial hyposensitization. *Int. Arch. Allergy & Appl. Immunol.* **3,** 189

HETHERINGTON L.H. (1943) Primary aspergillosis of the lungs. *Amer. Rev. Tuberc.* **47,** 107.

HOLLAND N.W., HONG R., DAVIS N.C. & WEST C.D. (1962) Significance of precipitating antibodies to milk proteins in the serum of infants and children. *J. Pediat.* **61,** 181.

HOUSTON J.C., DE NAVASQUEZ S. & TRAUNCE J.R. (1953) The clinical and pathological study of fatal cases of status asthmaticus. *Thorax* **8,** 207.

HYDE L. (1958) Coccidioidal pulmonary cavitation. *Amer. J. Med.* **25,** 890.

IKEDA K. (1937) *Monilia* infection of the lungs (broncho-moniliasis). *Amer. J. clin. Path.* **7,** 376.

ISHIZAKA K. (1963) Gamma-globulin and molecular mechanisms in hypersensitivity reactions. In *Progress in Allergy*, vol. 7, p. 32. Basel: S. Karger.

ISRAEL H.L. & SONES M. (1965) Immunologic defect in patients recovered from sarcoidosis. *New Engl. J. Med.* **273,** 1003.

JAMES L.P. & AUSTEN K.F. (1964) Fatal systemic anaphylaxis in man. *New Engl. J. Med.* **270,** 597.

JENKINS P.A. & PEPYS J. (1965) Fog-fever. Precipitin (F.L.H.) reactions to mouldy hay. *Vet. Rec.* **77,** 464.

JIMINEZ-DIAZ C., LAHOZ C. & CANTO G. (1947) The allergens of mill dust. Asthma in millers, farmers and others. *Ann. Allergy* **5,** 519.

JOHNSTON J.E. (1966) *Report of the Atomic Energy Research Establishment, Harwell, Berkshire.* H.M.S.O. AERE-PR/HPM 9.

JOHNSTONE J.M. & LAWY H.S. (1966) Role of infection in cot deaths. *Brit. med. J.* **i,** 706.

JUNG R.C. & PACHECO G. (1960) Use of hemagglutination test in visceral larva migrans. *Amer. J. trop. Med. Hyg.* **9,** 185.

KAGAN I.G. (1958) Hemagglutination tests with *Ascaris* antigens. *J. Immunol.* **80,** 396.

KAGAN I.G., JESKA E.L. & GENTZKOW C.J. (1958) Serum-agar double diffusion studies with *Ascaris* antigens. II. Assay of whole worm and tissue antigen complexes. *J. Immunol.* **80,** 400.

KALLOS P. & PAGEL W. (1937) Experimentelle untersuchungen über Asthma bronchiale. *Acta med. Scand.* **91,** 292.

KAUFMAN H.J., LANTZ J. & BURGIN-WOLFF A. (1963) Anaphylactic shock of the lungs triggered by microaspiration of cow's milk: a form of sudden unexpected death in early infancy. *Ann. Paediat.* **200,** 20.

KENT H.N. (1960) Isolation of specific antigens from *Ascaris lumbricoides* (var. suum). *Exp. Parasit.* **10,** 313.

KETTLE E.H. (1930) The relation of dust to infection. *Proc. roy. Soc. Med.* **24,** 79.

KOBAYASHI M., STAHMANN M.A., RANKIN J. & DICKIE H.A. (1963) Antigens in moldy hay as the cause of farmer's lung. *Proc. Soc. exp. Biol. Med.* **113,** 472.

LACEY J. & LACEY M.E. (1964) Spore concentrations in the air of farm buildings. *Trans. Brit. Mycol. Soc.* **47,** 547.

LAMSON R.W. (1924) Sudden death associated with the injection of foreign substances. *J. Amer. Med. Ass.* **82,** 109.

LAUCHE A. (1928) Über das problem der lobären pneumonie vom standpunkt des pathologen. *Klin. Wschr.* **7,** 2421.

LINDAU A. (1933) Studies on varying response to pneumococcal infections, especially lobar pneumonia. *Acta path. microbiol. scand.* **10,** 1.

LITT M. (1964a) Studies in experimental eosinophilia. VI. Uptake of immune complexes by eosinophils. *J. cell. Biol.* **23,** 355.

LITT M. (1964b) Eosinophils and antigen-antibody reactions. *Ann. N.Y. Acad. Sci.* **116,** 964.

LÖFFLER W. (1932) Zur Differential-Diagnose der Lungeninfiltrierungen. II. Über flüchtige Succedan-Infiltrate (mit Eosinophilie). *Beitr. Klin. Tuberk.* **79,** 368.

LÖFFLER W. (1936) Die flüchtigen Lungeninfiltrate mit Eosinophilie. *Schweiz. med. Wschr.* **17,** 1069.

LONGBOTTOM J.L. (1964) Immunological investigation of *Aspergillus fumigatus* in relation to disease in man. Thesis for the Ph.D., University of London.

LONGBOTTOM J.L. & PEPYS J. (1964) Pulmonary aspergillosis. Diagnostic and immunological significance of antigens and C-substance in *Aspergillus fumigatus*. *J. Path. Bact.* **88,** 141.

LONGBOTTOM J.L., PEPYS J. & TEMPLE-CLIVE F. (1964) Diagnostic precipitin test in *Aspergillus* pulmonary mycetoma. *Lancet* **i,** 588.

LONGCOPE W.T. (1943) Serum sickness and analogous reactions from certain drugs, particularly the sulfonamides. *Medicine (Baltimore)* **22,** 251.

LUNN J.A. (1966) Millworkers' asthma. Allergic responses to the grain weevil (*Sitophilus granarius*). *Brit. J. indust. Med.* **23,** 149.

LUNN J.A. & HUGHES D.T.D. (1966) Pulmonary hypersensitivity to the grain weevil. *Brit. J. indust. Med.* (In print.)

LUZ A.Q. & TODD R.H. (1964) Antigenicity of heated milk proteins. *Amer. J. Dis. Child* **108,** 479.

MAHON W.E., SCOTT D.J., ANSELL G., MANSON G.L. & FRASER R. (1967) Hypersensitivity lung reactions to pituitary snuff. *Thorax* **22,** 13.

MASSOUD A. & TAYLOR G. (1964) Byssinosis: antibody to cotton antigens in normal subjects and in cotton cardroom workers. *Lancet* **ii,** 607.

MAYER P.S., MOSKO M,M., SCHUTZ P.J., OSTERMAN F.A., STEEN L.H. & BAKER L.A. (1953) Penicillin anaphylaxis. *J. Amer. Med. Ass.* **151,** 351.

MELTZER S.J. (1910) Bronchial asthma as a phenomenon of anaphylaxis. *J. Amer. med. Ass.* **55,** 1021.

MINISTRY OF HEALTH REPORT (1965) Enquiry into sudden death in infancy. *Ministry of Health Report on Public Health*, No. 113 London: H.M.S.O.

MORRIS-EVANS W.H. & FOREMAN W.H. (1963) Smallpox handler's lung. *Proc. Roy. Soc. Med.* **56,** 274.

NAYLOR B. (1962) The shedding of the mucosa of the bronchial tree in asthma. *Thorax* **17,** 69.

NELSON T.L. (1964) Spontaneously occurring antibodies to milk in mongoloids. *Amer. J. Dis. Child* **108,** 494.

NOELLP-ESCHENHAGEN I. & NOELLP B. (1954) New contributions to experimental asthma. In *Progress in Allergy*, vol. 4, p. 361, ed. KALLOS P. Basel and New York: S. Karger.

OLIVER-GONZALEZ J. (1960) Histopathological and immunological observations after inoculation of substances isolated from the muscle and cuticle of *Ascaris lumbricoides*. *J. infect. Dis.* **107,** 94.

PALMER C.E. (1945) Nontuberculous pulmonary calcification and sensitivity to histoplasmin. *Publ. Hlth. Rep. Wash.* **60,** 513.

PARISH H.J. & OAKLEY C.L. (1940) Anaphylaxis after injection of tetanus toxoid. Report of a case. *Brit. med. J.* **1,** 294.

PARISH W.E., BARRETT A.M. & COOMBS R.R.A. (1960a) Inhalation of cow's milk by sensitized guinea-pigs in the conscious and anaesthetized state. *Immunology* **3,** 307.

PARISH W.E., BARRETT A.M., COOMBS R.R.A., GUNTHER, MAVIS & CAMPS F.E. (1960) Hypersensitivity to milk and sudden death in infancy. *Lancet* **ii,** 1106.

PARISH W.E. (1961) The response of normal and sensitized animals to products of mouldy hay. *Acta allerg. (Kbh.)* **16,** 78.

PARISH W.E., HALL L.W. & COOMBS R.R.A. (1963) The effect of anaesthesia on anaphylaxis in guinea-pigs. *Immunology* **6,** 462.

PARISH W.E. (1963) Farmer's lung. I. An immunological study of some antigenic components of mouldy foodstuffs. *Thorax* **18,** 83.

PARISH W.E., RICHARDS C.B., FRANCE N.E. & COOMBS R.R.A. (1964b) Further investigations on the hypothesis that some cases of cot death are due to a modified anaphylactic reaction to cow's milk. *Int. Arch. Allergy* **24,** 215.

PARISH W.E. & COOMBS R.R.A. (1968) Peripheral blood eosinophilia in guinea-pigs following implantation of anaphylactic guinea-pig and human lung. *Brit. J. Haemat.* **14,** 421.

PARISH W.E., AKESTER A.R. & GREGG D.McC. (1964) The demonstration of bronchospasm in anaphylaxis by radiography. *Int. Arch. Allergy* **25,** 89.

PARSONS R.J. & ZARAFONETIS C.J.D. (1945) Histoplasmosis in man. Report of seven cases and a review of seventy-one cases. *Arch. Int. Med.* **75,** 1.

PENDERGRASS H.P., TONDREAU R.L., PENDERGRASS E.P., RITCHIE D.J., HILDRETH E.A. & ASKOVITZ S.I. (1958) Reactions associated with intravenous urography: historical and statistical review. *Radiology* **71,** 1.

PEPYS J., FAUX J.A., LONGBOTTOM J.L., MCCARTHY D.S. & HARGREAVE D.E. (1967) Allergic reactions to *Candida albicans* in respiratory disease. (To be published.)

PEPYS J., RIDDELL R.W., CITRON K.M., CLAYTON Y.M. & SHORT E.I. (1959) Clinical and immunologic significance of *Aspergillus fumigatus* in the sputum. *Amer. rev. Resp. Dis.* **80,** 167.

PEPYS J., RIDDELL R.W., CITRON K.M. & CLAYTON Y.M. (1961) Precipitins against extracts of hay and fungi in the serum of patients with farmer's lung. *Acta allerg. (Kbh.)* **16,** 76.

PEPYS J., RIDDELL R.W., CITRON K.M. & CLAYTON Y.M. (1962) Precipitins against extracts of hay and moulds in the serum of patients with farmer's lung, aspergillosis, asthma and sarcoidosis. *Thorax* **17,** 366.

PEPYS J., JENKINS P.A., FESTENSTEIN G.N., GREGORY P.H., LACEY M.E. & SKINNER F.A. (1963) Farmer's lung. Thermophilic actinomycetes as a source of 'farmer's lung hay' antigen. *Lancet* **ii,** 607.

PEPYS J., LONGBOTTOM J.L. & JENKINS P.A. (1964) Vegetable dust pneumoconioses. Immunologic responses to vegetable dusts and their flora. *Amer. rev. Resp. Dis.* **89,** 842.

PEPYS J. & JENKINS P.A. (1965) Precipitin (F.L.H.) test in farmer's lung. *Thorax* **20,** 21.

PEPYS J., JENKINS P.A., LACHMANN P.J. & MAHON W.E. (1966) An iatrogenic autoantibody: immunological responses to 'pituitary snuff' in patients with diabetes insipidus. *Clin. exp. Immunol.* **1,** 377.

PETERSON R.A. & GOOD R.A. (1963) Antibodies to cow's milk proteins. Their presence and significance. *Pediatrics* **31,** 209.

POWELL D.E.B. & GOUGH J. (1959) The effect on experimental silicosis of hypersensitivity induced by horse serum. *Brit. J. exp. Path.* **40,** 40.

PUCKETT T.F. (1953) Pulmonary histoplasmosis. A study of twenty-two cases with identification of *H. capsulatum* in resected lesions. *Amer. Rev. Tuberc.* **67,** 453.

RACKEMANN F.M. & GREENE J.E. (1939) Periarteritis nodosa and asthma. *Trans. Ass. Amer. Phycns.* **54,** 112.

RAFFEL S. (1946) The relationship of acquired resistance, allergy antibodies and tissue reactivities to the components of the tubercle bacillus. *Amer. Rev. Tuberc.* **54,** 564.

RAFFEL S. (1948) The components of the tubercle bacillus responsible for the delayed type of 'infectious' allergy. *J. Infect. Dis.* **82,** 267.

RAFFEL S. (1950) Chemical factors involved in the induction of infectious allergy. *Experientia* **6,** 410.

RAMAZZINI B. (1713) *De Morbus Artificum Diatriba.* Published by University of Chicago Press, Chicago, 1940.

RATNER B. (1953) Temporal and quantitative factors influencing development of experimental asthma in the guinea-pig. *J. Allergy* **24,** 316.

RATNER B., JACKSON H.C. & GRUEHL H.L. (1927) Respiratory anaphylaxis; sensitization; shock; bronchial asthma and death induced in guinea-pigs by nasal inhalation of dry horse dander. *Amer. J. Dis. Child* **34,** 23.

READ J. (1958) The pathogenesis of the Hamman-Rich syndrome. A review from the standpoint of possible allergic etiology. *Amer. Rev. Tuberc.* **78,** 353.

REDMOND A., CARRÉ I.J., BIGGART J.D. & MACKENZIE D.W.R. (1965) Aspergillosis (*A. nidulans*) involving bone. *J. Path. Bact.* **89,** 391.

REED C.E., SOSMAN A. & BARBEE R.A. (1965) Pigeon breeder's lung. A newly observed interstitial pulmonary disease. *J.A.M.A.* **193,** 261.

RÉNON L. (1897) *Étude sur l'aspergillose chez les animaux et chez l'homme.* Paris: Masson et cie

RICH A.R. (1951) *The Pathogenesis of Tuberculosis,* 2nd ed., pp. 369, 377, 490, 575, 721, 729. Oxford: Blackwell Scientific Publications.

ROBERTS A.N. (1966) Rapid uptake of tritiated antigen by mouse eosinophils. *Nature (Lond.)* **210,** 266.

ROSA L.M. & MCDOWALL R.J.S. (1951) The action of local hormones on the isolated human bronchus. *Acta allerg. (Kbh.)* **4,** 293.

ROSE G.A. & SPENCER J. (1957) Polyarteritis nodosa. *Quart. J. Med.* N.S. **26,** 43.

SABESIN S.M. (1963) A function of the eosinophil; phagocytosis of antigen-antibody complexes. *Proc. Soc. exp. Biol. (N.Y.),* **112,** 667.

SADUM E.H., NORMAN LOIS & ALLAIN DOROTHY (1957) The detection of antibodies to infections with the nematode, *Toxocara canis,* a causative agent of visceral larva migrans. *Amer. J. trop. Med. Hyg.* **6,** 562.

SALVAGGIO J.E., BUECHNER H.A., SEABURY J.H. & ARQUEMBOURG P.C. (1966) Bagassosis. I. Precipitins against extracts of crude bagasse in the serum of patients with bagassosis. *Ann. int. Med.* **64,** 748.

SALVIN S.B. & FURCOLOW M.L. (1954) Precipitins in human histoplasmosis. *J. Lab. clin. Med* **43,** 259.

SANDS J.H., PALMER P.P., MAYCOCK R.L. & GREGER W.P. (1955) Evidence for serologic hyper-reactivity in sarcoidosis. *Amer. J. Med.* **19,** 401.

SAPERSTEIN S., ANDERSON D.W., GOLDMAN A.S. & KNIKER W.T. (1963) Milk allergy. III. Immunological studies with sera from normal and allergic children. *Pediatrics* **32,** 580.

SCHATTENBERG H.J. & FLINN M. (1939) Experimental studies on bronchomoniliasis. *Proc. Soc. exp. Biol. (N.Y.)* **41,** 557.

SCHENKEN J.R., TAMISIEA J. & WINTER F.D. (1953) Hypersensitivity to bee and wasp sting. Report of a fatal case and review of literature. *Amer. J. clin. Path.* **23,** 1216.

SCHILD H.O., HAWKINS D.F., MONGAR J.L. & HERXHEIMER H. (1951) Reactions of the isolated human asthmatic lung and bronchial tissue to a specific antigen. *Lancet* **ii,** 376.

SCHLOSS O.M. (1924–25) The intestinal absorption of antigenic protein. *Harvey Lectures, Baltimore,* Series XX, 156.

SCHWARTZ M. (1952) Heredity in asthma. *Acta allerg.* (*Kbh.*) Suppl. II.

SERAFINI U., TORRIGIANI G. & MASALA C. (1965) Organ specific autoantibodies in reagin containing sera from allergic patients. *Lancet* **ii,** 821.

SHARP E.A. & BLAKE F.G. (1930) Host factors in the pathogenesis of pneumococcus pneumonia. I. The reaction of the lungs to pneumococcus autolysate in sensitized rabbits. *J. exp. Med.* **52,** 501.

SHRAND H. (1964) Visceral larva migrans. *Toxacara canis* infection. *Lancet* **i,** 1357.

SINGH M.M., STOKES J.F., DRURY R.A.B. & WALSHE J.M. (1958) The natural history of malignant granuloma of the nose. *Lancet* **i,** 401.

SMITH D.T. (1947) *Fungus Diseases of the Lungs.* Springfield, Illinois: Charles C Thomas.

SPEIRS R.S. & SPEIRS E.E. (1963) Cellular localization of radioactive antigen in immunized and non-immunized mice. *J. Immunol.* **90,** 561.

STEWART I. (1957) Sudden natural death in infants. *Med. Offr.* **98,** 139.

STRAUB M. & SCHWARZ J. (1955) The heated primary complex in histoplasmosis. *Amer. J. clin. Path.* **25,** 727.

SUTHERLAND I., MITCHELL D.N. & HART P.D'ARCY (1965) Incidence of intrathoracic sarcoidosis among young adults participating in a trial of tuberculosis vaccines. *Brit. med. J.* **ii,** 497.

SUTLIFF W.D. & FINLAND M. (1932) Antipneumococcic immunity reactions in individuals of different ages. *J. exp. Med.* **55,** 837.

SYMPOSIUM. Farmer's lung (1965) *N.Y. State J. Med.* **65,** 3013.

TALLERMAN K.H. (1934) Sensitivity to cow's milk proteins in acute gastro-enteritis. *Arch. Dis. Child* **9,** 189.

TEN CATE H.J. (1961) Provocation tests with inhalants and exposure tests with foods. In *Test Procedures Used in the Examination of the Allergic Patient,* p. 101. Proc. Eur. Acad. Allergy. Leiden: H.E.Kroese N.V.

THOMSON J.G. (1945) Fatal bronchial asthma showing the asthmatic reaction in an ovarian teratoma. *J. Path. Bact.* **57,** 213.

TIFFENAU R. (1958) Analyse, mesure et rôle des facteurs régissant les perturbations fonctionelles pulmonaires et de l'asthmatique. *III*[eme] *Congres Int. d'allergologie,* p. 55. Editions Medicales Flammarion.

TRASOFF A. & SCARF M. (1940) Periarteritis nodosa and asthma. Case report. *J. allergy* **11,** 277.

TUCKER H.A. (1951) Histoplasmin sensitivity in the canal zone. A correlated clinicopathologic study of one thousand patients with speculations as to the present status of *Histoplasma capsulatum* on the Isthmus of Panama. *Arch. Derm. Syph.* (*N.Y.*) **64,** 713.

TURNER-WARWICK M. & DONIACH D. (1965) Auto-antibody studies in interstitial pulmonary fibrosis. *Brit. Med. J.* **1,** 886.

BACH F., SONES M. & ISRAEL H.L. (1952) Passive transfer of tuberculin sensitivity to patients with sarcoidosis. *New Engl. J. Med.* **247,** 794.

VANCE B.M. & STRASSMAN G. (1942) Sudden death following injection of foreign protein. *Arch. Path.* **34,** 849.

VAN ORDSTRAND H.S., HUGHES R., DE NARDI J.M. & CARMODY M.G. (1945) Beryllium poisoning. *J. Amer. med. Ass.* **129,** 1084.

VAUGHAN W.T. & PIPES D.M. (1936) On the probable frequency of allergic shock. *Amer. J. Digest. Dis.* **3,** 558.

VIGLIANI E.C. & PERNIS B. (1958) Immunological factors in the pathogenesis of the hyaline tissue of silicosis. *Brit. J. industr. Med.* **15,** 8.

WAGNER V., TOMSIKOVA A., SACH J., JANKOVA J. & NOVACKOVA D. (1965) Autoimmune mechanism in disease of the respiratory tract. *Acta allerg. (Kbh.)* **21,** 1.

WALTON E.W. (1958) Giant-cell granuloma of the respiratory tract (Wegener's granulomatosis). *Brit. med. J.* **ii,** 265.

WEDGWOOD R.J. & BENDITT E.P. (1965) *Sudden Death in Infants.* U.S. Department of Health, Public Health Service Publication No. 1412, Washington.

WEGELIN C. (1948) Anatomische befunde bei todlichem bienen und Wespenstich. *Schweiz. Med. Wochensch.* **78,** 1253.

WEGENER F. (1939) Über eine eigenartige rhinogene granulomatose mit besonderer Beteiligung des Arterian systems und der Nieren. *Beitr. path. Anat.* **102,** 36.

WENZEL F.J., EMANUEL D.A., LAWTON B.R. & MAGNIN G.E. (1964) Isolation of the causative agent of farmer's lung. *Ann. Allergy* **22,** 533.

WEILL H., BUECHNER H.A., GONZALEZ E., HERBERT S.J., AUCOIN E. & ZISKIND M.M (1966) Bagassosis. A study of pulmonary function in twenty cases. *Ann. int. Med.* **64,** 737.

WILLIAMS J.V. (1963) Inhalation and skin tests with extracts of hay and fungi in patients with farmer's lung. *Thorax* **18,** 182.

WILSON J.F., HEINER D.C. & LAHEY M.E. (1964) Milk-induced gastrointestinal bleeding in infants with hypochromic microcytic anaemia. *J.A.M.A.* **189,** 122.

WILSON K.S. & ALEXANDER H.L. (1945) The relation of polyarteritis nodosa to bronchial asthma and other forms of human hypersensitiveness. *J. Lab. clin. Med.* **30,** 195.

WOODRUFF A.W., BISSERU B. & BOWE J.C. (1966) Infection with animal helminths as a factor causing poliomyelitis and epilepsy. *Brit. Med. J.* **i,** 1576.

ZETTERGREN L. (1950) Thresher's lung (pulmonary moniliasis). An experimental investigation. *Acta Soc. Med. upsalien* **55,** 257.

CHAPTER 37

MUSCLE IN ALLERGIC DISEASE: MYASTHENIA GRAVIS

JAMES HOUSLEY

INTRODUCTION

Myasthenia gravis is a disease in which excessive weakness of voluntary muscles develops during prolonged or repeated use, apparently as a result of impairment of neuromuscular transmission (Osserman 1958). The aetiology of the disease is unknown but the occurrence of transient myasthenia in a proportion of infants born of affected mothers (Teng & Osserman 1956) suggests the presence of a circulating inhibitor of neuromuscular transmission which is transferred across the placenta. Exacerbations of symptoms during pregnancy and the puerperium and in relation to menstruation (Viets, Schwab & Brazier 1942; Fraser & Turner 1953; Keynes 1954), and the reports of hormonal abnormalities (Osserman 1958; Schrire 1959; Grashenkov & Perelman 1966) point to significant endocrine influences on myasthenia gravis but no clear evidence of an underlying endocrine disturbance has emerged so far. There is no convincing evidence, either, for hereditary factors, except perhaps in the uncommon juvenile cases, as distinct from transient neonatal cases (Teng & Osserman 1956). During recent years there have been numerous reports of immunological abnormalities in myasthenia

gravis, indicating that immunological factors might be involved in the pathogenesis of the disease. In this review, the relevant clinical, pathological and physiological features are described first, followed by an account of the immunological abnormalities and finally a summary of the attempts which have been made to fit these observations into theories of pathogenesis.

CLINICAL FEATURES

Diseases of autoallergic aetiology might be expected to share clinical features in common and it is noteworthy that myasthenia gravis resembles systemic lupus erythematosus with respect to its high incidence in young women, variable clinical course and precipitating factors (Harvey *et al* 1954; Osserman 1958; Simpson 1960). Similarly there is likely to be a significantly frequent association of two or more auto-allergic diseases in the same patient and in Simpson's (1960, 1964) series of 491 myasthenics there was a 17% incidence of thyroid disorders (a group of diseases in which auto-antibodies are frequently present, see chapter 35) and a 3.6% incidence of rheumatoid-like arthritis.

However, although at least fifteen cases of associated myasthenia gravis and systemic lupus erythematosus have been reported (Wolf & Burrows 1966), the frequency of the association in the largest myasthenia series reported has always been less than 1%, with the exception of the series of Downes, Greenwood & Wray (1966) who found two cases among their seventy-four patients (2.7%).

On the other hand the high incidence of thyroid diseases reported by Simpson is a fairly consistent finding; the figures in other series vary between 3% and 13% (Osserman 1958; Sahay, Blendis & Greene 1965; Downes, Greenwood & Wray 1966; Wolf *et al* 1966) and there are numerous case reports (Osserman 1958). The frequency of thyroid disease in the general population is not accurately established but it is probably less than 3% (Logan & Cushion 1958) so there seems little doubt that it is significantly more common in myasthenia gravis. When thyroid disease is present it usually takes the form of thyrotoxicosis. The number of reported cases of associated Hashimoto's disease or hypothyroidism and myasthenia gravis is small (Simpson 1964; Daly & Jackson 1964; Sahay, Blendis & Greene 1965) but post-mortem studies have shown a high incidence of histological evidence of thyroiditis (Becker *et al* 1964; Wolf *et al* 1966) suggesting that milder forms of Hashimoto's disease may not be uncommon.

The evidence for an association with rheumatoid arthritis is less convincing. There were three cases (6.2%) among the forty-eight myasthenic patients studied by Adner *et al* (1964) but in larger series the incidence has been less than 4%, which is probably no higher than would be expected by change alone (Lawrence 1963). See Table 37.1 for reported frequency of diseases possibly associated with myasthenia gravis.

Klein *et al* (1964) have reported two patients with polymyositis and thymomas who subsequently developed myasthenia gravis after resection of the thymic tumour. On the basis of these two cases and the published reports of associated polymyositis and thymoma Klein and his colleagues suggest that myasthenia gravis and polymyositis may be in some way related, especially in the presence of

TABLE 37.1

(*a*) Diseases possibly associated with myasthenia gravis

Series	No. of patients	Systemic lupus erythematosus No.	%	Thyroid diseases No.	%	Rheumatoid arthritis No.	%
Osserman 1958	325	0	0	11	3.3	4	1.2
Simpson 1964	491	1	0.2	87	17	18	3.6
Adner *et al* 1964	48	0	0	2	4.1	3	6.2
Downes, Greenwood & Wray 1966	74	2	2.7	13	13	2	2.7
Wolf *et al* 1966	399	2	0.5	22	5.5	3	0.75
Sahay, Blendis & Green 1965	260	—	—	8	3.0	—	—

(*b*) *Post-mortem studies*

Series	No. of patients	Thyroiditis No.	%
Becker *et al* 1964	32	6	18
Wolf *et al* 1966	34	4	12

a thymoma. The greater extent and severity of skeletal and cardiac muscle lesions in myasthenics with thymomas (see below) may be significant in this context.

PATHOLOGY

A striking feature of myasthenia gravis is the extremely high incidence of abnormalities of the thymus (Castleman 1955). In about 70% of all patients the medulla shows lymphoid hyperplasia in the form of germinal follicles resembling those found in other lymphoid tissues when reacting to antigenic stimulation; but in myasthenia there is no generalized lymphoid hyperplasia throughout the rest of the body. Abnormal medullary collections of plasma cells may also be present (Goldstein 1966a). However, neither plasma cells nor lymphoid hyperplasia are specific for myasthenia gravis since plasma cells are also found in the

thymus in septicaemia and systemic lupus erythematosus (Goldstein 1966a) and Sloan (1943) has described lymphoid hyperplasia with germinal follicle formation in autopsies on cases of sudden death; changes described by Sloan as similar to those found in myasthenia were also present in cases of Addison's disease and hyperthyroidism but germinal follicles were not mentioned.

In a further 10–15% of patients thymomas are present; these patients are commonly older males and the prognosis is poor (Wilkins, Edmunds & Castleman 1966). The microscopic appearance of the tumour is usually of varying proportions of lymphocytes and plump, ovoid epithelial cells, in contrast to the spindle-shaped epithelial cells usually found in the thymomas of non-myasthenics. In addition to the relationship to myasthenia, an interesting feature of thymomas is their low degree of malignancy; although local infiltration, implantation on serous membranes within the thorax and recurrence after resection are not uncommon, blood-borne metastases rarely occur (Mendelow 1958). It has even been suggested that thymomas in cases of myasthenia gravis are not true neoplasms but merely extreme forms of epithelial cell hyperplasia (Norris 1936).

Histological examination of skeletal muscle (Russell 1963) reveals three types of abnormality: (1) acute, coagulative necrosis of muscle fibres with inflammatory cellular reaction, (2) collections of lymphocytes and mononuclear cells known as lymphorrhages (Buzzard 1905) and (3) areas of simple atrophy affecting single fibres or groups of fibres. These changes are related to some extent to the degree and distribution of muscle weakness and are more severe in patients with thymomas. Lymphorrhages and areas of acute necrosis may also be found in the myocardium, again especially in patients with thymomas (Genkins *et al* 1961). However, as with the thymic changes, the skeletal muscle abnormalities are not specific for myasthenia gravis, similar lesions having also been described in Addison's disease, rheumatoid arthritis, thyrotoxicosis and other conditions (Clawson, Noble & Lufkin 1947; Cruikshank 1952; Mendelow 1958).

Marked abnormalities of terminal motor nerve fibres and motor endplates have been found (Bickerstaff & Woolf 1960; MacDermott 1960) using supravital staining and cholinesterase staining techniques. The motor endplates in myasthenics are frequently elongated, variable in size and multiple, and these changes, being entirely different to those found in motor endplates in degenerative muscle diseases, suggest either a primary developmental abnormality ('dysplasia') or a compensatory expansion of the synaptic region of the muscle fibre as a consequence of impairment of neuromuscular transmission. Swollen, irregular terminal nerve fibres showing abnormal branching and bizarre terminal expansions are also found but they probably represent a non-specific response to muscle inflammation as they are most marked in the vicinity of lymphorrhages and similar changes are found in other muscle diseases (Woolf 1966).

There are conflicting reports on the electron microscopic appearances of the motor endplate region. Zacks, Bauer & Blumberg (1962) have described extensive disorganization of the endplates with degenerative changes in the terminal nerve fibres, but others (Bickerstaff, Evans & Woolf 1960; Woolf 1966) have found merely poor development of the junctional folds of the motor endplates and other minimal changes.

NEUROMUSCULAR TRANSMISSION

It seems unlikely that the impairment of neuromuscular transmission in myasthenia gravis is due solely to the observed pathological changes in nerve and muscle fibres since the weakness is so readily reversible by anticholinesterase drugs (Remen 1932; Walker 1934). It has long been suggested that the impairment of transmission is produced by a circulating curare-like substance blocking the action of acetylcholine at the motor endplates. Efforts to detect a neuromuscular blocking agent in thymus gland extracts have met with little success (McArdle 1965) but successful results have been claimed using myasthenic serum or plasma (Wilson & Stoner 1944; Strüppler 1955). Other workers have failed to confirm these results and doubts have been cast on their validity (Bergh 1953; Nastuk, Strauss & Osserman 1959). The phenomenon of transient neonatal myasthenia is of more significance and it is interesting that the weakness in infants lasts up to 2 months, which would be the order of time required to catabolize maternal IgG antibodies. Moreover, Parkes & McKinna (1966) have recently produced further evidence for a neuromuscular inhibitor in the serum of myasthenics and have tentatively located it in the crude globulin fraction. However, there is so far no direct evidence of any immunological processes involving the neuromuscular junction (see below), and detailed speculation on the role of immunological factors in the production of the neuromuscular block is necessarily extremely tentative.

Similarities in the behaviour of muscles of myasthenics and newborn infants in response to pharmacological agents and nerve stimulation have prompted the alternative suggestion that the basic defect may be a state of arrested development of the motor endplate at a foetal or neonatal stage, the symptoms being precipitated by unknown factors in later years (Churchill-Davidson & Wise 1963). The extensive investigations of Grob and his colleagues (see Grob & Johns 1961; Grob, Namba & Feldman 1966) also suggest a post-synaptic defect, involving both endplate abnormalities and abnormal acetylcholine breakdown. Other experimental evidence points to presynaptic lesions, namely failure to synthesise adequate amounts of acetylcholine (Desmedt 1966) or diminished amounts of acetylcholine in the individual packets ('quanta') in which it is released from the terminal nerve fibres (Elmqvist *et al* 1964).

IMMUNOLOGICAL ABNORMALITIES

Serum Complement

During experiments to detect neuromuscular blocking agents in myasthenic serum using frog nerve-muscle preparations, Nastuk, Strauss & Osserman (1959) found a reduction in muscle contraction with some sera but noticed that it was associated with lysis of surface muscle fibres. Further experiments (Nastuk & Strauss 1961) suggested that serum complement was involved and investigation of serum complement levels in sixty-eight myasthenics (Nastuk, Plescia & Osserman 1960) revealed that fifty-four had values at some time outside the normal range (thirty-four patients had serial determinations). Furthermore, in the forty-six patients in whom complement levels and clinical status could be adequately compared the levels tended to be low during exacerbations of symptoms and to rise to normal or greater than normal during remissions. There was no evidence that anticholinesterase therapy had affected serum complement levels and *in vitro* tests showed no effect by anticholinesterases on the complement measuring system (haemolytic). On this basis it was suggested that an autoallergic reaction resulting in the consumption of complement might play a part in the pathogenesis of myasthenia gravis. The actual mechanism producing the observed alterations in serum complement levels is, however, still unknown. Further detailed analysis (Plescia, Segovia & Strampp 1966) indicates depression of the complement components C'_2 and C'_4 and also the presence of inhibitors of these same components but it is not clear whether these phenomena are causally related or whether both could result from the *in vivo* formation of antigen-antibody complexes.

Muscle and Thymus Antibodies

Further evidence of immunological abnormalities soon followed. Strauss *et al* (1960) using the direct fluorescent antibody technique, detected a component in the crude globulin fraction of pooled myasthenic sera which bound to alternate striations of normal and human myasthenic skeletal muscle and rat skeletal muscle, see Plates 37.1 and 37.2. Using a more complicated fluorescent antibody technique, the globulin component was also shown to fix complement and, applying this latter method to individual sera, thirteen of thirty-one myasthenics but only one of sixteen controls were found to have this muscle-binding, complement-fixing property.

Further studies on the reactions between myasthenic sera and muscle were reported by Beutner *et al* (1962). Employing direct, indirect and complement-fixing fluorescent antibody techniques and conventional complement fixation tests against human and monkey skeletal and heart muscle they found strongly positive reactions with two of ten myasthenic sera but with none of their control sera. The positive sera did not react with smooth muscle, liver or pancreas and

PLATES 37.1 AND 37.2. Photomicrographs of rat skeletal muscle sections stained by the indirect fluorescent antibody method using myasthenic sera containing muscle antibodies. ×400.
PLATE 37.1. Staining of edges of A-bands.

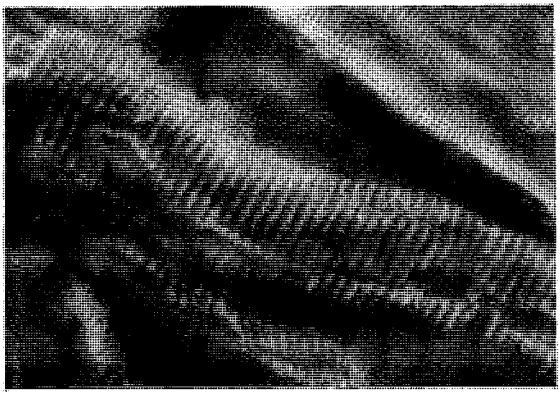


PLATE 37.2. Unsplit fluorescent bands—I-band type of staining. ×300.

facing p. 1002

the reactivity with skeletal and heart muscle was shown to be a property of the γ-globulin. This last observation, together with the organ and disease specificity indicated that the reaction was due to the presence of antibodies. It was also observed that although the myasthenic sera stained both skeletal and heart muscle in the direct and indirect fluorescent antibody tests, the tests involving complement fixation gave positive reactions with skeletal muscle only. On this evidence and the results of absorption studies with skeletal and heart muscle (Beutner, Witebsky & Leff 1963) it was suggested that *two* muscle antibodies were present in the myasthenic sera, one, the 'S' antibody, reacting with skeletal muscle alone and capable of fixing complement, and the other, the 'SH' antibody, reacting with skeletal and heart muscle, but non-complement fixing. Beutner and his colleagues have subsequently found the 'SH' antibody in 25% of myasthenic sera but the 'S' antibody in only 5% and then only in the presence of high 'SH' titres (Beutner, Witebsky & Djanian 1965). Reactions between muscle and serum from the same patient have demonstrated that these are auto-antibodies.

Antibodies to muscle may also be detected by the tanned red cell haemagglutination method, using an ammonium sulphate precipitated fraction of a physiological saline extract of skeletal muscle as the coating antigen (Djanian, Beutner & Witebsky 1964).

Shulman *et al* (1966) have reported precipitation reactions in agar and in a capillary tube fluid system between myasthenic sera and extracts of human and monkey skeletal muscle. Positive reactions were observed with three of ten sera known to have high fluorescent antibody and tanned cell haemagglutination muscle antibody titres. The three positive sera also fixed complement with muscle extracts and the precipitation reactions were thought to be due to 'S' antibodies. Considerable technical difficulties were encountered due to the instability of the muscle extracts.

The important discovery of an antigenic relationship between skeletal muscle and thymus was first reported by van der Geld and his colleagues (van der Geld *et al* 1963; van der Geld, Feltkamp & Oosterhuis 1964). Studies using the indirect fluorescent antibody technique showed that of ninety myasthenic sera, thirty-six contained antibodies to rat skeletal muscle striations and that all of these sera cross-reacted with the cytoplasm of calf thymus epithelial cells. Absorpton of the sera with skeletal muscle abolished reactivity with both tissues but absorption with thymus did not completely remove reactivity with either, possibly due to paucity of thymic epithelial cell antigen.

Further extensive and careful studies with the indirect fluorescent antibody technique by Strauss *et al* (1965) have elucidated the incidence, specificity and concurrence of antibodies to skeletal muscle striations and thymic epithelial cells cytoplasm in myasthenic patients and controls. With their technique and materials some reactivity for skeletal muscle was found in all sera (including normal sera), sometimes up to a dilution of 1 in 30; therefore a titre of 1 in 60 or

greater was interpreted as a positive result for muscle or thymus. Using these criteria, antibodies to *both* tissues were found in ninety-nine of 336 myasthenic sera (twenty-seven had muscle antibody titres of 1 in 1920 or greater) and none of the sera reacted with muscle or thymus alone. Complete cross-reactivity with heart muscle (bovine) was observed, thus agreeing with Beutner, Witebsky & Djanian (1965), but, using myasthenic sera with high muscle and thymus antibody titres, no cross-reactivity has been detected with epithelial cells in tissues other than the thymus, except for occasional reactions in low titres with human thyroid epithelium and also occasional low-titre reactions with human thyroid colloid, bovine pancreas islet cells and bovine adrenal zona glomerulosa cells (Strauss *et al* 1966). Muscle and thymus antibodies show wide species cross-reactivity with the tissues of other mammals and lower vertebrates (Beutner, Witebsky & Djanian 1965; Strauss, Kemp & Douglas 1966a).

Using the fluorescent antibody technique, the overall incidence of muscle and thymus antibodies in non-myasthenic sera is extremely low. In the series of Strauss *et al* (1965) only one of 129 normal sera and only one of 674 sera from patients with other diseases (including connective tissue diseases and various muscle diseases) were positive. The latter positive serum was from a patient with a thymoma and aplastic anaemia and prompted investigation of further patients with thymomas, or histories thereof, but not suffering from myasthenia gravis. Of fifty-one such patients, twelve (24%) had concurrent muscle and thymus antibodies (Strauss *et al* 1966); six of these scropositive patients were studied intensively for evidence of subclinical myasthenia gravis but none was found (McFarlin, Barlow & Strauss 1966). The possible significance of muscle and thymus antibodies in non-myasthenic thymoma patients will be discussed subsequently.

The results in the main myasthenic series investigated for muscle and thymus antibodies are set out in Table 37.2.

In a small number of myasthenics (and one non-myasthenic thymoma patient) apparent *in vivo* binding of γ-globulin to muscle striations and/or sarcolemma has been detected using the direct fluorescent antibody technique with biopsy or post-mortem tissues (Beutner, Witebsky & Leff 1963; Gordon, Hess & Frederick 1965; Beutner *et al* 1966; Namba & Grob 1966). It is by no means certain, however, that this represents a specific antigen-antibody reaction at these sites.

Muscle and thymus antibodies are certainly found in the IgG class (Strauss *et al* 1965) but no detailed studies of their distribution among the immunoglobulin classes have been made apart from a series of six myasthenic patients shown to have exclusively IgG antibodies by Oosterhuis, Feltkamp & van der Geld (1966).

Myoid Cells

The cross-antigenicity displayed by thymic epithelial cells and striated muscle fibres is probably explained by the so-called myoid cells in the thymus (Barg-

TABLE 37.2

Antibodies to muscle and thymus in myasthenia gravis

(*a*) Indirect fluorescent antibody method*

Series	No. of patients	Muscle antibodies No.	%	
Strauss *et al* 1960	30	13	43	Complement-fixing technique
Beutner *et al* 1962	10	2	20	
Adner *et al* 1964	48	11	23	
Vetters 1965	68	—	16	'A-band' staining only
Beutner, Witebsky & Djanian 1965	80	20	25	'SH' antibody
		4	5	'S' antibody
Osserman & Weiner 1965	256	77	30	
Namba & Grob 1966	60	37	62	
Downes, Greenwood & Wray 1966	69	36	53	
		Concurrent muscle and thymus antibodies No.	%	
van der Geld, Feltkamp & Oosterhuis 1964	90	36	40	
Strauss *et al.* 1965	336	99	29	

(*b*) Tanned cell haemagglutination method

Series	No. of patients	Muscle antibodies No.	%
Djanian, Beutner & Witebsky 1964	45	10	22

* Lowest titre regarded as positive varies in different series, depending on results with control sera.

mann 1943). These have the features of striated muscle cells both on light microscopy and on electron microscopy and have been described in humans and many other species (van der Velde & Friedman 1966; Henry 1966; Feltkamp-Vroom 1966). Myasthenic sera containing muscle and thymus antibodies have been shown to react with the myoid cells of turtle thymus (Strauss, Kemp & Douglas

1966b) and reaction with myoid cells in adult human thymus has also been described (Feltkamp-Vroom 1966). It seems very likely, therefore, that the 'epithelial' cells of mammalian thymus which react with myasthenic serum are in fact morphologically altered striated muscle cells which have retained antigenic determinants reacting with antibodies to muscle.

Localization of Antibodies in Muscle

Strauss, Deitch & Hsu (1961) compared fluorescent antibody views with polarized light and phase contrast views of identical muscle sections and located the fluorescence in the lateral parts of the A-bands of the muscle sarcomere; a narrow central area, corresponding to the H-zones, remained unstained (see Fig. 37.1 and Plate 37.1). Using isolated myofibrils, Nastuk *et al* (1966) noted that some myasthenic sera in addition to the lateral A-band staining also produced even brighter staining of the Z-lines between the individual sarcomeres. In extremely contracted myofibrils, when the I-bands became very narrow, the fluorescent lateral parts of adjacent A-bands sometimes appeared to fuse with an intervening stained Z-line producing a single fluorescent band which could be mistaken for an I-band. Normal sera produced similar patterns but the staining was much weaker than that produced by many myasthenic sera.

On the other hand, Vetters (1965) considers that although a few myasthenic sera (16% of his series of sixty-eight) strongly stain the lateral parts of the A-band, the majority do in fact stain the I-band, although relatively weakly. The appearance is as shown in Plate 37.2. Furthermore, he reported that the weak striational staining produced by the majority of normal sera was also of the I-band type. In addition, Strauss (1967) has now also found that many myasthenic sera stain the I-band.*

Thus, although there is no doubt that a proportion of myasthenic sera contain antibodies reacting with muscle striations as shown by the disease and organ specificity at high serum dilutions using the fluorescent antibody technique, further work is needed to elucidate the staining patterns produced by myasthenic and normal sera and to establish their significance. (Beutner and his colleagues did not identify the striations with which the 'S' and 'SH' antibodies reacted.)

The reactions of the muscle antibodies with the striations, whether A-band or I-band, contrasts with the physiological and pharmacological evidence of a defect at the neuromuscular junction but it is possible, of course, that striations and motor endplate regions could share a common antigen against which the antibodies are directed. However, careful studies with myasthenic sera with antibodies to muscle and thymus (using the fluorescent antibody technique together with histochemical staining of cholinesterase on the same section in

*Strauss now states that *all* reactive sera (including normal sera at high concentrations) in fact stain I-bands predominantly (Straus and Kemp 1967),

order to identify the motor endplates) have failed to detect any antibody localization at the neuromuscular junction (McFarlin, Engel & Strauss 1966).

Clinical Relationships of Muscle and Thymus Antibodies

In a series of 256 myasthenic patients investigated by Osserman & Weiner (1965a), the incidence of muscle antibodies, detected by the fluorescent antibody method, was highest in patients with more severe disease and in those whose disease was of recent onset. The same authors (1965b) investigated serial serum specimens from 117 patients over periods ranging up to 9 years (average 1 year) for possible relationships between the presence of muscle antibodies and clinical progress. Twenty-six patients (22%) displayed reversibility of antibody findings, changing from positive to negative or vice versa, and in twenty-one of these the changes correlated well with alterations in clinical status; positive results tended to disappear with improvement and negative sera tended to become positive during exacerbation. But of thirty patients with muscle and thymus antibodies submitted to detailed quantitative studies of antibody titres and clinical status only fifteen revealed the expected correlation, with titres rising during exacerbations and falling during remissions (Weiner & Ossermann 1966).

An interesting finding has been that almost all myasthenic patients with thymomas have antibodies to muscle and thymus (van der Geld, Feltkamp & Oosterhuis 1964; Strauss 1966). The failure of Hoffbrand (1966), using the fluorescent antibody technique, to detect muscle antibodies in a series of three myasthenics with thymomas is difficult to evaluate as he did not include a positive control serum to demonstrate that his methods were adequate. It seems then that the absence of muscle and thymus antibodies in a case of myasthenia gravis is a useful indication of the absence of a thymoma.

A further curious feature of the relationship between myasthenia gravis, thymomas and antibodies to muscle and thymus is that the average age at detection of the thymoma in patients *with* myasthenia (this group are almost all seropositive as mentioned above) is in the fourth decade, while the corresponding age of seropositive thymoma patients *without* myasthenia is significantly higher, in the sixth decade (Strauss *et al* 1966). While this may merely reflect earlier radiological studies in the myasthenic group, leading to detection of the tumour at an earlier stage, it could also be that the age at which a thymoma develops is of significance in the possible development of myasthenia gravis.

There is no evidence that neonatal myasthenia bears any relationship to the placental transfer of muscle antibodies from the mother. Cases have been described both of neonatal myasthenia without detectable serum antibodies and of infants with muscle antibodies derived from the mother but not suffering from myasthenia (Stern, Hall & Robinson 1964; Oosterhuis, Feltkamp & van der Geld 1966; Namba & Grob 1966; Strauss 1967).

Nature of Muscle Antigen(s)

The localization of muscle antibodies to the A-bands in some myasthenic sera indicates that the antigen against which they are directed could be the 'contractile' protein myosin, which is confined to this region (Hanson & Huxley 1953), see Fig. 37.1. Moreover, experimental fluorescent antibody studies of myosin distribution in skeletal muscle fibres show a similar pattern of A-band staining (Marshall *et al* 1959).* However, until the various myasthenic and normal serum staining patterns have been worked out it is impossible to speculate further on this sort of evidence.

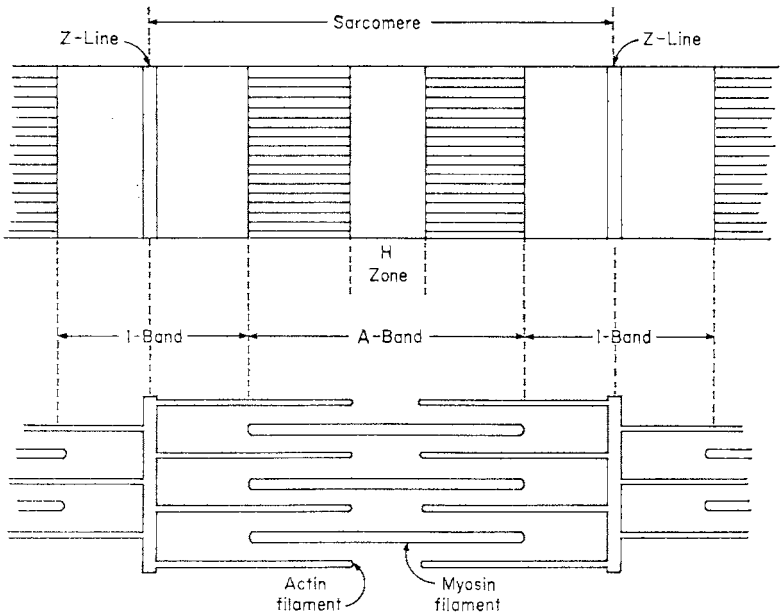


FIG. 37.1. Diagram of light microscope appearance and ultrastructure of striated muscle myofibril

Another possible means of identifying the muscle antigen(s) is to locate the binding sites of the antibodies on electron microscopy using seropositive myasthenic globulin labelled with an electron-dense marker. Attempts to do this using a ferritin label have so far been unsuccessful owing to non-specific binding of ferritin-conjugated serum proteins to muscle structures (Gottlieb *et al* 1966).

The only other evidence on the nature of the muscle antigen is conflicting. Ricken (1966) tested myasthenic sera against preparations of myosin, actomyosin and actin from human skeletal muscle in agar precipitation and complement

*Reports of I-band staining (see page 1006), on the other hand, suggest antibodies to actin or trypomyosin.

fixation tests and reported reactions with myosin, and to a lesser degree with actomyosin, but not with actin. Other work, in which muscle antibodies in myasthenic sera were absorbed out with preparations of muscle proteins, indicates that myosin is not the antigen against which the antibodies are directed (Hale & Beutner 1965) and this is supported by the failure of myasthenic globulin to inhibit the ATPase activity of myosin (Nastuk *et al* 1966). Furthermore, Namba & Grob (1966) have isolated from normal human skeletal muscle a ribonucleoprotein which may be involved in neuromuscular transmission, and they suggest, mainly on the evidence of complement fixation tests and absorption tests on myasthenic sera, that this is the antigen corresponding to myasthenic muscle antibodies.

Other Antibodies

Multiple auto-antibody production is a feature of diseases of possible auto-allergic aetiology (Doniach & Roitt 1962) and the same feature appears to be

Table 37.3

Antibodies to tissues other than muscle and thymus in myasthenia gravis

Series	No. of patients	Antinuclear factor		Thyroid		Rheumatoid factor		Gastric	
		No.	%	No.	%	No.	%	No.	%
White & Marshall 1962	16	6	37	—		—		—	
van der Geld *et al* 1963	111	11	10	36	32	5	4.5	—	
Sturgill *et al* 1964	44	15	37	17	35	3	6	—	
Downes, Greenwood & Wray 1966	74	22	30	31	42	—	9		12*

	No. abnormal/No. studied			
	Antinuclear factor	Thyroid	Rheumatoid factor	Gastric
Simpson 1964	8/40 (20%)	10/39 (25%)	—	31/38 (8%)
Wolf *et al* 1966	4/35 (11%)	4/26 (15%)	6/58 (10%)	

* Not significantly higher than in controls.

true of myasthenia gravis. In addition to antibodies to muscle and thymus there is a high incidence of antinuclear factor, variously reported at between 10% and 37%, and between 15% and 42% of patients have been found to have antibodies

to thyroid (White & Marshall 1962; van der Geld *et al* 1963; Sturgill *et al* 1964; Simpson 1964; Wolf *et al* 1966; Downes, Greenwood & Wray 1966). The incidence of rheumatoid factor is much lower, the highest reported being 10% by Wolf *et al* (1966), and it is doubtful whether the incidence of gastric parietal cell antibodies is any higher than normal (Simpson 1964; Downes, Greenwood & Wray 1966). See Table 37.3 for details of the main series.

Thymectomy, Thymic Pathology and Immune Response

The place of thymectomy in the treatment of myasthenia gravis has long been a source of controversy (see Osserman 1958) but it now seems adequately established that the operation is of value in one group of patients at least, namely women with severe disease, without thymomas and less than 40 years old (Perlo *et al* 1966). The most marked improvement seems to occur in patients with glands showing florid hyperplastic changes (Smith 1966). In men and in older women the beneficial effects of thymectomy are less certain and in patients with thymomas the prognosis for the myasthenia does not seem to be improved by removal of the tumour.

There is evidence that in patients with hyperplastic glands who undergo thymectomy, muscle antibodies tend to disappear and abnormal preoperative serum complement levels tend to revert to the normal range. But in thymoma patients there is no significant change in the frequency of muscle antibodies and the changes in serum complement levels are inconsistent (Nastuk *et al* 1966).

Considering the role of the thymus in immunological processes (Miller 1965a and b; Good *et al* 1965), myasthenics might be expected to show abnormalities of immunological responsiveness either as a result of the thymic abnormalities or as a late result of thymectomy even in adult life. The evidence to date for any such alteration is conflicting. Adner *et al* (1964) found normal humoral antibody production but depression of capacity to develop delayed hypersensitivity. The latter could not be attributed to thymectomy or thymic irradiation although a relationship to thymic pathology (tumour or hyperplasia) was possible. On the other hand Kornfeld *et al* (1965) reported normal delayed hypersensitivity reactions but found some evidence of impairment of antibody production in that myasthenics showed poorer secondary responses to antigenic stimulation than control subjects and their antibody titres were less well sustained. The impairment was more marked in thymectomized patients, but this could not be attributed definitely to the absence of the thymus as these patients were also those most severely affected by myasthenia. There was no relationship between depression of antibody production and presence or type of thymic pathology in the thymectomized group. The differences in antibody response between the patients of Adner *et al* and Kornfeld *et al* may be explained by differences in antigens used, length of follow-up period and clinical material.

There is no evidence of impaired immunological capacity in myasthenics, either thymectomized or non-thymectomized, as measured by the capacity of lymphocytes cultured *in vitro* to respond to phytohaemagglutinin or bacterial products by blast transformation and increased DNA synthesis; nor on the other hand are abnormal blast transformation and increased DNA synthesis observed when lymphocytes from myasthenics are cultured in the presence of skeletal muscle or thymus tissue (Housley & Oppenheim 1967).

Finally, there are no consistent abnormalities of γ-globulins, either on paper electrophoretic analysis (Kornfeld 1964) or on quantitative estimation of individual immunoglobulins (Housley & Rowe 1966).

Immunosuppressive Therapy

In diseases of unknown aetiology it can be argued that improvement with adrenocorticotrophin (ACTH) or corticosteroid therapy, unexplained by other means, is evidence in favour of auto-allergic factors in the causation of the disease. Reports of the use of ACTH and cortisone in the treatment of myasthenia appeared soon after the introduction of these drugs into clinical practice and some benefit was observed in that, after an initial exacerbation of muscle weakness, temporary remissions of symptoms sometimes occurred (Torda & Wolff 1951; Millikan & Eaton 1951). However, the more widespread use of this form of treatment was discouraged by other reports of lack of beneficial effect and by the initial deterioration in muscle strength which, in bulbar cases especially, could be dangerous or even fatal (Grob & Harvey 1952; Torda & Wolff 1951; Mount 1964). Recent trials on the other hand indicate that high dosage ACTH therapy may have a place in the management of myasthenia, although the risks are recognized as considerable (Freydberg 1959; von Reis *et al* 1966; Osserman & Genkins 1966; Grob & Namba 1966), but it is difficult to use this as an argument in favour of an auto-allergic aetiology in myasthenia as the beneficial effects are by no means striking and in any case the manifold actions of ACTH and steroid hormones include effects on normal muscle and motor endplates (Ellis 1956; Perkoff *et al* 1959; Tuncbay & Boshes 1966).

There have been a few attempts to induce remissions in myasthenia using the immunosuppressive drug 6-mercaptopurine but they have been unsuccessful (Wolf *et al* 1966).

PATHOGENESIS

The abnormalities of the thymus in myasthenia gravis must presumably be of fundamental importance if the disease is the result of aberration of immunological mechanisms. Cases of myasthenia gravis developing after thymectomy for thymoma have occurred (Klein *et al* 1964; Fisher 1964) and at first sight these are difficult to reconcile with any theory involving the thymus closely in the

pathogenesis of the disease, but, as Fisher has pointed out, all such cases who have subsequently had a second operation or come to autopsy have been found to have remaining tumour or hyperplastic gland.

Smithers (1959) speculating on the relationships of the thymus and other lymphoid tissues to diseases showing immunological abnormalities and noting the resemblance of the lymphoid hyperplasia of the thymus in myasthenia to the changes in the thyroid in Hashimoto's disease, was the first to suggest that myasthenia gravis might be due to an auto-allergic response.

The following year Nastuk, Plescia & Osserman (1960) reported their finding of abnormal serum complement levels and suggested that this might be due to consumption of complement in auto-allergic reactions involved in the pathogenesis of the disease, this suggestion being supported by the demonstration of circulating antibodies to muscle fibres by Strauss *et al* (1960). At the same time, but independently, Simpson (1960) also put forward the hypothesis that myasthenia gravis is an auto-allergic disease, based chiefly on clinical features. He postulated that antibodies to motor endplates may be formed as a result either of an abnormal thymus reacting to endplate protein as if it were antigenically foreign or of an auto-allergic response involving the motor endplate following an upper respiratory infection by mechanisms such as are suggested in rheumatic fever and acute glomerulonephritis.

The normal thymus, in contrast to other lymphoid tissues, shows little evidence of lymphoid hyperplasia or of antibody production in response to circulating antigens (Askonas & White 1956; Marshall & White 1961; Metcalf 1964). The failure to produce an immune response does not seem to be due to a lack of immunologically competent cells as some thymic cells, at least, are capable of graft-versus-host reactions (Fiore-Donati *et al* 1964, Hilgard *et al* 1965) and of antibody production (Stoner & Bond 1963). Furthermore, injection of antigens directly into the thymus (Marshall & White 1961; Sherman, Adner & Damashek 1965) leads to germinal follicle and plasma cell formation (although it is possible that immunologically competent cells may have entered the gland at the site of injury).

Marshall & White (1961) have suggested that there may be some form of barrier to the entry of circulating antigens into the thymus and have produced some direct evidence for this. They further suggest that if such a barrier exists, then the histological changes in the thymus (together with the absence of generalized lymphoid hyperplasia) indicates that in myasthenia gravis an immune response has arisen *within* the gland. The immune response could be a spontaneous reaction of lymphoid cells, i.e. a 'forbidden clone' (Burnet 1959), which if capable of reacting with nerve endings or muscle components could possibly produce the disease phenomena. Alternatively, the immune response could arise as the result of the release of segregated antigen within the thymus, in which case the disease process would have to be explained by the sharing of

common antigenic determinants by thymus and nerve or muscle structures.

Thymus and skeletal muscle do in fact share antigenic determinants, as described above, but it is difficult to see how the antibodies to thymic epithelial cells and muscle striations could produce the neuromuscular block. As already mentioned, they apparently do not react with structures at the neuromuscular junction, they are present in only a minority of patients, are found in a substantial proportion of thymoma patients *without* myasthenia and do not produce myasthenia in infants when transmitted across the placenta. It may well be that the auto-antibodies in myasthenia gravis are merely immunological markers of the underlying disease process, and, indeed, the apparent harmlessness of many auto-antibodies is a striking feature of diseases of presumed auto-allergic aetiology (Anderson 1963). A secondary pathogenic role, however, perhaps synergism with cellular mechanisms, cannot be ruled out. Furthermore, although the muscle antibodies have not been shown to react at the neuromuscular junction, which is the apparent site of the physiological defect, a concomitant defect of muscle contractility, in which the muscle antibodies could play a part, has not been entirely excluded (Osserman 1958).

Goldstein & Whittingham (1966) claim to have produced '*autoimmune thymitis*' (collections of lymphocytes around Hassall's corpuscles in the thymic medulla) in guinea-pigs immunized with heterologous (calf) muscle or thymus in complete Freund's adjuvant. In addition they report that some of these animals showed a myasthenic type of neuromuscular block on electromyography but that no such block was produced in animals immunized similarly but previously thymectomized. On this evidence it is suggested that the neuromuscular block was due to the release of an inhibitor of neuromuscular transmission from the diseased thymus itself; the alternative explanation that thymectomy had prevented the development of an auto-allergic reaction involving structures at the neuromuscular junction directly being rejected on the results of experiments in control animals showing that prior thymectomy did not prevent the development of another auto-allergic disease, allergic encephalomyelitis. Relating this animal model to the thymic pathology and immunological abnormalities in myasthenia gravis in man, Goldstein (1966b) postulates that the basic lesion in the human disease also is auto-allergic thymitis. He suggests that the thymitis results from autosensitization by the thymoma in the 10% of cases in which a tumour is present and from unknown causes in the rest. As in the animal model, the auto-allergic reaction then causes the release of a neuromuscular blocking agent producing the symptoms of the disease. In this hypothesis again, the muscle and thymus antibodies have no pathological role, being merely an inconstant (except in thymoma cases) accompaniment of the immune response to the 'myoid' antigen in epithelial cells. The development of myasthenia depends on the release of a sufficient amount of neuromuscular blocking substance.

A further hypothesis, which attempts to relate the thymic pathology and immunological findings in myasthenia gravis to the concept of a circulating inhibitor of neuromuscular transmission and current views on the immunological functions of the thymus, has been advanced by Strauss *et al* (1966). According to this hypothesis, thymic disease could result in the elaboration and release of a substance, possibly a sub-unit of a normal thymic epithelial cell constituent, which gains entry to the A-band* regions of skeletal muscle fibres. In this situation, it might, because of configurational similarities to A-band structures, produce a metabolic disturbance the retrograde effects of which could result in impairment of neuromuscular transmission. The majority of patients would maintain immunological tolerance to this hypothetical substance but in a minority it might function as an antigen stimulating the formation of antibodies cross-reacting with thymic cells and skeletal muscle A-bands. Such antibodies could, therefore, be entirely incidental to the development of the disease, but on the other hand, in view of the suggestion by Good & Papermaster (1964) that the thymus and other lymphoid tissues may exercise a controlling influence on aberrant cellular proliferation by producing immunological reactions in response to antigenic changes on the cell surface, it is also conceivable that the antibody response in myasthenia gravis represents a defensive mechanism serving to inactivate the 'neuromuscular' blocking substance. The presence of muscle and thymus antibodies in thymoma patients *without* myasthenia gravis could, then, represent the successful immunological destruction of this agent.

Acknowledgment

I wish to thank Dr A.J.L.Strauss for helpful criticism of the manuscript.

REFERENCES

Adner M.M., Sherman J.D., Isé, Caroline, Schwab R.S. & Damashek W. (1964) An immunologic survey of forty-eight patients with myasthenia gravis. *New Eng. med. J.* **271,** 1327.

Anderson J.R. (1963) Auto-antibodies in diseases of man. *Brit. med. Bull.* **19,** 251.

Askonas B.A. & White R.G. (1956) Sites of antibody production in the guinea-pig. The relation between *in-vitro* synthesis of anti-ovalbumin and γ-globulin and distribution of antibody-containing plasma cells. *Brit. J. exp. Path.* **37,** 61.

Bargman W. (1943) In *Handbuch der Mikroskopischen Anatomie des Menschen*, vol. VI, sect. 1, ed. von Moellendorf W. Berlin.

Becker K.L., Titus J.L., McConahey W.M. & Woolner L.B. (1964) Morphologic evidence of thyroiditis in myasthenia gravis. *J. Amer. med. Ass.* **187,** 994.

Bergh N.P. (1953) Biologic assays in myasthenia gravis for any agents causing a neuromuscular block. *Scand. J. lab. clin. Invest.* **5,** suppl. 5, 1.

Beutner E.H., Witebsky E., Ricken D. & Adler R.H., (1962) Studies on auto-antibodies in myasthenia gravis. *J. Amer. med. Ass.* **182,** 156.

*or I-band; See page 1006.

BEUTNER E.H., WITEBSKY E. & LEFF I. (1963) Auto-immune responses in myasthenia gravis: observations on S and SH antigens, on species specificity and on *in vivo* reactions. *Fed. Proc.* **22,** 217.

BEUTNER E.H., WITEBSKY E. & DJANIAN, AIDA Y. (1965) Serologic studies of myasthenia gravis. *Ann. N.Y. Acad. Sci.* **124,** 709.

BEUTNER E.H., FAZEKAS G., SCOTT A. & WITEBSKY E. (1966) Direct fluorescent antibody studies of gamma globulin localization in muscle of patients with myasthenia gravis. *Ann. N.Y. Acad. Sci.* **135,** 588.

BICKERSTAFF E.R. & WOOLF A.L. (1960) The intramuscular nerve endings in myasthenia gravis. *Brain* **83,** 10.

BICKERSTAFF E.R., EVANS J.V. & WOOLF A.L. (1960) The ultrastructure of human myasthenic and non-myasthenic motor end plates. *Brain* **86,** 638.

BURNET F.M. (1959) *The Clonal Selection Theory of Acquired Immunity.* Cambridge: University Press.

BUZZARD E.F. (1905) The clinical history and post-mortem examination of five cases of myasthenia gravis. *Brain* **28,** 438.

CASTLEMAN B. (1955) Tumours of the thymus gland. In *Atlas of Tumor Pathology,* Section V—Fascicle 19. Washington, D.C.: Armed Forces Institute of Pathology.

CHURCHILL-DAVIDSON H.C. & WISE R.P. (1963) Neuromuscular transmission in the newborn infant. *Anesthesiology* **24,** 271.

CLAWSON B.J., NOBLE J.F. & LUFKIN N. (1947) Nodular inflammation and degenerative lesions of muscles from 450 autopsies. *Arch. Path.* **43,** 479.

CRUIKSHANK B. (1952) Focal lesions in skeletal muscles and peripheral nerves in rheumatoid arthritis and other conditions. *J. Path. Bact.* **44,** 21.

DALY J.J. & JACKSON E. (1964) Case of Hashimoto's disease with myasthenia gravis. *Brit. med. J.* **i,** 748.

DFSMEDT J.E. (1966) Presynaptic mechanism in myasthenia gravis. *Ann. N.Y. Acad. Sci.* **135,** 209.

DJANIAN, AIDA Y., BEUTNER E.H. & WITEBSKY E. (1964) Tanned-cell haemagglutination test for detection of antibodies in sera of patients with myasthenia gravis. *J. lab. clin. Med.* **63,** 1.

DONIACH, DEBORAH & ROITT I.M. (1962) Autoantibodies in disease. *Ann. Rev. Med.* **13,** 213.

DOWNES J.M., GREENWOOD B.M. & WRAY S.H. (1966) Auto-immune aspects of myasthenia gravis. *Quart. J. Med.* N.S. XXXV, No. 137, 85.

ELLIS J. (1956) Necrosis and regeneration of skeletal muscle in cortisone treated rabbits. *Amer. J. Path.* **32,** 993.

ELMQVIST D., HOFFMAN W.W., KUGELBERG J. & QUASTEL D.M.J. (1964) An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. *J. Physiol.* **174,** 417.

FELTKAMP-VROOM, THEA (1966) Myoid cells in human thymus. *Lancet* **i,** 1320.

FIORE-DONATI L., CHIECO-BIANCHI L., DE BENEDICTIS G. & TRIDENTE G. (1964) Graft-versus-host reaction in F_1 hybrid mice injected with pre-immunized parental thymus cells. *Blood* **24,** 770.

FISHER E.R. (1964) Pathology of the thymus and its relation to human disease. In *The Thymus In Immunobiology,* ch. 36, ed. GOOD R.A. & GABRIELSON A. New York: Hoeber.

FRASER D. & TURNER J.W.A. (1953) Myasthenia gravis and pregnancy. *Lancet* **ii,** 417.

FREYDBERG L.D. (1959) The place of corticotrophin in the treatment of myasthenia gravis. *Ann. intern. Med.* **52,** 806.

GENKINS G., MENDELOW H., SOBEL H.J. & OSSERMAN K.E. (1961) Myasthenia gravis. Analysis of 31 consecutive post-mortem examinations. In *Myasthenia Gravis. The Second International Symposium Proceedings*, part IV, ch. 11. ed. VIETS H.R. Springfield, Illinois: C. Thomas.

GOLDSTEIN G. (1966a) Plasma cells in the human thymus. *Aust. J. exp. med. Sci.* **44,** 695.

GOLDSTEIN G. (1966b) Thymitis and myasthenia gravis. *Lancet* **ii,** 1164.

GOLDSTEIN G. & WHITTINGHAM S. (1966) Experimental autoimmune thymitis. An animal model of human myasthenia gravis. *Lancet* **ii,** 315.

GOOD R.A. & PAPERMASTER B.W. (1964) Ontogeny and phylogeny of adaptive immunity. In *Advances in Immunology*, vol. 4. ed. DIXON F.J. & HUMPHREY J.H. New York and London: Academic Press.

GOOD R.A., PETERSON R.D.A., MARTINEZ C., SUTHERLAND D.R.E., KELLUM M.J. & FINSTAD, JOANNE (1965) The thymus in immunobiology. *Ann. N.Y. Acad. Sci.* **124,** 73.

GORDON S.V., HESS J.W. & FREDERICK R.J. (1965) Myasthenia gravis. Studies on *in vivo* binding of γ-globulin to muscle. *Arch. intern. Med.* **115,** 405.

GOTTLIEB A.J., DOUGLAS S.D., STRAUSS A.J.L. & SPICER S.S. (1966) Ultrastructural localization of gamma globulin binding to skeletal muscle. *Ann. N.Y. Acad. Sci.* **135,** 638.

GRASHENKOV N. & PERELMAN L.B. (1966) Some aspects of myasthenia gravis. *Ann. N.Y. Acad. Sci.* **135,** 398.

GROB D. & HARVEY A.M. (1952) Effect of adrenecorticotrophic hormone (ACTH) and cortisone in patients with myasthenia gravis and report of onset of myasthenia gravis during prolonged cortisone administration. *Bull. Johns Hopkins Hosp.* **91,** 124.

GROB D. & JOHNS R.J. (1961) Further studies on the mechanism of the defect in neuromuscular transmission in myasthenia gravis with particular reference to the acetylcholine-insensitive block. In *Myasthenia Gravis. The Second International Symposium Proceedings*, part II, ch. 2. ed. VIETS H.R. Springfield, Illinois: C. Thomas.

GROB D. & NAMBA T. (1966) Corticotrophin in generalized myasthenia gravis. Effect of short intensive courses. *J. Amer. med. Ass.* **198,** 703.

GROB D., NAMBA T. & FELDMAN D.S. (1966) Alteration in reactivity to acetylcholine in myasthenia gravis and carcinomatous myopathy. *Ann. N.Y. Acad. Sci.* **135,** 247.

HALE W.L. & BEUTNER E.H. (1965) Studies of the serological relation of myosin and actomyosin to antigens reactive with muscle antibodies in the sera of myasthenia gravis patients. *Fed. Proc.* **24,** 693.

HANSON, JEAN & HUXLEY H.H. (1953) The structural basis of the cross-striations in muscle. *Nature (Lond.)* **172,** 530.

HARVEY A.M., SCHULMAN L.E., TUMULTY P.A., CONLEY C.L. & SCHOENRICH, EDITH M. (1954) Systemic lupus erythematosus. Review of the literature and clinical analysis of 138 cases. *Medicine* **33,** 291.

HENRY K. (1966) Mucin secretion and striated muscle in the human thymus. *Lancet* **i,** 183.

HILGARD H.R., SOSIN H., MARTINEZ C. & GOOD R.A. (1965) Specifically increased graft-versus-host reactivity of thymus cells from immunized mice. *Nature (Lond.)* **207,** 208.

HOFFBRAND B.I. (1966) A serological study of primary thymic neoplasms. *Thorax* **21,** 263.

HOUSLEY J. & ROWE D.S. (1966) Unpublished observations.

HOUSLEY J. & OPPENHEIM J.J. (1967) Lymphocyte transformation in thymectomized and non-thymectomized patients with myasthenia gravis. *Brit. med. J.* **2,** 679

KEYNES G. (1954) Surgery of the thymus gland. Second (and third) thoughts. *Lancet* **i,** 1197.

KLEIN J.J., GOTTLIEB A.J., MONES R.J., APPEL S.H. & OSSERMAN K.E. (1964). Thymoma and polymyositis. *Arch. Int. Med.* **113,** 142.

KORNFELD P. (1964) Serum proteins in myasthenia gravis. *J. Amer. med. Ass.* **190,** 463.

KORNFELD P., SIEGAL S., WEINER L.B. & OSSERMAN K.E. (1965) Studies in myasthenia gravis. Immunologic response in thymectomized and non-thymectomized patients. *Ann. int. Med.* **63,** 416.

LAWRENCE J. (1963) The prevalence of arthritis. *Brit. J. clin. Pract.* **17,** 699.

LOGAN W.P.D. & CUSHION A.A. (1958) Studies on medical and population subjects. In *Morbidity Statistics from General Practice*, vol. 1. *General*. London: H.M.S.O.

MCARDLE B. (1965) Biochemical aspects of myasthenia. In *Biochemical Aspects of Neurological Disorders*, 2nd series, ch. 3, ed. CUMINGS J.N. & KREMER M. Oxford: Blackwell.

MCDERMOTT, VIOLET (1960) The changes in the motor endplates in myasthenia gravis. *Brain* **83,** 24.

MCFARLIN D.E., BARLOW M. & STRAUSS A.J.L. (1966) Anti-muscle, anti-thymus serum reactivities in non-myasthenic patients with thymoma: clinical evaluation. *New. Eng. J. Med.* **275,** 1321.

MCFARLIN D.E., ENGEL W.K. & STRAUSS A.J.L. (1966) Does myasthenic serum bind to the neuromuscular junction? *Ann. N.Y. Acad. Sci.* **135,** 656.

MARSHALL J.M., HOLTZER H., FINCK H. & PEPE F. (1958) The distribution of protein antigens in striated myofibrils. *Exp. Cell. Res.*, Suppl. **7,** 219.

MARSHALL A.H.E. & WHITE R.G. (1961). The immunological reactivity of the thymus. *Brit. J. Exp. Path.* **42,** 379.

MENDELOW H. (1958) Pathology. In *Myasthenia Gravis*, ch. 2, *ed.* OSSERMAN K.E. New York and London: Grune & Stratton.

METCALF D. (1964) The thymus and lymphopoiesis. In *The Thymus in Immunobiology*, ed. GOOD R.A. & GABRIELSON, ANN. New York: Hoeber-Harper.

MILLER J.F.A.P. (1965a) The role of the thymus in immune processes. *Internat. Arch. Allergy* **28,** 61.

MILLER J.F.A.P. (1965b) Effect of thymectomy in adult mice on immunological responsiveness. *Nature* (*Lond.*) **208,** 1337.

MILLIKAN C.H. & EATON L. (1951) Clinical evaluation of ACTH and cortisone in myasthenia gravis. *Neurol.* **1,** 145.

MOUNT F.W. (1964) Corticotrophin in treatment of ocular myasthenia. *Arch. Neurol.* **11,** 114.

NAMBA T. & GROB D. (1966) Autoantibodies and myasthenia gravis with special reference to muscle ribonucleoprotein. *Ann. N.Y. Acad. Sci.* **135,** 606.

NASTUK W.L., STRAUSS A.J.L. & OSSERMAN K.E. (1959) Search for a neuromuscular blocking agent in the blood of patients with myasthenia gravis. *Amer. J. Med.* **26,** 394.

NASTUK W.L., PLESCIA O.J. & OSSERMAN K.E. (1960) Changes in serum complement activity in patients with myasthenia gravis. *Proc. Soc. exp. Biol. Med.* **105,** 177.

NASTUK W.L. & STRAUSS A.J.L. (1961) Further developments in the search for a neuromuscular blocking agent in the blood of patients with myasthenia gravis. In *Myasthenia Gravis. The Second International Symposium Proceedings*, p. 229, ed. VIETS H.R. Springfield, Illinois: C. Thomas.

NASTUK W.L., KESSLER H.J., GRYNBAUM A. & SMITH M. (1966) Immunological changes following thymectomy in myasthenia gravis. *Arch. Neurol.* **15,** 1.

NORRIS E.H. (1936) The thymoma and thymic hyperplasia in myasthenia gravis, with observations on the general pathology. *Amer. J. Cancer* **27,** 421.

OOSTERHUIS H.J.G.H., FELTKAMP T.E.W. & VAN DER GELD H.R.W. (1966) Muscle antibodies in myasthenic mothers and their babies. *Lancet* **ii,** 1226.

OSSERMAN K.E. (1958) *Myasthenia Gravis*. New York and London: Grune & Stratton.

OSSERMAN K.E. & WEINER L.B. (1965a) Studies in myasthenia gravis: immunofluorescent tagging of muscle striations with antibody from serums of 256 myasthenic patients. *Ann. N.Y. Acad. Sci.* **124,** 730.

OSSERMAN K.E. & WEINER L.B. (1965b) Studies in myasthenia gravis. Correlation of antibody immunofluorescence with clinical course. *New Eng. J. Med.* **273,** 615.

OSSERMAN K.E. & GENKINS G. (1966) Studies in myasthenia gravis, short term massive corticotrophin therapy. *J. Amer. med. Ass.* **198,** 699.

PARKES J.D. & MCKINNA J.A. (1966) Neuromuscular blocking activity in the blood of patients with myasthenia gravis. *Lancet* **i,** 338.

PERKOFF G.T., SILBER R., TYLER E.H. & CARTWRIGHT G.E. (1959). Studies in disorders of muscle. XII. Myopathy due to administration of therapeutic amounts of 17 hydroxycorticosteroids. *Amer. J. Med.* **26,** 891.

PERLO V.P., POSKANZER D.C., SCHWAB R.S., VIETS H.R., OSSERMAN K.E. & GENKINS G. (1966) Myasthenia gravis: evaluation of treatment in 1355 patients. *Neurol.* **16,** 431.

PLESCIA O.J., SEGOVIA J.M. & STRAMPP, ALICE (1966) An assessment of changes in the complement level of myasthenic sera. *Ann. N.Y. Acad. Sci.* **135,** 580.

REMEN L. (1932) Zur Pathogenese und Therapie der Myasthenia gravis pseudoparalytic. *Dtsch. Ztschr. f. Nervenheilkunde* **128,** 66.

RICKEN D. (1966) Myasthenia gravis and humoural antibodies against human skeletal muscle proteins. *Germ. med. Monthly* **XI,** 134.

RUSSELL, DOROTHY S. (1953) Histological changes in the striped muscles in myasthenia gravis. *J. Path. Bact.* **65,** 279.

SAHAY B.M., BLENDIS L.M. & GREENE R. (1965). Relation between myasthenia gravis and thyroid disease. *Brit. med. J.* **i,** 762.

SCHRIRE I. (1959) Progesterone metabolism in myasthenia gravis. *Quart. J. Med.* **28,** 59.

SHERMAN J.D., ADNER M.M. & DAMASHEK W. (1965) Experimental production of germinal follicles in the thymus. Relationship of Hassall's corpuscles to germinal follicle formation *Ann. N.Y. Acad. Sci.* **124,** 105.

SHULMAN S., LANG R., BEUTNER E. & WITEBSKY E. (1966) Precipitation of autoantibody in serum from patients with myasthenia gravis. *Immunology* **10,** 289.

SIMPSON J. (1960) Myasthenia gravis: a new hypothesis. *Scot. med. J.* **5,** 419.

SIMPSON J. (1964) Immunological disturbances in myasthenia gravis with a report of Hashimoto's disease developing after thymectomy. *J. Neurol. Neurosurg. Psychiat.* **27,** 129.

SLOAN H.E. JR. (1943) Thymus in myasthenia gravis, with observations on the normal anatomy and histology of the thymus. *Surgery* **13,** 154.

SMITH W.T. (1966) Personal communication.

SMITHERS D.W. (1959) Tumours of the thyroid gland in relation to some general aspects of neoplasia. *J. Fac. Radiol. (Lond.)* **10,** 3.

STERN G.M., HALL J.M. & ROBINSON D.C. (1964) Neonatal myasthenia gravis. *Brit. med. J.* **ii** 284.

STONER R.D. & BOND V.P. (1963) Antibody formation by transplanted bone marrow, spleen, lymph node and thymus cells in irradiated recipients. *J. Immunol.* **91,** 185.

STRAUSS A.J.L. (1966) Muscle antibodies in myasthenia gravis. *Lancet* **ii,** 749.

STRAUSS A.J.L. (1967) Personal communication.

STRAUSS A.J.L., SEEGAL, BEATRICE C., HSU K.C., BURKHOLDER P.M., NASTUK W.L. & OSSERMAN K.E. (1960) Immunofluorescence demonstration of a muscle-binding, complement-fixing serum globulin fraction in myasthenia gravis. *Proc. Soc. exp. Biol. Med.* **105,** 184.

STRAUSS A.J.L., DEITCH, ARLENE & HSU K.C. (1961) Further observations on the localization of a muscle-binding, complement-fixing serum globulin fraction in myasthenia gravis. *Fed. Proc.* **20,** 38.

STRAUSS A.J.L., VAN DER GELD H.R.W., KEMP P.G., EXUM E.D. & GOODMAN H.C. (1965) Immunological concomitants of myasthenia gravis. *Ann. N.Y. Acad. Sci.* **124,** 744.

STRAUSS, A.J.L. & Kemp, P.G., (1967) *J. Immunol.* **99,** 945.

STRAUSS A.J.L., KEMP P.G. & DOUGLAS S.D. (1966a) Phylogenetic and ontogenetic studies with striated muscle and thymic cell-binding gamma globulin. *Fed. Proc.* **25,** 679.

STRAUSS A.J.L., KEMP P.G. & DOUGLAS S.D. (1966b) Myasthenia gravis. Immunological relationship between striated muscle and thymus. Thymic myoid cells. *Lancet* **i,** 772.

STRAUSS A.J.L., SMITH C.W., CAGE G.W., VAN DER GELD H.W.R., MCFARLIN D.E. & BARLOW M. (1966) Further studies on the specificity of presumed immune associations of myasthenia gravis and consideration of possible pathogenic implications. *Ann. N.Y. Acad. Sci.* **135,** 557.

STRÜPPLER A. (1955). Experimentelle Untersuchungen zur Pathogenese der Myasthenie. *Ztschr. ges. exp. Med.* **125,** 244.

STURGILL B.C., CARPENTER E.R., STRAUSS A.J.L. & GOODMAN H.C. (1964) Antibodies in systemic lupus erythematosus and myasthenia which react with thermally denatured DNA-coated bentonite. *Proc. Soc. exp. Biol. Med.* **115,** 246.

TENG P. & OSSERMAN K.E. (1956) Studies in myasthenia gravis, neonatal and juvenile types. *J. Mt. Sinai Hosp.* **23,** 711.

TORDA, CLARA & WOLFF H.G. (1951) Effects of administration of ACTH to patients with myasthenia gravis. *A.M.A. Arch. Neurol. Psychiat.* **65,** 534.

TUNCBAY T.O. & BOSHES B. (1966) Studies in muscle and motor endplate enzymes. I. Neuromuscular disorders. *Ann. N.Y. Acad. Sci.* **135,** 98.

VAN DER GELD H.R.W., FELTKAMP T.E.W., VAN LOGHEM J.J., OOSTERHUIS H.J.G.H. & BIEMOND A. (1963) Multiple antibody production in myasthenia gravis. *Lancet* **ii,** 373.

VAN DER GELD H., FELTKAMP T.E.W. & OOSTERHUIS H.J.G.H. (1964) Reactivity of myasthenia gravis serum γ-globulin with skeletal muscle and thymus demonstrated by immunofluorescence. *Proc. Soc. exp. Biol. Med.* **115,** 782.

VAN DER VELDE R.L. & FRIEDMAN N.B. (1966) The thymic 'myoidzellen' and myasthenia gravis. *J. Amer. med. Ass.* **198,** 287.

VETTERS J.M. (1965) Immunofluorescence staining patterns in skeletal muscle using serum of myasthenic patients and normal controls. *Immunology* **9,** 93.

VIETS H.R., SCHWAB R.S. & BRAZIER, MARY A.B. (1942) Effects of pregnancy on the course of myasthenia gravis. *J. Amer. med. Ass.* **119,** 236.

VON REIS G., LILJESTRAND A. & MATELL G. (1966) Treatment of severe myasthenia gravis with large doses of ACTH. *Ann. N.Y. Acad. Sci.* **135,** 409.

WALKER, MARY B. (1934) Treatment of myasthenia gravis with physostigmine. *Lancet* **i,** 1200.

WEINER L.B. & OSSERMAN K.E. (1966) Studies in myasthenia gravis. Demonstration of presence of immunofluorescence in serums correlated with clinical findings. *Ann. N.Y. Acad. Sci.* **135,** 644.

WHITE R.G. & MARSHALL A.H.E. (1962) The autoimmune response in myasthenia gravis. *Lancet* **ii,** 120.

WILKINS E.W. JR., EDMUNDS L.H. & CASTLEMAN B. (1966). Cases of thymoma at the Massachusetts General Hospital. *J. thorac. cardiovasc. Surg.* **52,** 322.

WILSON A. & STONER H.B. (1944) Myasthenia gravis. A consideration of its causation in fourteen cases. *Quart. J. Med.* **13,** 1.

WOLF S.M. & BURROWS H.S. (1966) Myasthenia gravis and systemic lupus erythematosus. *Arch. Neurol.* **14,** 254.

WOLF S.M., ROWLAND L.P., SCHOTLAND D.L., MCKINNEY A.S., HOEFER P.F.A. & ARANOW H. JR. (1966) Myasthenia gravis as an autoimmune disease: clinical aspects. *Ann. N.Y. Acad. Sci.* **135,** 517.

WOOLF A.L. (1966) Morphology of the myasthenic neuro-muscular junction. *Ann. N.Y. Acad. Sci.* **135,** 35.

ZACKS S.I., BAUER W.C. & BLUMBERG J.M. (1962) The fine structure of the myasthenic neuromuscular junction. *J. Neuropath. exp. Neurol.* **21,** 335.

CHAPTER 38

THE STOMACH IN ALLERGIC DISEASES

I. CHANARIN

The common lesion of the stomach is an atrophic process involving the mucosa. This lesion is associated with the production of antibodies against the parietal cell and against one of the products of this cell, viz. intrinsic factor.

Gastric atrophy was described by Handfield Jones (1855) and Fenwick (1870). Austin Flint (1860) noted the association with pernicious anaemia. However, the development of a simple instrument for obtaining gastric biopsy specimens during life by Woods, Doig, Motteram & Hughes (1949) and Tomenius (1950) ushered in the modern era in the study of the gastric mucosa.

THE GASTRIC LESION

There is general agreement concerning the classification of the histological changes seen in biopsy specimens. These changes fall into three groups:

(*a*) superficial gastritis
(*b*) atrophic gastritis
(*c*) gastric atrophy.

SUPERFICIAL GASTRITIS

Here there is no loss of secreting cells and the mucosa remains of normal thickness. There is a patchy and often heavy infiltration of plasma cells and

polymorphs beneath the surface epithelium. This infiltration does not extend to the deeper layers.

Atrophic Gastritis

Here there is loss of secreting cells and disorganization of the regular columns of chief and parietal cells that constitute the deeper layers of the normal body mucosa. There is infiltration with lymphocytes, plasma cells, macrophages and eosinophils. Where many secreting cells remain it is convenient to term the process moderate in degree. Where surviving chief and parietal cells are relatively few the degree of atrophy is graded as severe. Intestinal metaplasia may be present.

Gastric Atrophy

Here there is virtually complete loss of parietal and chief cells often with metaplasia of the epithelium to a mucus cell or intestinal cell type containing goblet and paneth cells. There is infiltration with plasma cells and lymphocytes.

On the whole the lesion is a slowly progressive one and further biopsy in patients who some years earlier had superficial gastritis often discloses atrophic gastritis of varying severity. Both lesions may be present in the same stomach. There is a relatively high incidence of superficial gastritis in young subjects and a greater frequency of atrophic gastritis in older age groups.

Correlation Between Structure and Function

The body of the stomach secretes hydrochloric acid, pepsin and intrinsic factor. Pepsin is produced by the chief cell whereas the other two, acid and intrinsic factor, appear to be a product of the parietal cell. Atrophic changes in the gastric mucosa are associated with a progressive decline in the number of secreting cells and with a fall in the volume of their secretions. Acid production fails first followed by pepsin and finally by intrinsic factor. Overall the correlation between structure and function is a good one when a dose of 40 μg of histamine per kg of body weight is used as the stimulant to gastric secretion. Nevertheless, about 10% of patients with achlorhydria appear to have a normal gastric biopsy appearance and a further 20% only superficial gastritis. A rather better correlation exists with intrinsic factor secretion. Either impaired vitamin B_{12} absorption or a decline in intrinsic factor output by direct assay is invariably associated with marked histological changes.

GASTRIC ANTIBODIES

Two distinct antibodies may be found directed against gastric antigens. One is the parietal cell antibody and the other the intrinsic factor antibody.

Parietal Cell Antibodies

Markson & Moore (1962), Irvine, Davies, Delamore & Williams (1962) and Taylor, Roitt, Doniach, Couchman & Shapland (1962) demonstrated that antibodies against gastric mucosa were present in sera from a high proportion of patients with pernicious anaemia. One technique was a complement fixation method using as antigen a microsomal fraction of extracts of the mucosa from the body of the stomach. The other was a fluorescent technique using a fluorescein-labelled antiglobulin serum and frozen sections of human stomach. The latter method demonstrated that the antibody reacted with an antigen in the cytoplasm of the gastric parietal cell.

Antibodies against the gastric parietal cell do not cross-react with other tissue antigens such as those from other parts of the gastro-intestinal tract or the thyroid. The antigen is inactivated by alcohol and other fixatives, by detergents, by heat and is destroyed by trypsin and papain. Rat gastric mucosa is not as suitable as human stomach in the performance of the test and indeed positive reactions with human antisera are obtained with sections of stomach from the monkey, mouse, cat and guinea-pig.

Not only is the parietal cell antibody present in serum but its identification in the gastric secretion indicates its presence in the gastric mucosa and indeed local production by lymphoid cells in the mucosa is likely. The parietal cell antibody is an IgG, occasionally an IgA globulin.

Intrinsic Factor Antibody

The discovery of antibodies against gastric intrinsic factor followed various lines of investigation. One of the methods of treating pernicious anaemia is by the oral administration of preparations containing both vitamin B_{12} and hog intrinsic factor. In time, however, many patients with pernicious anaemia maintained on this therapeutic regime relapsed both clinically and haematologically. The relapse was associated with the appearance in their sera of a factor (γ-globulin) which interfered with the action of hog intrinsic factor in promoting the absorption of vitamin B_{12} (Schwartz 1958). Thus here was a demonstration not only of the development of a probable antibody against intrinsic factor but also evidence that the antibody interfered with the physiological function of the antigen in promoting vitamin B_{12} absorption. At the same time Taylor & Morton (1959) produced antibodies to human intrinsic factor by immunizing rabbits with human gastric juice, and these rabbit antisera too were able to block intrinsic factor mediated vitamin B_{12} absorption in man. The technique consisted of the simultaneous oral administration to a patient with pernicious anaemia of the serum under test, radioactive vitamin B_{12} and a hog intrinsic factor preparation. If the serum had no antibody the intrinsic factor was able to potentiate vitamin B_{12} absorption; if the serum had such an antibody there was a significant reduction in the amount of vitamin B_{12} absorbed.

It was soon apparent that an antibody (against intrinsic factor) was not only present in about 70% of patients who had relapsed on oral hog intrinsic factor therapy, but was also found in about one-third of patients with pernicious anaemia who were untreated or who had received only injections of vitamin B_{12} (Taylor 1959). Simpler and more sensitive methods for the detection of intrinsic factor antibodies followed (Ardeman & Chanarin 1963), and these depended on the fact that exposure of intrinsic factor to the antibody prevented the subsequent attachment of vitamin B_{12}. With these methods antibodies were demonstrated in the sera of some 57% of patients with pernicious anaemia. These antibodies which are IgG globulins are found not only in serum but, like the parietal cell antibody, also appear in the gastric secretion.

The Frequency of Gastric Atrophic Changes and of Gastric Antibodies in the General Population

Joske, Finckh & Wood (1955) reported the results of 336 gastric biopsies carried out in subjects without any complaints referable to the gastro-intestinal tract. The biopsy was normal in appearance in only 16% of this group; 37% showed

Table 38.1

Age of patients (years)	Abnormal gastric biopsy appearance (%)
0–20	22.2
21–30	49.4
31–40	53.7
41–50	61.1
51–60	69.5
61–70	81.4
71 and over	86.5

superficial gastritis, another 37% atrophic gastritis and the remaining 10% had gastric atrophy.

When the data on 840 biopsies were grouped according to the age of the patients the results shown in Table 38.1 were obtained.

Thus, after the age of 30 more than half the population have histological changes in the gastric mucosa. Incidentally two-thirds of the patients in this study were males. These findings, of course, correlate with the increased frequency of achlorhydria with advancing age.

What is the frequency of gastric antibodies in the general population? Doniach & Roitt (1964) found that the incidence of parietal cell antibodies was 2% below

the age of 20, between 6% and 8% in the 30–60 age group and reached 16% after the age of 60. The figure was 19% in women over the age of 70. Irvine, Davies, Teitelbaum, Delamore & Williams (1965) found that 8% of 372 hospital controls and 5% of 629 blood donors had parietal cell antibodies. Intrinsic factor antibodies are only rarely found in conditions other than Addisonian pernicious anaemia.

Thus the frequency of gastric antibodies in the general population is far smaller than the frequency of atrophic changes in the gastric mucosa. This suggests that these antibodies are unlikely to be the primary factor in initiating such changes in the stomach but that they might be a consequence of these changes in an important minority of patients with a gastric lesion.

The Frequency of Gastric Antibodies in Patients with Atrophic Changes in Gastric Mucosa

When sera from patients who are known to have atrophic changes in the gastric mucosa are tested, parietal cell antibodies are present in about 60% of the women and only about 12% of the men. The number of patients who have parietal cell antibodies increases with the severity of the gastric lesion (Wright, Whitehead, Wangel, Salem & Schiller, 1966)—Table 38.2. Two-thirds of patients with the

Table 38.2

Gastric lesion	Parietal cell antibodies present (%)
Superficial gastritis	20
Atrophic gastritis	44
Gastric atrophy	67

most advanced lesion, viz. gastric atrophy, have parietal cell antibodies although if this group is divided according to sex, males show a much lower incidence (20%) as compared to the females (71%). With very rare exceptions intrinsic factor antibodies are not found in patients with gastric atrophy unless the atrophy is part of the picture of pernicious anaemia.

Do all patients with parietal cell antibodies have histological changes in the gastric mucosa? Adams, Glen, Mackenzie, Morrow, Anderson, Gray & Middleton (1964) obtained a gastric biopsy on twenty such patients and all showed changes which varied from superficial gastritis in two, atrophic gastritis in nine and gastric atrophy in nine. On the other hand te Velde, Abels, Anders, Arends, Hoedemaeker & Nieweg (1964) reported that the gastric biopsy was

normal in appearance in four out of twenty relatives of pernicious anaemia patients all of whom had parietal cell antibodies. More evidence is required on this point, i.e. can the parietal cell antibody precede the appearance of changes in the gastric mucosa and, if so, does this apply to only the pernicious anaemia group?

The increased incidence of parietal cell antibodies in those with a more severe gastric lesion can be interpreted in two ways. It may be that prolongation of the inflammatory process in the gastric mucosa gives rise to greater opportunity for the release of antigen and of stimulating an antibody response. Alternatively, it may be that the development of a more severe lesion in the gastric mucosa follows the appearance of the parietal cell antibody. There is evidence to suggest that the latter hypothesis may be correct.

Iron-deficiency Anaemia and the Gastric Mucosa

The gastric mucosa is normal in only 20% of patients with chronic iron-deficiency anaemia. About 40% have superficial gastritis and the remainder have a more advanced lesion. Witts (1956) has suggested that the changes in the gastric mucosa are the result of iron deficiency. It has been known for some time that in subjects below the age of 30 iron deficiency may be associated with loss or reduction of acid in the gastric juice (Leonard 1954) and that correction of the iron deficiency may lead to a return of acid secretion. What is not clear at the present time is whether the transient failure of acid production is due to a functional change in the gastric mucosa or whether it is due to loss of parietal cells which regenerate in these young subjects when iron deficiency has been corrected. Lees & Rosenthal (1958) failed to demonstrate any improvement in the appearance of the gastric biopsy 1 year after the correction of the iron-deficiency anaemia. The frequency of parietal cell antibodies among 210 patients with iron-deficiency anaemia was 24%.

Thus there is an increased incidence of achlorhydria, of gastric atrophy and of parietal cell antibodies in patients with iron-deficiency anaemia. Long-standing iron deficiency may be a factor in bringing about permanent atrophic changes in the stomach, and an increased frequency of pernicious anaemia in such patients has been noted (Dagg, Goldberg, Gibbs & Anderson 1966).

Thyroid Disease and Gastric Atrophy

The end result of gastric atrophy is a loss of all the normal secretions of the gastric mucosa, and in some this is accompanied by a failure to absorb vitamin B_{12}, except when this is given with an additional source of intrinsic factor. This is the gastric lesion of Addisonian pernicious anaemia. The clinical association between disease of the thyroid gland and pernicious anaemia is well known. Thus pernicious anaemia is present in 2–3% of patients with Graves' disease and

in about 10% of patients with myxoedema. Thyroid disease is present in about 15% of pernicious anaemia patients; thirteen out of 157 had Graves' disease and ten myxoedema (Doniach, Roitt & Taylor 1963).

Gastric parietal cell antibodies are present in one-third of patients with either hypo- or hyperthyroidism. Absent or reduced acid production is frequent among those with thyroid disease and gastric biopsy in forty-one thyrotoxic patients showed atrophic changes in almost 80% (Siurala & Lamberg 1959). Similarly, the high frequency of thyroid disease among pernicious anaemia patients is paralleled by a high incidence (55%) of thyroid cytoplasmic antibodies in the pernicious anaemia group.

When simple atrophic gastritis is considered, thyroid antibodies were found in half the females, but male patients did not show a higher incidence than did matched controls (Coghill *et al* 1965).

Doniach *et al* (1965) reported a study among relatives of thyroid patients and pernicious anaemia patients—Table 38.3. The results showed an increased

TABLE 38.3

	Relatives of patients with			
	Thyroid disease		Pernicious anaemia	
Parietal cell antibodies (%)	20	(8)*	36	(6)
Thyroid antibodies (%)	46	(15)*	50	(15)

* The figures in brackets indicate the values for matched controls.

frequency of antibodies against thyroid and stomach in relatives of patients with either of these diseases. The familial incidence of both these diseases is well known.

What do patients with these disorders of the thyroid gland or of the stomach have in common? The answer appears to be that these individuals and their as yet unaffected relatives have an unusual tendency to form organ-specific antibodies against antigens from these two organs. This answer also implies that these antibodies are important in the pathogenesis of the disease process and are not merely by-products.

Thus in summary, atrophic changes in the gastric mucosa are common in the general population. Some patients, however, react to these changes in the gastric mucosa (and to similar changes in the thyroid gland) by an auto-immune response which tends to potentiate the damage in the gastric mucosa. The greater frequency of these diseases (pernicious anaemia and thyroid disease) in certain families and the high incidence of the appropriate antibodies in their families suggest that the tendency to develop such antibodies is to some extent determined

genetically. The much higher incidence of parietal cell antibodies in those with more severe gastric lesions suggests that the antibodies may be responsible for the progression of the gastric lesion. The greater tendency for women to form such antibodies is paralleled by the preponderance of females among patients with thyroid disease and pernicious anaemia.

Gastric Antibodies in Other Diseases

Parietal cell antibodies occur in 5 to 11% of patients with rheumatoid arthritis and in about 12% of patients with diabetes mellitus (12%). In both these conditions it has been suggested that there is an increased frequency of gastric atrophy and pernicious anaemia (Partridge & Duthie 1963).

There is some doubt about the incidence of antibodies in patients with gastric ulceration although accompanying atrophic changes in the mucosa (usually the antrum) are usual; nor is there much information about parietal cell antibodies after partial gastrectomy. Doniach & Roitt (1964) reported parietal cell antibodies in 22% of patients with gastric ulcers but failed to find an increase in those with duodenal ulceration. Patients with disseminated lupus erythematosus tend to have a lower incidence of gastric antibodies than the rest of the population. Irvine (1963) found an increased incidence of parietal cell antibodies in adrenal insufficiency (Addison's disease).

PERNICIOUS ANAEMIA

The stomach in pernicious anaemia shows either severe atrophic gastritis or gastric atrophy. Residual parietal cells are present in a third of patients. The serum contains parietal cell antibodies in 86% using an immunofluorescent technique. With a complement fixation technique the percentage of positive results falls to 62%. Thus the frequency of parietal cell antibodies is higher than in simple gastric atrophy although the appearance of the gastric biopsy is the same in both conditions. Possibly the reason for this is that the functional lesion of pernicious anaemia develops in the 'best-antibody formers' and therefore this group will show the highest frequency of such antibodies. Taylor *et al* (1962) have reported that the percentage of positive results by the immunofluorescent method is 93% among patients below the age of 60 and 76% in those older than 60 years of age. However, further observations on this point are required since in many patients the apparent titre of the parietal cell antibody remains unchanged over a period of years. If confirmed, it might suggest that, in some, the parietal cell antibodies disappeared from serum with time.

The gastric lesion in pernicious anaemia in many cases is a reversible one and under appropriate circumstances regeneration of the columns of secreting cells takes place with a return of both acid and intrinsic factor to the gastric juice. This can be brought about by treatment with steroids in about half the patients

with pernicious anaemia. Withdrawal of steroids results in a reversal of the process in the gastric mucosa within a few weeks. Thus it is possible that the persistence of parietal cell antibodies over many years is of importance in preventing regeneration of parietal cells. Although little information is available about the titre of parietal cell antibodies on steroid therapy, there is a steady decline in the titre of intrinsic factor antibody and this titre rises again after steroids are withdrawn.

It is equally important to note that in about half the patients with Addisonian pernicious anaemia who respond to steroid therapy (and almost all do respond) there is no change in the gastric secretion and therefore in these it is likely that steroids produce an improvement in vitamin B_{12} absorption by an action elsewhere than on the stomach. The likely site is the ileum where vitamin B_{12} is absorbed.

What of the small number of patients with pernicious anaemia in whom examination of the serum fails to reveal a parietal cell antibody? It may be that in these, as in all patients with pernicious anaemia, such antibodies are present at cellular level, i.e. in the gastric mucosa, although they may be absent from the serum.

Most patients with so-called 'simple' gastric atrophy secrete more intrinsic factor in the gastric juice than do patients with pernicious anaemia, but a few secrete as little intrinsic factor as do those with proven pernicious anaemia. Nevertheless, these patients still absorb enough vitamin B_{12} to maintain a normal serum vitamin B_{12} concentration and tests for vitamin B_{12} absorption are either normal or border-line. A transition from this type of case into one of pernicious anaemia is only rarely seen and it cannot be assumed on the evidence available at this time that such a transition takes place with any degree of frequency.

What determines whether the sequence of events leading to gastric atrophy results in pernicious anaemia in a few cases or to simple atrophy in the majority? It has been suggested that pernicious anaemia will develop in those patients in whom the appearance of a parietal cell antibody is followed by the appearance of an antibody against gastric intrinsic factor.

Intrinsic factor antibodies are found in 57% of patients with pernicious anaemia. Fisher, Rees & Taylor (1966) were able to demonstrate intrinsic factor antibody in the gastric juice of a patient with pernicious anaemia who did not have such an antibody in serum. Thus it raises the possibility that indeed all patients with pernicious anaemia have intrinsic factor antibodies at cellular level. Such antibodies in the gastric secretion will prevent intrinsic factor from combining with vitamin B_{12} and hence will prevent the absorption of vitamin B_{12}. Tenfold concentration of the IgG fraction of pernicious anaemia sera apparently lacking intrinsic factor antibody, failed to bring out any activity against intrinsic factor. Thus it is not likely that current techniques for the detection of intrinsic factor antibody are failing to find such antibodies.

Antibodies to intrinsic factor may react with determinants at the site of vitamin B_{12} binding or with determinants elsewhere on the glycoprotein molecule. Antibodies blocking the vitamin B_{12} binding site prevent vitamin

B_{12} uptake. With rare exceptions, virtually all antisera have this type of antibody. Some two-thirds of sera also have antibodies which still react with intrinsic factor in the presence of vitamin B_{12}, that is, they react with the intrinsic factor-vitamin B_{12} complex as well as with free intrinsic factor. Vitamin B_{12} itself does not function as an antigenic determinant. Tests for intrinsic factor antibodies in serum which require the preformed intrinsic factor-vitamin B_{12} complex give about 30–40% positive results as opposed to 57% when intrinsic factor alone is used as antigen in the test.

The presence of intrinsic factor antibody in the gastric secretion may in part account for the observation that hog intrinsic factor is more effective in improving vitamin B_{12} absorption in pernicious anaemia patients than is human intrinsic factor. Hog intrinsic factor shows reaction with human intrinsic factor antibody and is not inactivated by the human antibody to the same extent as is human intrinsic factor. The superiority of hog intrinsic factor in promoting vitamin B_{12} absorption was noted in both patients with circulating antibodies and in those without demonstrable antibodies in serum. This would lend further support to the view that intrinsic factor antibody at cellular level is present in all pernicious anaemia patients.

Normal gastric juice contains a vast excess of intrinsic factor; indeed, about a hundred times more than is required for vitamin B_{12} absorption. Thus the rare cases where intrinsic factor antibody has been found in the absence of pernicious anaemia have generally had adequate amounts of intrinsic factor and normal vitamin B_{12} absorption. It is only when the amount of intrinsic factor secreted is just adequate to sustain the absorption of vitamin B_{12} as in gastric atrophy that an intrinsic factor antibody is capable of producing a significant effect and tipping the balance in favour of vitamin B_{12} malabsorption. Once this occurs a slow and steady depletion of body stores of vitamin B_{12} follows.

There is some evidence that the intrinsic factor antibody may influence vitamin B_{12} absorption in the ileum. The intrinsic factor-vitamin B_{12} complex becomes adsorbed at specific receptor sites on the surface of the villi. Thereafter there appears to be a slow separation of vitamin B_{12} from intrinsic factor and vitamin B_{12} enters the blood reaching a peak 8–12 hr after the time of administration of the oral dose of vitamin B_{12}. The reason for supposing that the ileum may be the site of an intrinsic factor antibody effect is that some cases of pernicious anaemia who respond to therapy with steroids do not show any increase in the amount of intrinsic factor in the gastric juice. In these it must be supposed that the improvement in vitamin B_{12} absorption is effected elsewhere than via the stomach and hence the ileum where vitamin B_{12} is absorbed seems the likely site.

An experimental model of what may happen in pernicious anaemia is provided by studies of the development of resistance to hog intrinsic factor in man. It was found that the resistance to hog intrinsic factor could be overcome temporarily by giving a large dose of dried hog stomach extract by mouth. This

suggested that an antibody at small gut level had been neutralized. Production of circulating antibodies by parenteral injections of hog intrinsic factor in pernicious anaemia patients had no effect on hog intrinsic factor mediated vitamin B_{12} absorption since presumably such antibodies although present in blood were not present in the gut wall. Thus it may be that intrinsic factor antibody is produced locally in the stomach wall and appears in the gastric secretion and is also produced in the ileal mucosa. The antibody produced in the stomach reacts locally and in the gastric lumen with any intrinsic factor present. In some, intrinsic factor is still present in excess in the gastric secretion despite the effect of local antibody and absorption of this intrinsic factor-vitamin B_{12} complex may be blocked further downstream by antibodies in the ileal wall.

There are two important intrinsic factor-deficiency states in man, viz. pernicious anaemia and that due to surgical removal of the stomach. In both, megaloblastic anaemia due to vitamin B_{12} deficiency develops and in both there is malabsorption of vitamin B_{12} corrected by the addition of intrinsic factor. In both, the amount of intrinsic factor left in the gastric secretion is similar (Ardeman & Chanarin 1966). Nevertheless, patients with post-gastrectomy megaloblastic anaemia on the average absorb twice as much vitamin B_{12} from a test dose as do patients with pernicious anaemia. Although there may be a small increase in the incidence of parietal cell antibodies in post-gastrectomy patients, intrinsic factor antibodies are not found. Thus this difference in the amount of vitamin B_{12} absorbed may be due to the effect of intrinsic factor antibodies which are absent from the gastrectomized group.

Further evidence implicating the intrinsic factor antibody as a probable factor in producing vitamin B_{12} malabsorption is found in the pathogenesis of the so-called 'Juvenile auto-immune pernicious anaemia' group. In these patients pernicious anaemia appears between the ages of about 10 and 18 and is accompanied by disorders affecting either the parathyroids, the thyroid or the adrenal glands. The evidence is that these organs are affected by an auto-allergic process. The stomach shows gastric atrophy as in adult cases with pernicious anaemia. Parietal cell antibodies, at least in serum, have been found in only one case (Doniach and Roitt: personal communication) but all have antibodies against intrinsic factor. It thus seems likely that the development of vitamin B_{12} malabsorption at this early age has been conditioned by the early appearance of intrinsic factor antibodies.

Intrinsic Factor Antibodies without Pernicious Anaemia

A few patients with intrinsic factor antibodies, atrophic change in the gastric mucosa but adequate gastric secretion and normal vitamin B_{12} absorption have been reported. Th iswas found in 2–3% of patients with either Graves' disease or myxoedema (Ardeman, Chanarin, Krafchik & Singer 1966), among pernicious

anaemia relatives (te Velde *et al* 1964) and amongst the patients with disorders known to be associated with a higher incidence of parietal cell antibodies than is found in the rest of the population. A 3 year follow-up in four such patients failed to show any progression of the lesion. All but one of these patients had adequate amounts of intrinsic factor in the gastric juice. It may be that these patients differ from pernicious anaemia patients in that the intrinsic factor antibody appeared at a relatively early stage in the progression of the gastric lesion, and, secondly, that the antibody though present in serum is absent from the gut where it can make contact with intrinsic factor.

REFERENCES

ADAMS J.F., GLEN A.I.M., KENNEDY E.H., MACKENZIE I.L., MORROW J.M., ANDERSON J.R. GRAY K.G. & MIDDLETON D.G. (1964) The histological and secretory changes in the stomach in patients with autoimmunity to gastric parietal cells. *Lancet* **i,** 401.

ARDEMAN S. & CHANARIN I. (1963) A method for the assay of human gastric intrinsic factor and for the detection and titration of antibodies against intrinsic factor. *Lancet* **ii,** 1350.

ARDEMAN S. & CHANARIN I. (1966) Gastric intrinsic factor secretion after partial gastrectomy. *Gut* **7,** 217.

ARDEMAN S., CHANARIN I., KRAFCHIK B. & SINGER W. (1966) Addisonian pernicious anaemia and intrinsic factor antibodies in thyroid disorders. *Quart. J. Med.* **35,** 421.

COGHILL N.F., DONIACH D., ROITT I.M., MOLLIN D.L. & WILLIAMS A.W. (1965) Autoantibodies in simple atrophic gastritis. *Gut* **6,** 48.

DAGG J.H., GOLDBERG A., GIBBS W.N. & ANDERSON J.R. (1966) Detection of latent pernicious anaemia in iron-deficiency anaemia. *Brit. med. J.* **ii,** 619.

DONIACH D. & ROITT I.M. (1964) An evaluation of gastric and thyroid auto-immunity in relation to hematologic disorders. *Seminar in Haemat.* **1,** 313.

DONIACH D., ROITT I.M. & TAYLOR K.B. (1963) Autoimmune phenomena in pernicious anaemia. Serological overlap with thyroiditis, thyrotoxicosis and S.L.E. *Brit. med. J.* **i,** 1347.

DONIACH D., ROITT I.M. & TAYLOR K.B. (1965) Autoimmunity in pernicious anaemia and thyroiditis: a family study. *Ann. N.Y. Acad. Sci.* **124,** 605.

FENWICK S. (1870) On atrophy of the stomach. *Lancet* **ii,** 78.

FISHER J.M., REES C. & TAYLOR K.B. (1966) Intrinsic factor antibodies in gastric juice of pernicious-anaemia patients. *Lancet* **ii,** 88.

FLINT A. (1860) A clinical lecture on anaemia delivered at the Long Island College Hospital. *Amer. Med. Times* **1,** 181.

HANDFIELD JONES C. (1855) *Pathological and Clinical Observations Respecting the Working Conditions of the Stomach.* London: Churchill.

IRVINE, W.J. (1963) A clinical and immunological study of adrenal insufficiency. *J. Leudocsis* **26,** 32

IRVINE W.J., DAVIES S.H., DELAMORE I.W. & WILLIAMS A.W. (1962) Immunological relationship between pernicious anaemia and thyroid disease. *Brit. med. J.* **ii,** 254.

IRVINE W.J., DAVIES S.H., TEITELBAUM S., DELAMORE I.W. and WILLIAMS A.W. (1965) The clinical and pathological significance of gastric and parietal cell antibody. *Ann. N.Y. Acad. Sci.* **124,** 657.

JOSKE R.A., FINCKH E.S. & WOOD I.J. (1955) Gastric biopsy. A study of 1000 consecutive successful gastric biopsies. *Quart. J. Med.* **24,** 269.

LEES F. & ROSENTHAL F.D. (1958) Gastric mucosal lesions before and after treatment in iron deficiency anaemia. *Quart. J. Med.* **27,** 19.

LEONARD B.J. (1954) Hypochromic anaemia in R.A.F. recruits. *Lancet* **i,** 899.

MARKSON J.L. & MOORE J.M. (1962) Autoimmunity in pernicious anaemia and iron deficiency anaemia. *Lancet* **ii,** 1240.

PARTRIDGE R.E.H. & DUTHIE J.J.R. (1963) Incidence of macrocytic anaemia in rheumatoid arthritis. *Brit. med. J.* **i,** 89.

SCHWARTZ M. (1958) Intrinsic factor inhibiting substance in serum of orally treated patients with pernicious anaemia. *Lancet* **i,** 61.

SIURALA M. & LAMBERG B.A. (1959) Stomach in thyrotoxicosis. *Acta med. Scand.* **165,** 181.

TOMENIUS J. (1950) An instrument for gastrobiopsies. *Gastroenterology* **15,** 498.

TAYLOR K.B., ROITT I.M., DONIACH D., COUCHMAN K.G. & SHAPLAND C. (1962) Autoimmune phenomena in pernicious anaemia: gastric antibodies. *Brit. med. J.* **ii,** 1347.

TAYLOR K.B. & MORTON J.A. (1958) An antibody to Castle's intrinsic factor. *Lancet* **i,** 29.

TAYLOR K.B. (1959) Inhibition of intrinsic factor by pernicious anaemia sera. *Lancet* **ii,** 106.

TE VELDE K., ABELS J., ANDERS J.G., ARENDS A., HOEDEMAEKER PH.J. & NIEWEG H.O. (1964) A family study of pernicious anaemia by an immunological method. *J. Lab. clin. Med.* **64,** 177.

WITTS L.J. (1956) Anaemia and the alimentary tract: the relationship between changes in the alimentary tract and deficiencies of iron, folic acid and vitamin B_{12}. The Sydney Watson Smith Lecture, Edinburgh. The Royal College of Physicians.

WOOD I.J., DOIG R.K., MOTTERAM R. & HUGHES A. (1949) Gastric biopsy, report of fifty-five biopsies using the new flexible gastric biopsy type. *Lancet* **i,** 18.

WRIGHT R., WHITHEAD R., WANGEL A.G., SALEM S.N. & SCHILLER K.F.R. (1966) Autoantibodies and microscopic appearance of gastric mucosa. *Lancet* **i,** 618.

CHAPTER 39

THE INTESTINE IN ALLERGIC DISEASES

S.C.TRUELOVE & R.WRIGHT

INTRODUCTION

The gastro-intestinal tract is a part of the body which comes into intimate contact with a great variety of foreign substances, many of which can act as antigens under suitable circumstances. The possible antigens fall into three main categories. First, there are the proteins contained in foods. Secondly, in civilized communities, many chemical agents are liable to be ingested, as medicinal drugs, as preservatives added to food or as contaminants following the use of insecticides; for the most part, these agents are ingested in small amounts but some of them, such as the food preservatives, are consumed over long periods and there must be the possibility that they can act as haptens and thus be potentially dangerous. Thirdly, there are bacterial and viral antigens; some of these come from ingested micro-organisms but in addition the large intestine is

teeming with bacteria and viruses and it is conceivable that the antigens of the normal intestinal flora may play a role in disease.

Apart from immunological reactions to these exogenous antigens, the gastro-intestinal tract may be involved in auto-allergic processes. This may occur either in diseases which are primarily those of the gastro-intestinal tract itself or alternatively when the gastro-intestinal tract becomes affected during a generalized auto-allergic disease, such as systemic lupus erythematosus.

PHYSIOLOGICAL ASPECTS

Profound changes occur in the gastro-intestinal tract soon after birth and these changes are of great immunological significance. In mammals during foetal development there are very few lymphocytes and plasma cells in the lamina propria of the mucosa, and the localized collections of lymphoid tissue represented by the Peyer's patches and small lymphoid follicles are ill-developed. After birth, the lymphoid structures develop and the mucosa acquires an infiltration of the lamina propria with lymphocytes, plasma cells and a few eosinophils. The lymphoid development and mucosal infiltration are almost certainly an immunological response to ingested bacteria because animals maintained in a germ-free state fail to show these changes (Thorbecke 1959). The full development of the localized collections of lymphoid tissue, such as Peyer's patches, is dependent upon the thymus because in animals thymectomized before birth this process is impeded. As far as the lymphocytes and plasma cells which infiltrate the mucosa are concerned, it appears likely that they are derived from the lymphocytes circulating in the blood. Gowans (1962) collected lymph from the thoracic duct of rats and labelled the lymphocytes with tritiated adenosine or with tritiated thymidine. When labelled lymph was transfused into the femoral vein of another animal of the same highly inbred strain, about 70% of the small lymphocytes could be recovered from the lymph during the next few days. The labelled small lymphocytes were found in large numbers in the spleen, in lymph nodes and in Peyer's patches, but only occasional ones were found in the mucosa of the gut. With labelled large lymphocytes, the picture was different. They left the blood but did not stay long in the lymphatic circulation; evidently they 'homed' into the mucosa of the gut where they appeared with the morphological characteristics of plasma cells. The reason for this predilection of the large lymphocytes for the intestinal mucosa is uncertain, but two main possibilities have been suggested. The first is simply that the gut is the richest reservoir of antigenic material and the large lymphocytes are attracted to it. The second is that, as the large lymphocytes were obtained from the thoracic duct, many of them would already have passed through the intestinal lymphatics and might have become sensitized in some way so as to influence their subsequent migration when transfused into another animal. In either event, the experiment demon-

strates one way in which the mucosa of the gastro-intestinal tract may become populated with lymphocytes and plasma cells when the newborn animal comes into contact with a normal germ-laden environment. This infiltration of the normal intestinal mucosa has been called 'physiological inflammation' by Sprinz (1962) but this seems an unfortunate choice of words to describe a normal process.

It seems likely that the lymphocytes and plasma cells of the intestinal mucosa are immunologically competent and are concerned with the production of immunoglobulins. It is known that the germ-free animal synthesizes much smaller quantities of γ-globulin than a normal animal (Wostmann 1962; Saquet, Varques & Charlier 1961; Sell & Fahey 1964), and it is possible that globulin synthesis is defective in the intestinal mucosa as well as elsewhere.

The immunoglobulins contained in the plasma cells of the gastro-intestinal tract have been studied by immunofluorescence by a number of workers, such as Rubin, Fauci, Sleisenger & Jeffries (1965), Crabbé, Carbonara & Heremans (1965) and Crabbé & Heremans (1966c). By using specific fluorescent antisera directed against each type of human immunoglobulin (γA, γM and γG) it has been shown that the majority of the plasma cells present in the normal mucosa of the entire gastro-intestinal tract contain γA-immunoglobulin. The recently discovered γD-immunoglobulin (Rowe & Fahey 1965), which occurs in trace amounts in normal serum, is found in very few plasma cells of the normal gastro-intestinal tract. Some biopsy specimens show cells which are morphologically plasma cells but which fail to react with any of the antisera for the four immunoglobulins already mentioned. Crabbé & Heremans (1966c) suggest three possibilities to account for this finding:

1. The cells in question contain only the light polypeptide chains of immunoglobulins.
2. These cells contain a still unidentified fifth type of immunoglobulin.
3. These cells contain no immunoglobulin.

The likelihood that the plasma cells of the gastro-intestinal tract are actually producing and liberating immunoglobulins is suggested by the finding that γA-immunoglobulin is present in large amounts in mucous secretions of the gastro-intestinal tract (Crabbé & Heremans 1966c). Antibody activity in human intestinal secretions was first demonstrated by Davies (1922) when studying the serological properties of the faeces in bacillary dysentery. More recently, the term 'coproantibodies' was introduced by Burrows, Elliott & Havens (1947) to embrace all those antibodies which can be recovered from faeces. The production of coproantibodies against cholera and other enteric infections has been studied in various laboratory animals and also in human volunteers and there is strong evidence that they are produced locally in the gastro-intestinal tract (Freter & Gangarosa 1963).

In foetal life, γ-globulins are transmitted from the maternal circulation to the foetus, in some animals by way of the yolk-sac and in others (including man)

by passage across the placenta. After birth, in certain animals γ-globulins are absorbed in considerable amounts by the small intestine from the colostrum and milk, although this process becomes minimal or ceases after the neonatal period. The intestine absorbs the γ-globulin by a process of pinocytosis and there is evidence that there is a controlled release of the protein from the intestinal epithelial cell so that the amount reaching the general circulation is largely independent of the concentration in the intestinal lumen. Apart from this controlling mechanism, there is also evidence of selection of particular γ-globulins. Brambell, Hemmings, Oakley & Porter (1960) found that Fragment III of a papain digest of rabbit globulin was transmitted to the foetal circulation as was whole γ-globulin, whereas Fragments I and II were transmitted far less readily; this suggests that Fragment III is capable of combining with cellular receptors. Morris (1963) extended these observations to the intestinal absorption of immunoglobulin in suckling mice and showed that the absorption of guinea-pig antibodies to *S. pullorum* was prevented by prior feeding with whole rabbit serum or the papain Fragment III, but not by papain Fragments I and II. This is interpreted as evidence of competition for cellular receptors. Another example of differential absorption of antibodies has been provided by Locke, Segre & Meyers (1964), who have found that 6.6 S globulin of ovine origin is much better absorbed by baby pigs than is 18 S globulin in the same preparation, a difference which is apparently due to the 18 S globulin lacking a structure corresponding to the Fragment III of the 6.6 S globulin. In some animals, post-natal intestinal absorption from the colostrum is the sole mechanism by which the young obtain antibodies; for example, an immunoelectrophoretic study has shown that calves are born with no γ-globulin in the serum but that it is present 2 hr after they have ingested colostrum, and neutralizing antibody to foot-and-mouth disease is transmitted during this process (Graves 1963). Another interesting feature is that the immunoglobulin may be modified during the process of transfer; for example, Brambell, Halliday & Hemmings (1961) found that bovine γ-globulin passes into the circulation when it is fed to young rats but bovine anti-brucella antibodies are not transmitted, suggesting that the antibody activity may be destroyed in transit, while Morris (1965) found that ingested complete antibodies are either not transmitted or are converted to incomplete antibodies during transmission. Comparable *in vitro* studies have been made by Bamford (1966) with everted intestinal sacs of young rats. By using antibodies and isotopically labelled preparations of γ-globulin, he demonstrated transmission across the intestinal wall and showed that the process is selective, homologous antibodies or γ-globulin being transmitted more readily than heterologous. The whole subject is covered in a recent review by Brambell (1966).

The fact that dietary proteins are antigenic has wide implications. Insufficient whole protein is absorbed from the gastro-intestinal tract to have any significance from a nutritional standpoint, but enough may be absorbed to actuate im-

munological mechanisms. Many years ago, it was shown that normal human infants being artificially fed gave positive precipitin and complement fixation reactions to cow's milk proteins (Anderson, Schloss & Myers 1925; Lippard, Schloss & Johnson 1936). Recently, Gunther *et al* (1962) have studied the effects of feeding cow's milk to healthy children during infancy. Taking babies with a low titre of antibodies to milk proteins in the cord blood (derived from the mother) it was found that the majority developed higher titres after being fed on cow's milk. Very high titres were found in babies who were fed on cow's milk early in infancy. There was no evidence of immunological tolerance no matter how early in infancy the cow's milk was fed to the babies; conversely, the possession of fairly high titres of antibodies in the cord blood did not encourage an enhanced response to exogenous cow's milk. Babies maintained on the breast for some months before being fed on cow's milk developed lower titres than those fed with it in early infancy. Gunther *et al* make the interesting suggestion that the susceptibility of bottle-fed babies to infection may sometimes be due to the diversion of the normal immune response against infective agents because of the overwhelming stimulus of the protein antigens in cow's milk. By contrast with the results obtained with cow's milk proteins, Gunther *et al* were unable to detect any antibody to human milk in 100 sera obtained from these infants, but there is no information to let one decide whether this negative finding represents failure to absorb whole protein of the mother's milk or whether such proteins are absorbed but provoke no immunological response.

These circulating antibodies to cow's milk proteins commonly persist throughout life, although the titres found in adults are usually lower than those in children (Taylor *et al* 1961). In the case of casein, there is evidence that antibodies are present in everybody if sought for at low titre (Wright *et al* 1962). Circulating antibodies to other dietary antigens (such as gluten and egg albumen) can be demonstrated in a considerable proportion of normal subjects. The only reasonable interpretation of this finding is that enough dietary protein is absorbed to evoke an immunological response and that this process continues throughout life. A large-scale serial study of dietary antibodies in ulcerative colitis has shown that the titres stay remarkably constant in any one individual over the course of a year (Wright & Truelove, 1965b) and our more limited observations on normal subjects are in agreement.

ALIMENTARY ALLERGY AND GASTRO-INTESTINAL ALLERGY

It is convenient for the purposes of clinical description to distinguish between alimentary allergy and gastro-intestinal allergy. Alimentary allergy denotes allergic responses to foods which are manifested by disturbances outside the gastro-intestinal tract, with symptoms such as asthma, migraine and skin

eruptions. Gastro-intestinal allergy is the term applied when the allergic response results in a gastro-intestinal disturbance, such as abdominal pain and diarrhoea. Sometimes both types of response may follow ingestion, so that distinction is not always clear cut.

Examples of alimentary allergy are numerous. Some individuals have atopic reactions to various shellfish, fruits such as strawberries or to some nuts, and develop urticarial eruptions. The reaction follows quickly upon the ingestion of the particular foodstuff so that the victim usually pinpoints the responsible agent. Other reactions, such as migraine, may not be traced to the offending foodstuff so easily. Skin tests appear to be of limited value and the use of elimination diets is probably the most effective approach to diagnosis if alimentary allergy is suspected. Desensitization can sometimes be achieved, once the offending foodstuff is recognized, by introducing it in tiny quantities into the diet and gradually stepping up the amounts ingested. The subject is covered in more detail by Miller & Keeney (1965).

Whenever a case of apparent alimentary allergy is encountered, it is worth considering whether the allergic response is to a food itself or whether a contaminant is the causative agent. For example, some patients have reacted to ingested milk and it has been demonstrated that their allergic response is to minute quantities of penicillin secreted into the milk by cows receiving it therapeutically (Vickers, Bagratuni & Alexander 1958). This raises the possibility that chemical additives in foods may act as haptens and provoke hypersensitivity reactions without the cause being readily recognized.

Gastro-intestinal allergy has been invoked as a possible cause of several important diseases and these we shall be discussing in detail in succeeding sections. However, it is worth mentioning here that considerable caution must be exercised before labelling a gastro-intestinal disturbance as allergic. For example, it has long been known that some infants cannot tolerate milk and respond to its ingestion by developing abdominal colic and diarrhoea. It is only in the last few years that it has been recognized that many of these infants suffer from deficiency of lactase in the small-intestinal epithelium (Dahlqvist 1962). Removal of lactose from the diet cures them. Since that time, the study of disaccharidase deficiency has progressed greatly and it has become plain that a number of adult cases of 'irritable colon syndrome' are examples of disaccharidase deficiency (Weser, Rubin, Ross & Sleisenger 1965).

COELIAC DISEASE AND IDIOPATHIC STEATORRHOEA

General Considerations

Coeliac disease typically makes its appearance in early childhood when the infant goes on to a mixed diet. It is characterized by failure to thrive, loose

motions containing excessive fat, and other evidence of faulty absorption such as vitamin deficiencies. The observations of Dicke and other Dutch workers established that wheat gluten is harmful to children with coeliac disease and that its strict exclusion from the diet results in clinical and biochemical reversion to normality (Dicke, Weijers & van de Kamer 1953; Weijers & van de Kamer 1953). Wheat gluten is a mixture of proteins and it is the gliadin fraction which is harmful. Complete hydrolysis of gliadin to amino acids renders it innocuous but partial hydrolysis to polypeptides leaves its harmful properties intact.

Idiopathic steatorrhoea (non-tropical sprue) is the name given to a similar disease occurring in adults. The present view is that most cases of idiopathic steatorrhoea represent coeliac disease manifesting itself overtly during adult life, so that the term adult coeliac disease is sometimes applied. Careful inquiry will often reveal that symptoms compatible with coeliac disease have been in existence since childhood, though not severe enough to have resulted in medical attention. A considerable proportion of the patients respond favourably when treated with a gluten-free diet. The term gluten-induced enteropathy is sometimes used for the illness, whether occurring in a child or in an adult, when its relationship to gluten is firmly established.

Tropical sprue is a separate though clinically similar disease which only occurs in certain areas of the tropics and which is unaffected by a gluten-free diet. It will not be discussed here.

Pathology

In coeliac disease and idiopathic steatorrhoea, the intestine shows no gross or naked-eye abnormalities but Paulley (1954) showed by obtaining surgical biopsy specimens from patients undergoing abdominal surgery that the small-intestinal mucosa showed atrophy of the villi. On the pathological side, the real progress followed the introduction of peroral small-intestinal biopsy by Shiner (Doniach & Shiner 1957; Sakula & Shiner 1957) since when extensive biopsy studies have been made. Both in children and in adults, the small-intestinal mucosa shows a virtually complete loss of villi, while the crypts are lengthened and the lamina propria is heavily infiltrated with lymphocytes, plasma cells and a sprinkling of eosinophils. Prolonged treatment with a gluten-free diet is associated with reversion towards normality, a process which appears to occur more readily in children than in adults. Histological deterioration follows the reintroduction of gluten into the diet.

One of the most convincing demonstrations of the harmful effect of gluten in idiopathic steatorrhoea was the experiment by Rubin *et al* (1962) in which they instilled a slurry of wheat gluten into the relatively normal ileum of patients on a gluten-free diet and observed rapid clinical, biochemical and histological relapse.

Theories of Aetiology

Two main theories have been advanced to account for the disease. The first postulates that there is lack of a specific enzyme in the intestinal epithelial cells and that in its absence polypeptides are absorbed and cause the pathological lesion of the mucosa. The second theory is that the disease represents a hypersensitivity reaction to gluten or one of its components.

As far as the enzyme theory is concerned, attempts have been made to isolate the specific polypeptide responsible for the harmful action of gluten but so far without success. Conversely, studies of biopsy specimens have failed to demonstrate any deficiency of a specific peptidase in the small-intestinal mucosa (Parkins 1966).

The other theory regards the lesion as an allergic response to ingested gluten.

Immunological Aspects

Berger (1958) administered gluten to some children with coeliac disease and found that this was followed by levels of circulating antibodies to gluten as measured by complement fixation which were much higher than those occurring in normal children. Employing haemagglutination, Taylor, Truelove, Thomson & Wright (1961) found that children with coeliac disease showed higher levels of circulating antibodies to a water-soluble peptic-tryptic digestion of gluten (Fragment III) than did healthy children. Similarly, adults with idiopathic steatorrhoea showed higher levels than normal adults. However, the patients with coeliac disease or idiopathic steatorrhoea also showed high titres of antibodies to other dietary proteins, namely, the three main proteins of cow's milk, and subsequently we have obtained analogous results with purified ovalbumin. Closely similar results were obtained by Alarcón-Segovia *et al* (1964). Heiner *et al* (1962) obtained positive precipitin tests against gluten and cow's milk proteins so frequently in children with coeliac disease that they suggested that the precipitin test might be useful in diagnosis. Precipitin tests were also included in a study by Kivel, Kearns & Leibowitz (1964).

By immunofluorescence, Rubin *et al* (1965) were able to demonstrate gliadin binding by the jejunal epithelium in patients with adult coeliac disease, whereas control subjects did not show this effect; in addition, they were unable to demonstrate any comparable binding of other dietary proteins, such as casein and ovalbumin. They were unable to demonstrate any evidence of gliadin being bound to immunoglobulin-containing cells in the lamina propria; the gliadin appeared to be deposited on the surface of the epithelial cells and apparently this phenomenon was not due to the gliadin being involved in any complement-fixing antigen-antibody complexes.

Attempts to demonstrate auto-antibodies have chiefly been negative. Malik *et al* (1964), using the indirect immunofluorescent technique and a fluorescinated

antiserum to human α- and β-globulins, obtained a reaction between fresh sera from patients with untreated coeliac disease and the cytoplasm of human jejunal mucosa and also monkey jejunum, the fluorescence appearing especially in the crypts of Lieberkühn. However, Rubin *et al* (1965) were unable to reproduce these results. We ourselves have studied a considerable number of children with coeliac disease and adults with idiopathic steatorrhoea and have compared the findings with those in control groups of healthy subjects matched for age and sex. Fluorescence in the crypts was often seen, but reading the results blind revealed no appreciable difference between the two groups of patients and their respective control groups.

It has already been mentioned that the abnormal small-intestinal mucosa in coeliac disease and idiopathic steatorrhoea is heavily infiltrated with lymphocytes and plasma cells. Rubin *et al* (1965) have demonstrated the presence of immunoglobulins in many of these mononuclear cells of the small-intestinal mucosa, and these immunoglobulins were present in control subjects as well as in coeliac disease, with A-immunoglobulin predominating in both. We have already referred, when dealing with physiological aspects, to the finding of Crabbé & Heremans (1966c) that cells containing A-immunoglobulin were the type most commonly found throughout the mucosa of the intestine. In a recent study, the same authors (1966b) have found scarcity of A-immunoglobulin in the serum of three patients with steatorrhoea (with the features of idiopathic steatorrhoea, including subtotal villous atrophy on jejunal biopsy). In addition, plasma cells containing A-immunoglobulin were relatively scarce in the jejunal biopsy specimens, while the number of cells containing M-immunoglobulin was greatly increased. On the other hand, Hobbs & Hepner (1966) found low serum levels of M-immunoglobulin in fourteen out of fifty-two patients with coeliac disease and no examples of severe deficiency of A-immunoglobulin.

Several associations with idiopathic steatorrhoea are of great interest in an immunological context. They are splenic atrophy, hypogammaglobulinaemia and lymphoma of the small intestine. The spleen is frequently very small in idiopathic steatorrhoea, whereas it is usually normal in coeliac disease in childhood. Various non-immunological explanations for the splenic atrophy have been suggested, such as that it may be an expression of chronic folic acid deficiency, but there is the possibility that it may represent an immunological disturbance such as exhaustion of the lymphoreticular system (McCarthy *et al* 1966). Steatorrhoea and other features resembling those of idiopathic steatorrhoea not infrequently occur in patients with hypogammaglobulinaemia, both of the congenital and of the acquired type (Allen & Hadden 1964; Gitlin, Cross & Janeway 1959), but the syndrome may be a separate entity as these patients often fail to respond to a gluten-free diet. It now seems fairly well established that lymphoma of the small intestine can arise as a complication of idiopathic steatorrhoea. Gough, Read & Naish (1962) described three cases of small-

intestinal lymphomata complicating chronic idiopathic steatorrhoea and subsequently the association has been confirmed by Harris *et al* (1966). It has been suggested by McCarthy *et al* (1966) that the relatively high risk of lymphoma may be related to immunological insufficiency, the suggestion being based on the theory of Burnet (1964) that the high incidence of malignancy in infancy and old age is due to immunological insufficiency.

CROHN'S DISEASE (REGIONAL ENTERITIS)

General Considerations

Crohn's disease is a chronic inflammatory disease of the intestine of unknown aetiology. Any part of the intestine may be affected, but the commonest site is the terminal ileum, and the condition is then often called regional ileitis. However, other parts of the small intestine may be affected, either as an isolated phenomenon or in association with regional ileitis, the separate lesions then being known as 'skip' lesions. The colon is not infrequently affected, sometimes in continuity with typical regional ileitis (the term regional ileocolitis then being used), sometimes with a 'skip' lesion in any part of the colon and sometimes when the colon alone is affected (primary Crohn's disease of the colon). The disease has a variety of effects and modes of presentation. Diarrhoea is common. Loss of weight and general malaise are frequent. Acute attacks of pain occur, often resembling acute appendicitis, and the lesions of Crohn's disease are not infrequently first found at laparotomy for suspected acute appendicitis. Disturbance of small-intestinal function is a common effect and any of the manifestations of the malabsorption syndrome may be seen. Anaemia, usually due to blood loss, but occasionally macrocytic, is fairly common. In children, failure to grow may be a prominent feature. Fistula formation is an important aspect of the disease, and may occur between the diseased segment and adjacent loops of intestine or may be peri-anal.

A number of complications occur remote from the intestine, such as uveitis, arthritis, ankylosing spondylitis, erythema nodosum, aphthous ulceration of the mouth and liver disease. These complications follow the same pattern as the complications of ulcerative colitis and will be discussed jointly for the two diseases.

Pathology

The affected part of the intestine is obviously inflamed to naked-eye examination. All coats of the intestinal wall are at first thickened and oedematous, although later fibrosis may predominate and fibrous stricture develop. In the active inflammatory stage, the serosal surface is obviously inflamed with diffuse hyperaemia and many petechial spots; the corresponding part of the mesentery

is thickened and oedematous, and enlarged mesenteric lymph nodes are often present. The mucosal surface is often marked by deep ulcerations which may penetrate into the muscle layers. Microscopically, there is widespread mucosal infiltration with lymphocytes, plasma cells and eosinophils. This cellular infiltration may be found in all coats of the intestinal wall, sometimes with very pronounced lymphoid hyperplasia. Characteristic granulomata may also be present in the submucosa, in the muscle coat, in the serosa and in the mesenteric lymph nodes. Hadfield (1939) considered that the earliest, and possibly the specific, pathological feature was lymphadenoid hyperplasia with the formation of non-caseating giant-cell systems in the submucosa. He regarded ulceration and fistula formation as secondary to the lesion in the submucosa and to the obstructive lymphoedema produced by it. Other pathologists have favoured a non-granulomatous inflammation as the primary lesion (Schepers 1945; Meyer 1960). Recently, Williams (1964) has described three main types of inflammation. Non-specific inflammation, characterized by chronic inflammatory cells, oedema, superficial ulceration and fissures, is the only finding in about 20%. Diffuse granulomatous inflammation with scattered histiocytes and foreign body type giant cells is found in about 30%. In the remaining 50%, focal granulomata are present in addition to the non-granulomatous inflammation. The granulomata themselves are histologically similar to those occurring in sarcoidosis.

Theories of Aetiology

The same main theories of aetiology have been advanced for Crohn's disease as for ulcerative colitis and we shall discuss them somewhat more fully when dealing with the latter disease.

In Crohn's disease, the frequent presence of granulomata has excited attention. The gastro-intestinal tract can react to a variety of stimuli by the formation of granulomata. Many types of foreign body will induce them, and granulomata have been seen in relation to starch, talc, suture materials and barium. Certain infections also produce this type of response, and tuberculosis is the important example of this category; although it should be noted that central caseation is common with the tuberculous lesion whereas it is exceptional in Crohn's disease. Eosinophilic granulomata may occur as a reaction to the herring parasite, *Eustoma rotundatum* (Voorhuis & Eijlers 1961).

Lesions resembling those of Crohn's disease have been reproduced experimentally by methods which block the intestinal lymphatics. Reichert & Mathes (1936) made repeated injections of crystalline silica into the mesenteric and subserosal lymphatics following an intravenous injection of *E. coli* and produced oedema of the submucosa and muscle layers with a thickening of the bowel wall. Chess *et al* (1953) fed dogs regularly with finely divided silica or talc powder and produced lesions resembling those of Crohn's disease. They developed the idea

that any ingested material with the property of piezo-electricity might produce the lesion because substances with this property produce a proliferative cellular response which may cause lymphatic blockage. Cholesterol is one such substance.

Immunological Aspects

The close similarity between the sarcoid granuloma and that seen in Crohn's disease raised the possibility that the one was the intestinal version of the other. However, Longcope & Freiman (1952) brought forward evidence to show that the gastro-intestinal tract is only occasionally involved in sarcoidosis. Williams (1965) found that the Kveim test was uniformly negative in regional enteritis even though he was careful to use an antigen of proven potency. Similarly, when tissue extracts obtained from patients with Crohn's disease have been used for skin testing, the results have been negative (Sanders 1964; Williams 1965). Sarcoidosis and Crohn's disease resemble one another in so far as the patients frequently are Mantoux-negative. In the case of Crohn's disease, this applies especially when the inflammation is of the granulomatous type (Williams 1965). On the other hand, patients with Crohn's disease show normal delayed hypersensitivity reactions to mumps skin antigen and to extracts of *Candida albicans* and of *Trichophytoa gypseum* (Binder, Spiro & Thayer 1966). We do not know of any attempts to measure the delayed hypersensitivity response by *in vitro* methods such as lymphocyte transformation.

Attempts to demonstrate circulating antibodies to intestinal mucosa appear to have yielded uniformly negative results with the immunofluorescent and precipitin techniques (Koffler, Minkowitz, Rothman & Garlock 1962; Kyle *et al* 1963; Harrison 1965a; Williams 1965). With haemagglutination, Lagercrantz, Hammerström, Perlmann & Gustafsson (1966) found high-titre reactions to rat faecal antigen in some patients with Crohn's disease, particularly when the colon was involved. Studies of other circulating antibodies, such as thyroid, gastric parietal cell and antinuclear factor have shown no preponderance in Crohn's disease over the findings in a healthy control group (Wright 1968).

Circulating antibodies to certain proteins have been measured in Crohn's disease. The patients with Crohn's disease showed no significant difference from the control group in respect of antibodies to casein and gluten Fraction III but were much more likely to show negative results in respect of antibodies to α-lactalbumin and β-lactoglobulin (Taylor, Truelove & Wright 1964).

ULCERATIVE COLITIS

General Considerations

This is a chronic inflammatory disease of the large intestine in which there is diffuse mucosal inflammation of a part or the whole of the organ. The disease most frequently presents as diarrhoea with blood and mucus present in the stools.

The faeces do not contain pathogenic bacteria such as shigellae which can also produce diffuse inflammation. The onset may be either acute or gradual, and the attack may vary from a mild illness to one of devastating severity. The usual course of the disease is for attacks of bloody diarrhoea to alternate with periods of complete freedom from symptoms (chronic intermittent type). Less commonly, the patient is never free from symptoms once the disease has become manifest (chronic continuous type) although the symptoms vary in severity from time to time. Some patients have a single attack and never have another; but the longer patients are followed, the fewer the number found with only the one attack.

Usually the diagnosis is easy because the rectum is almost always involved and diffuse inflammation is visible through the sigmoidoscope. A particular point worth remembering is that sigmoidoscopic and mucosal biopsy evidence of inflammation is often present when the patient is entirely symptom free between attacks.

Many complications occur. Some of them arise in and around the inflamed colon and are classed as the local complications. But, as with Crohn's disease, there are also many complications occurring remote from the colon, some of the most common ones being uveitis, arthritis, ankylosing spondylitis, erythema nodosum and liver disease.

Pathology

The mucosa of the affected part of the colon is diffusely inflamed and, in the more severe cases, gross ulceration is present. The lesions are characteristically more superficial than those in Crohn's disease, although in severe cases the ulceration may become deep and may even cause perforation. Microscopically, the mucosa is heavily infiltrated with lymphocytes, plasma cells and a few eosinophils; and another striking feature is the pronounced dilatation of the tiny blood vessels of the mucosa itself. With more severe disease, polymorphs become a feature of the cellular infiltrate, and they may accumulate in dilated crypts of Lieberkühn, giving rise to crypt abscesses. With gross ulceration, areas of the mucosa are lost and replaced by granulation tissue. In the healing phase, lymphoid aggregates become a prominent feature, both in the mucosa and in the submucosa.

Theories of Aetiology

The aetiology of ulcerative colitis is essentially unknown but several theories have been suggested, the chief ones being:

Infective

The diffuse inflammation of the colon closely resembles that seen in acute bacillary dysentery. It is therefore not surprising that repeated attempts have

been made to find a specific infective agent. Various bacteria and fungi were suspected and in turn discarded as causative. Recently, several studies have examined the possibility of the disease being a virus infection, but with negative results. The possibility of a virus infection cannot be definitely excluded but at the moment there is no positive support for it.

Nutritional

Deficiencies of various vitamins may cause inflammation of mucosal surfaces, A diet deficient in folic acid, for example, will cause diarrhoea in monkeys, and diarrhoea is also a prominent symptom of pellagra. However, there is no firm evidence to support the view that ulcerative colitis is a deficiency disorder.

Psychosomatic

Many physicians regard ulcerative colitis as a psychosomatic disorder. It has been established that attacks of the disease frequently follow closely upon a disturbing emotional event, such as the loss of the patient's mother or of some other person acting as a mother substitute. Personality studies have shown that the patient with ulcerative colitis frequently has a distinctive character, being shy, tidy and self-restrained. It is difficult to obtain scientific proof of the importance of psychological factors and it is possible that they act as no more than precipitants of the overt attack.

Immunological

The possibility that ulcerative colitis may be an allergic disease has been entertained for the past 40 years, but it is only during the last few that the development of modern immunological techniques has made possible any precise scientific evaluation of the possibility. The evidence will be considered in the next section.

Immunological Aspects

It has been established experimentally that the colon can be involved in immunological responses, such as the Schwartzmann and the Auer reactions (Kirsner 1961, 1965). However, it has so far proved impossible to create by immunological methods a chronic disease in animals with the precise features of ulcerative colitis. The acquisition of knowledge has been hampered by this lack of a satisfactory experimental model of the disease.

The pathological changes in the mucosa in ulcerative colitis are compatible with the disease being an expression of a hypersensitivity reaction. We have already mentioned that lymphocytes, plasma cells and eosinophils are found in the normal colonic mucosa; these cells, which are involved in the development of hypersensitivity reactions, show a pronounced increase in ulcerative colitis.

When dealing with physiological aspects, we mentioned that the three main immunoglobulins are found in the plasma cells of the colon, with A-immuno-

globulin predominating. The recently described D-immunoglobulin is found in very few plasma cells of the normal intestine, but Crabbe & Heremans (1966a) have found many plasma cells containing this immunoglobulin in rectal biopsy specimens taken from a patient with ulcerative colitis. Whether this is a common feature in ulcerative colitis and, if so, what significance it may have are issues for further research.

The eosinophil is a cell which appears to be implicated in many examples of allergic disease. It is therefore of interest that in ulcerative colitis there may be many eosinophils circulating in the blood, located in the colonic mucosa and present in the colonic exudate. Following isolated reports of eosinophilia in ulcerative colitis, Riisager (1959) made a systematic study of a few patients over a period of time and demonstrated that eosinophilia commonly accompanied frank attacks of the disease. Tissue eosinophilia has been noted by many observers who have examined large numbers of histological sections of the colon in ulcerative colitis. Eosinophilia has been found to be more common in the inflammatory exudate than in the blood (Riis & Anthonisen 1964). Recently we have made systematic studies of circulating and tissue eosinophils in seventy-seven patients who were the subjects of a controlled therapeutic trial of various diets over the course of a year (Wright & Truelove 1966b). In some patients there was a rise in the circulating eosinophil count during a clinical relapse of the disease and the count was significantly higher when there was histological evidence of inflammation in rectal biopsy specimens than when the biopsy appearances were normal. The findings with tissue eosinophils in the rectal biopsy specimens were similar, although there was only a rough correlation between circulating and tissue eosinophils.

There is good evidence that eosinophils antagonize histamine and this might explain the accumulation of these cells at the site of antigen-antibody reaction since histamine is liberated in the process. Speirs believes that the function of the eosinophil is not simply that of detoxification but that it is directly concerned in antibody synthesis by transporting antigen to antibody-forming cells (Speirs 1960; Speirs & Speirs 1964). However, Sabesin (1963) used electron microscopy to demonstrate phagocytosis of ferritin-antibody complexes by eosinophils and concluded that this may merely represent a specific attraction of eosinophils by immune complexes and that they need not be implicated in the production of antibody or in the mediation of the hypersensitive state. Whatever may be the exact role of the eosinophils in hypersensitivity reactions, there seems no doubt that they are concerned with the removal of histamine, and Welsh & Greer (1959) have demonstrated phagocytosis by eosinophils of the granules of mast cells which are known to contain histamine. This is of special interest in relation to ulcerative colitis because mast cells as well as eosinophils are increased in the colon in this disease (McAuley & Sommers 1961). According to Archer (1963) the mast cells and eosinophils are not distributed in parallel unless the mast cells

have become degranulated and are releasing histamine. McGovern & Archer (1957) attempted to link observations on the presence of mast cells in the colon to the psychosomatic theory of the causation of ulcerative colitis, postulating that emotional stress could result in the colon being bombarded with nervous impulses which in turn would act by disrupting mast cells. Whether the prevalence of mast cells has any connection with psychosomatic aspects of ulcerative colitis must be regarded as problematical but there is certainly the possibility that the mast cells are intimately involved in the inflammatory process and this is discussed in detail by Sommers (1966).

The possibility that the proliferation of mast cells in the colon may be the consequence of an immunological disturbance receives some support from the finding that circulating basophils (which are closely related to mast cells) are increased during a relapse of ulcerative colitis (Juhlin 1963). It has also been found that basophils become numerous in the skin of patients with ulcerative colitis in response to non-specific local antigenic stimulation (Priest, Rebuck & Havey 1960).

Detailed studies of the serum protein patterns by electrophoresis have yielded somewhat conflicting results. Most workers have found an increase in α_2-globulins, a change which is regarded as non-specific. Some workers have also found a high γ-globulin (Lagercrantz, Winberg & Zetterström 1958; Bicks, Kirsner & Palmer 1959) but this observation has not been confirmed by Soergel & Ingelfinger (1961) who found a relative, but not an absolute, increase. It appears to us that a difficulty in interpreting the findings in ulcerative colitis comes from the loss of proteins which may occur from the inflamed mucosa of the bowel, so that an increased production of proteins might be masked.

Reactions to Dietary Antigens

Andresen (1925) was the first to claim that ulcerative colitis is an allergic disease when he reported a case which he judged to be the result of allergy to cow's milk. He continued to study this possibility and much later reported on fifty patients with the disease in whom he judged food allergy had been demonstrated in two-thirds, basing his results on the effects of withdrawal of various foods and of their reintroduction into the diet. Among the foodstuffs he incriminated, cow's milk was the most important, but a number of others were sometimes judged to be at fault, such as wheat, eggs, fish, tomatoes, oranges and potatoes (Andresen 1942). Closely similar conclusions were reached by Rowe (1942).

Though long neglected, these views have met with revived interest during the last few years. Some patients were found to become symptom free on a strict milk-free diet and to relapse when milk was reintroduced (Truelove 1961). It was then found that circulating antibodies to the individual proteins of cow's milk were frequently found at higher titre in patients with ulcerative colitis than

in healthy control subjects (Taylor & Truelove 1961). These studies led on to a controlled therapeutic trial in which patients were allotted at random to a diet which they maintained for 1 year while being kept under medical supervision (Wright & Truelove 1965a). The patients on a milk-free diet fared better than those on a control diet which was essentially normal, with more being symptom free throughout the year and fewer who suffered repeated relapses. The best estimate appeared to be that roughly one patient in every five was symptom free on a milk-free diet but would have had repeated relapses on a normal diet. These clinical observations were supported by the findings obtained by serial rectal biopsy, which showed that a milk-free diet favourably influenced the severity of the inflammatory process (Wright & Truelove 1966c).

While the dietary trial was in progress, samples of blood were taken regularly for various observations, including immunological tests. Circulating antibodies to a number of dietary proteins were studied by passive haemagglutination. Employing whole cow's milk as the antigen, a strong correlation was found between initial high titres and an unfavourable course of the disease. No close correlation could be found between the results for the three main proteins of cow's milk tested individually and the clinical results obtained in the trial. Likewise, no correlation was found with gluten Fraction III and ovalbumin, which were also tested (Wright & Truelove 1965b). In the individual case, the levels of circulating antibodies gave no clear indication of whether a patient was likely to do well on a milk-free diet. This suggests that, if the harmful effect of milk is mediated immunologically, circulating antibodies do not provide the correct measure. Alternatively, the harmful effect may be non-immunological, such as due to lactose intolerance, which has been shown to exist in some patients with ulcerative colitis (Frazer *et al* 1966; Binder, Gryboski, Thayer & Spiro 1966).

Another approach has been made by Rider, Moeller, Devereux & Wright (1960). They injected various dietary antigens into the rectal mucosa of patients with ulcerative colitis and observed a sharp local reaction to some of them, which was confirmed by taking biopsy specimens from the affected sites; control subjects did not show these local reactions. The authors claim that dietary exclusion of the items causing local reactions was followed by a favourable clinical course. Unfortunately, no long-term follow-up study has been published on these patients and it is therefore necessary to view the study with a certain amount of scepticism, especially as it is known that the rectal mucosa in ulcerative colitis reacts readily to non-specific traumata (Mirvish 1960).

Reactions to Other Exogenous Antigens

Rowe, Rose & Uyeyama (1955) have claimed that some cases of ulcerative colitis represent an allergic response to pollen but there is no general agreement with this view.

Bucks & Rosenberg (1964) have created an inflammatory condition of the colon by daily intrarectal injections of 2,4-dinitrochlorobenzene (DNCB) in guinea-pigs already sensitized by dermal application. The relationship of this induced illness to ulcerative colitis in man remains obscure, but the experiment appears to show that the colon can react to a hapten.

Auto-allergic Reactions

Broberger & Perlmann (1959) were the first to describe auto-allergic reactions in ulcerative colitis. Using a phenol-water extract of human foetal colon to coat red blood cells, they demonstrated circulating anti-colon antibodies in a large proportion of children with ulcerative colitis, whereas control children gave negative results. In adults with the disease, this reaction is less frequently shown (Asherson & Broberger 1961). Other workers have studied circulating auto-antibodies by a variety of techniques, such as haemagglutination, coated collodion particles and complement fixation, and have obtained variable results; but some of those with negative findings employed adult human colon as the source of antigen and the possibility that bacterial contamination disturbed the reaction cannot be discounted.

The colonic antigens are lipopolysaccharides which appear to be chemically similar to the lipopolysaccharides extractable from Gram-negative bacteria and are usually macroglobulins although some sera contain antibodies of the 7S type as well. Colonic antigens, immunologically similar to those obtained from human foetal colon, can be extracted from the colon of adult germ-free rats and also from their faeces (Perlmann, Hammarström, Lagercrantz & Gustafsson 1965).

Using the immunofluorescent technique, it has been shown that the antigen is located in the cytoplasm of the colonic epithelial cell as well as being present in colonic mucus (Broberger & Perlmann 1962; Klavens 1962; Koffler *et al* 1962). By haemagglutination it has also been shown to be present in lymph nodes draining the colon in ulcerative colitis (Perlmann & Broberger 1960). In addition, it has been found by immunofluorescence in proliferating bile ducts and the goblet cells of the small intestine but not in a number of other tissues including liver, spleen, kidney, gall bladder and uvea (Koffler *et al* 1962; Wright & Truelove 1966a). The sera from some patients with ulcerative colitis have shown reactions with gastric glands, according to Harrison (1965a).

The immunofluorescent test is more specific but less sensitive than haemagglutination and the presence of colonic auto-antibodies can be demonstrated in only a minority of the patients with ulcerative colitis, there being 13.5% positive in 200 patients studied by Harrison (1965a) and 16% positive in 273 patients studied by Wright & Truelove (1966a). The antibody is a true auto-antibody as positive reactions are obtained with the patient's own colon. An interesting point is that colonic biopsy specimens obtained from a patient with a high titre of antibody in the serum gave strongly positive results with a much greater

proportion of ulcerative colitis patients than was obtained with normal colonic tissue and in addition a small proportion of healthy control subjects gave positive reactions. In other words, the use of this particular specimen of colon made the test much more sensitive and the results then resembled those obtained by haemagglutination. A formal comparison of the results obtained by haemagglutination and by immunofluorescence has been made by Lagercrantz *et al* (1966).

Whether measured by haemagglutination or by immunofluorescence, the colonic auto-antibody does not correlate closely with important clinical features of ulcerative colitis, such as clinical severity, duration, extent of colonic involvement and the development of extra-colonic complications (Harrison 1965a; Wright & Truelove 1966a; Lagercrantz *et al* 1966). An additional point is that the colonic auto-antibody persists after total colectomy.

The colonic auto-antibody has been found by some workers in conditions other than ulcerative colitis, such as Crohn's disease of the colon and amoebic dysentery (Lagercrantz *et al* 1966), whereas others have not demonstrated its occurrence (Harrison 1965a; Wright & Truelove 1966a). The discrepancies may be due to the use of different sources of antigen (human or rat) and the picture is not likely to be clarified until pure antigens are available.

Ulcerative colitis sometimes occurs in association with other diseases deemed to be auto-allergic in nature. They include systemic lupus erythematosus, lupoid hepatitis, thyroiditis, myasthenia gravis and pernicious anaemia (see Wright & Truelove 1966a, for references). There are several studies of the occurrence in ulcerative colitis of the auto-antibodies frequently found in some of the diseases just listed. Calabresi, Thayer & Spiro (1961) found a high incidence of antinuclear factor in ulcerative colitis but our studies and those of Harrison (1965b) show only a slight excess over the findings in matched healthy control subjects. Gastric parietal cell antibody is also somewhat more prevalent in ulcerative colitis than in health but the difference is not striking. The incidence of thyroid antibodies closely resembles that in healthy controls.

Another approach to the study of auto-allergy in ulcerative colitis has involved the use of colonic epithelial cells growing in tissue culture. When these cells are labelled with ^{32}P, the amount of radioactive isotope liberated can be used as a measure of cytotoxicity. The sera from patients with ulcerative colitis had no specific cytotoxic effect. However, the leucocytes from such patients liberated more ^{32}P than did leucocytes from control subjects, and were therefore cytotoxic as judged by this test (Broberger & Perlmann 1963; Perlmann & Broberger 1963). Confirmation of these findings has been provided by Watson, Quigley & Bolt (1966). It is also of interest that there have recently been reports that patients with ulcerative colitis show a local reaction when autologous leucocytes are injected intradermally (Long & Uesu 1964; Stoebner & Patterson 1965; Watson, Styler & Bolt 1965).

Reactions to Bacterial Antigens

Although, as already mentioned in the section on aetiology, there is no evidence that ulcerative colitis is directly due to infection, the possibility remains that bacterial antigens play a role in the disease. Felsen (1936) followed the progress of a large number of subjects involved in an outbreak of bacillary dysentery in Jersey City and found that some of them subsequently developed ulcerative colitis. It is commonplace to find that an infective gastroenteritis is often the trigger which fires off a patient's first attack of ulcerative colitis; an outbreak of diarrhoea and vomiting may affect a community and virtually all the affected members will be well again in a few days but in the occasional individual the diarrhoea persists and becomes bloody, and ulcerative colitis is diagnosed.

On the laboratory side, little recent work appears to have been done. One observation of great immunological interest is the discovery by Perlmann, Hammarström & Lagercrantz (1965) of a close relationship between a crude lipopolysaccharide extract from *E. coli* O14 and colonic antigens. This lipopolysaccharide contains, in addition to the type-specific O antigen, large amounts of a heterogenetic antigen (Kunin) known to be present in most members of the family *Enterobacteriaceae*. The sera of patients with ulcerative colitis frequently give high-titre reactions to this lipopolysaccharide whereas sera of control subjects only exceptionally do so. Another experiment showing the similarity between colonic antigen and bacterial antigens was performed by Asherson & Holborow (1966). They injected rabbits with *E. coli* and found that, as judged by immunofluorescence, the rabbits developed the same type of auto-antibody to colon as appeared after injecting them with rat colon. It must, however, be noted that the rabbits developed no colonic lesions.

THE REMOTE COMPLICATIONS OF ULCERATIVE COLITIS AND CROHN'S DISEASE

Ulcerative colitis and Crohn's disease differ from other diseases of the gastrointestinal tract in the pattern of manifestations remote from the tract itself. Whereas in idiopathic steatorrhoea virtually all the extra-intestinal complications can be attributed to malabsorption of nutrients, minerals or vitamins, the common extra-intestinal manifestations of ulcerative colitis and Crohn's disease are not so readily explained. Certain of these remote complications are likely to be clustered together, so that the same patient may suffer from two or more of them. As far as ulcerative colitis is concerned, the situation has been summed up as follows: 'The remote complications which are specially likely to occur in the same subject, either concurrently or consecutively, are arthritis, ankylosing spondylitis, erythema nodosum, certain skin eruptions, aphthous ulceration of the mouth, and eye lesions' (Edwards & Truelove 1964). As far as Crohn's

disease is concerned, no comparable information has been published but our own clinical observations make us believe that the situation is exactly the same. A more precise study has been made of the frequency of sacroiliac abnormalities and uveitis in ulcerative colitis, and both of these were shown to be much more common than in a control group; and, in addition, the two abnormalities were powerfully associated with one another in the ulcerative colitis group (Wright, Lumsden, Luntz, Sevel & Truelove 1965).

These features of ulcerative colitis and of Crohn's disease raise interesting possibilities on the immunological side. It is conceivable that the same antigen is responsible for the abnormalities in the colon and in the remote organs which are frequently affected. If this is so, ulcerative colitis and Crohn's disease may be generalized diseases in which the brunt of the illness falls on the gastro-intestinal tract. Another possibility is that the lesions in remote organs are truly complications of the intestinal disease and are a consequence of bacterial antigens being absorbed from the diseased intestine.

One approach to the problem has been to look for a common antigen in the colon and in other organs of the body. Broberger (1961) has shown that sera from children with ulcerative colitis contain haemagglutinating antibodies which react with multiple antigens in colon, liver and kidney, but that some of the antigens are confined to the colon. Using immunofluorescence, Koffler *et al* (1962) found that sera from patients with ulcerative colitis which reacted with colonic epithelial cell cytoplasm also reacted with mucosal cells in the ileum and proliferating hepatic bile ductules, which is of interest as the small intestine is sometimes affected in ulcerative colitis and as chronic liver disease is an accepted complication. We have confirmed these findings but have found that human and rabbit sera containing antibodies to colon fail to react with human or rabbit uvea; and, conversely, that sera from patients with uveitis or from rabbits immunized with uvea fail to react with colonic epithelium (Wright & Truelove 1966a).

The whole issue is complex. In patients with certain remote complications of ulcerative colitis, total colectomy will effect a cure; this is seen with pyodermia gangrenosum, a lesion which has features suggestive of a Schwartzmann reaction. But with some other complications, total colectomy has no effect; for example, we have a patient with ankylosing spondylitis which has progressed steadily despite total colectomy.

CARCINOMA OF THE COLON

Loss of organ-specific antigens has been shown to occur in experimental and naturally occurring carcinomata. Nairn, Fothergill, McEntegart & Richmond (1962) showed that a gastro-intestinal specific antigen demonstrable in mucoid cells by immunofluorescence was totally absent from the large majority of colonic carcinomata but was uniformly present in benign polyps.

GENERALIZED DISEASES AFFECTING THE INTESTINE

A number of generalized diseases, in which immunological disturbances occur, may affect the intestine. Systemic lupus erythematosus frequently gives rise to gastro-intestinal symptoms (Dubois & Tuffanelli 1964) and sometimes there are frank pathological changes. Any part of the intestine may be involved, the lesions usually being the result of a vasculitis of the smaller arteries supplying the intestinal wall. Rarely, there is widespread ulcerative colitis indistinguishable from the idiopathic variety discussed earlier (Brown, Shirley & Haserick 1956; Kurlander & Kirsner 1964). Ulcerative colitis occurs not infrequently in chronic active hepatitis, whether or not this is associated with the presence of LE cells in the blood (Mackay, Weiden & Hasker 1965).

Polyarteritis nodosa frequently gives rise to abdominal symptoms which are the result of the arteritis of the vessels supplying the gastro-intestinal tract. Involvement of large branches of the mesenteric arteries may produce massive bowel necrosis, involvement of the smaller arteries may cause a patchy gangrene of the bowel wall, and submucosal arteriolitis results in mucosal ulceration (Finkbiner & Decker 1963).

Other types of allergic vasculitis frequently involve the viscera, including the gastro-intestinal tract (Winkelmann & Ditto 1964).

Dermatomyositis is a rare disease characterized by inflammation and degeneration of the skin and skeletal muscles, but fairly often affecting the gastro-intestinal tract because of the vasculitis which occurs (Couris, Block & Rupe 1964). Dermatomyositis is powerfully associated with malignant neoplasm, with the stomach and intestine prominent as the site of the neoplasm. The reason for the association is at present unknown.

Systemic sclerosis, sometimes known as scleroderma, as the skin may be heavily involved, is an unusual condition in which any part of the gastro-intestinal tract may be affected.

Henoch-Schönlein purpura (anaphylactoid purpura) frequently affects the gastro-intestinal tract and attacks of severe abdominal pain are a common feature.

Amyloid disease may occur more commonly than is generally supposed in association with Crohn's disease (Werther, Schapira, Rubinstein & Janowitz 1960). One theory is that the amyloid material may result from the accumulation of antigen–antibody complexes in the tissues (Koletsky & Stecher 1939).

DISCUSSION

The absence of satisfactory experimental models of the important diseases which we have been discussing means that most of the evidence bearing on the

immunological aspects of these diseases comes at present from the study of circulating antibodies in human beings. This has shown considerable differences between the patients with these diseases and healthy control subjects but the significance of the findings is still a matter for dispute.

Dietary Antibodies

In coeliac disease and idiopathic steatorrhoea, the titres of circulating antibodies to all the dietary antigens so far tested have proved to be frequently much higher than those in control subjects. The only other diseases of the digestive tract frequently associated with high-titre reactions are severe aphthous ulceration of the mouth and ulcerative colitis, but in neither of these is there such a preponderance of high-titre reactions to all dietary antigens (Taylor, Truelove & Wright 1964). The following are some of the possible explanations.

Increased Absorption of Protein

An increased absorption of whole protein or of antigenic moieties might be encouraged by defective digestion or by a damaged mucosa. As far as defective proteolysis is concerned, in pernicious anaemia (in which gastric proteolysis is negligible) and in pancreatic steatorrhoea (in which small-intestinal proteolysis may be grossly defective) the levels of circulating antibodies to common dietary antigens are not strikingly different from those in control subjects. It is therefore unlikely that defective digestion in itself predisposes to the development of high titres of circulating antibodies.

As far as mucosal damage is concerned, it is tempting to invoke this mechanism in coeliac disease and idiopathic steatorrhoea, where the mucosa of the entire upper small intestine is diffusely abnormal and could conceivably allow whole protein or antigenic moieties to leak through and thus set up high titres of circulating antibodies. Against this is the fact that the titres of circulating antibodies to the various dietary antigens do not run in parallel; thus, in individual patients, one or two of the circulating antibodies may be at very high titres while others are low, and every conceivable combination is seen. A further point against increased absorption of protein through a damaged mucosa being the sole explanation for the serological findings is the fact that Crohn's disease is not associated with high-titre reactions even though it is usual for mucosal ulceration and damage to be present.

High Titres as an Index of Hypersensitivity

Experimentally, high titres of circulating antibodies can be shown to reflect a state of hypersensitivity. For example, guinea-pigs injected with dietary antigens, such as purified cow's milk proteins, develop high titres of circulating antibodies against the particular dietary antigen injected. When a further injection of the antigen is given, the animals are likely to die rapidly in anaphylactic shock.

As far as coeliac disease and idiopathic steatorrhoea are concerned, the high levels of circulating antibodies to gluten Fraction III may be of pathogenic significance as it is well established that a gluten-free diet is highly beneficial to most of the patients with these diseases. Moreover, in collaboration with Frazer, Schneider, Morgan & Robinson (1963) we have found that fractions of gluten which yield serological reactions are also fractions which will induce clinical relapse when ingested. It has been shown that introduction of gluten into the distal small intestine is followed by rapid clinical relapse, accompanied by biochemical relapse and histological deterioration (Rubin *et al* 1962). The speed of relapse following such introduction of gluten is compatible with the relapse being due to an immunological mechanism. Likewise, the favourable and rapid response to corticosteroid therapy in these diseases is more in favour of an allergic response than of an enzyme deficiency.

On the other hand, coeliac disease and idiopathic steatorrhoea are frequently associated with high titres of circulating antibodies to dietary proteins other than gluten even though these proteins do not appear to be implicated in the pathogenesis of the disease in the majority of the patients.

Auto-antibodies

Of the diseases we have discussed in some detail, only in ulcerative colitis are auto-antibodies found in a considerable proportion of the patients. However, even in this disease, the significance of auto-immune reactions remains problematical. It is disappointing, if one is looking for some causal connection between the auto-antibodies and the progress of the disease, to find no obvious correlations between the finding of auto-antibodies and such important clinical aspects as severity of the illness, extent of the inflammatory process, the long-term course and the liability to remote complications. This lack of correlation between auto-antibodies and the clinical picture does not entirely rule out the possibility that the auto-antibodies play an important role in the pathogenesis of the disease. It is conceivable, for example, that they act synergistically with cellular antibodies.

Removal of the entire colon leaves any circulating auto-antibodies persisting, so that presumably their production is being stimulated from the small intestine. This raises the possibility that the stimulus to auto-antibody formation is bacterial, particularly as it is known that some strains of *E. coli* share a common antigen with colonic epithelial cells.

CONCLUSION

It is plain that there is ample evidence of altered immunological responses in several important diseases of the intestine, but at present the significance of these changes in terms of pathogenesis or of perpetuation of the diseases remains

obscure. As virtually all the data at present available have come from the study of circulating antibodies, it is possible that further developments may depend on the study of cellular aspects of hypersensitivity.

REFERENCES

ALARCÓN-SEGOVIA D., HERSKOVIC T., WAKIN K.G., GREEN P.A. & SCUDAMORE H.H. (1964) Presence of antibodies to gluten and milk fractions in patients with non-tropical sprue. *Am. J. Med.* **36,** 485.

ALLEN G.K. & HADDEN D.R. (1964) Congenital hypogammaglobulinaemia with steatorrhoea in two adult brothers. *Brit. med. J.* **ii,** 486.

ANDERSON A.F., SCHLOSS O.M. & MYERS C. (1925) The intestinal absorption of antigenic protein by normal infants. *Proc. Soc. exp. Biol. (N.Y.)* **23,** 180.

ANDRESEN A.F.R. (1925) Gastrointestinal manifestations of food allergy. *Med. J. & Rec.* (Suppl.) **122,** 271.

ANDRESEN A.F.R. (1942) Ulcerative colitis—an allergic phenomenon. *Am. J. Digest. Dis.* **9,** 91.

ARCHER R.K. (1963) *The Eosinophil Leukocytes.* Oxford: Blackwell.

ASHERSON G.L. & BROBERGER O. (1961) Incidence of haemagglutinating and complement-fixing antibodies. *Brit. med. J.* **i,** 1429.

ASHERSON G.L. & HOLBOROW E.J. (1966) Autoantibody production in rabbits. VII. Auto-antibodies to gut produced by the injection of bacteria. *Immunology* **10,** 161.

BAMFORD D.R. (1966) Studies *in vitro* of the passage of serum proteins across the intestinal wall of young rats. *Proc. R. Soc., B* **166,** 30.

BERGER E. (1958) Zur allergischen Pathogenese der Cöliakie, mit Versuchen über die Spaltung pathogener Antigene durch Fermente. *Bibliotheca Paediatrica* [supplementa ad Annales Paediatrici (Basel)], No. 67, 1.

BICKS R.O., KIRSNER J.B. & PALMER W.L. (1959) Serum proteins in ulcerative colitis. II. The effects of therapy correlated with electrophoretic patterns. *Gastroenterology* **37,** 263.

BICKS R.O. & ROSENBERG E.W. (1964) A chronic delayed hypersensitivity reaction in the guinea-pig colon. *Gastroenterology* **46,** 543.

BINDER H.J., GRYBOSKI J.D., THAYER W.R. & SPIRO H.M. (1966) Intolerance to milk in ulcerative colitis: a preliminary report. *Am. J. digest. Dis.* **11,** 858.

BINDER H.J., SPIRO H.M. & THAYER W.R. JR. (1966) Delayed hypersensitivity in regional enteritis and ulcerative colitis. *Am. J. dig. Dis.* **11,** 572

BRAMBELL F.W.R., HALLIDAY & HEMMINGS W.A. (1961) Changes in ^{131}I-labelled immune bovine γ-globulin during transmission to the circulation after oral administration to the young rat. *Proc. R. Soc., B* **153,** 477.

BRAMBELL F.W.R., HEMMINGS W.A., OAKLEY C.L. & PORTER R.R. (1960) The relative transmission of the fractions of papain hydrolyzed homologous γ-globulin from the uterine cavity to the foetal circulation in the rabbit. *Proc. R. Soc., B* **151,** 478.

BRAMBELL F.W.R. (1966) The transmission of immunity from mother to young and the catabolism of immunoglobulins. *Lancet* **ii,** 1087.

BROBERGER O. & PERLMANN P. (1959) Autoantibodies in human ulcerative colitis. *J. exp. Med.* **110,** 657.

BROBERGER O. (1961) Studies of the immunological relationship between the antigens from different organs in human ulcerative colitis. *Acta Paediatr., stockh.* **50,** 577.

BROBERGER O. & PERLMANN P. (1962) Demonstration of an epithelial antigen in colon by means of fluorescent antibodies from children with ulcerative colitis. *J. exp. Med.* **115,** 13.

BROBERGER O. & PERLMANN P. (1963) *In vitro* studies of ulcerative colitis. I. Reactions of patients' serum with human fetal colon cells in tissue cultures. *J. exp. Med.* **117,** 705.

BROWN C.H., SHIRLEY E.K. & HASERICK J.R. (1956) Gastrointestinal manifestations of systemic lupus erythematosus. *Gastroenterology* **31,** 649.

BURNET M. (1964) Immunological factors in the process of carcinogenesis. *Brit. med. Bull.* **20,** 154.

BURROWS W., ELLIOTT M.E. & HAVENS I. (1947) Studies on immunity to Asiatic cholera. IV. The excretion of coproantibody in experimental enteric cholera in the guinea-pig. *J. infect. Dis.* **81,** 261.

CALABRESI P., THAYER W.R. JR. & SPIRO H.M. (1961) Demonstration of circulating antinuclear globulins in ulcerative colitis. *J. clin. Invest.* **40,** 2126.

CHESS S., OLANDER G., PRESTON C.B., BENNER W. & CHESS D. (1950) Regional enteritis. Clinical and experimental observations. *Surg. Gynec. Obstet.* **91,** 343.

COURIS C.D., BLOCK M.A. & RUPE C.E. (1963) Gastrointestinal complications of collagen diseases. *Arch. Surg.* **89,** 695.

CRABBÉ P.A., CARBONARA O.A. & HEREMANS J.F. (1965) The normal human intestinal mucosa as a major source of plasma cells containing γ-A-immunoglobulin. *Lab. Invest.* **14,** 235.

CRABBÉ P.A. & HEREMANS J.F. (1966a) Presence of large numbers of plasma cells containing IgD in the rectal mucosa of a patient with ulcerative colitis. *Acta clin. belg.* **21,** 73.

CRABBÉ P.A. & HEREMANS J.F. (1966b) Lack of gamma A-immunoglobulin in serum of patients with steatorrhoea. *Gut.* **7,** 119.

CRABBÉ P.A. & HEREMANS J.F. (1966c) The distribution of immunoglobulin-containing cells along the human gastrointestinal tract. *Gastroenterol.* **51,** 305.

DAHLQVIST A. (1962) The intestinal disaccharidases and disaccharide intoleranoe. *Gastroenterology* **43,** 694.

DAVIES A. (1922) An investigation into the serological properties of dysentery stools. *Lancet* **ii,** 1009.

DICKE W.K., WEIJERS H.A. & VAN DE KAMER J.H. (1953) Coeliac disease; presence in wheat of factor having deleterious effect in cases of coeliac disease. *Acta paediat., Uppsala* **42,** 34.

DONIACH I. & SHINER M. (1957) Duodenal and jejunal biopsies. II. Histology. *Gastroenterology* **33,** 71.

DUBOIS E.L. & TUFFANELLI D.L. (1964) Clinical manifestations of systemic lupus erythematosus. Computer analysis of 520 cases. *J. Am. med. Ass.* **190,** 104.

EDWARDS F.C. & TRUELOVE S.C. (1964) The course and prognosis of ulcerative colitis. Part III. Complications. *Gut* **5,** 1.

FELSEN J. (1936) The relationship of bacillary dysentery to distal ileitis, chronic ulcerative colitis and non-specific intestinal granuloma. *Ann. intern. Med.* **10,** 645.

FINKBINER R.B. & DECKER J.P. (1963) Ulceration and perforation of the intestine due to necrolizing arteriolitis. *New Engl. J. Med.* **268,** 14.

FRAZER A.C., SCHNEIDER R., MORGAN D.B. & ROBINSON G.B. (1963) Malabsorption syndrome: biochemical aspects. *Proc. R. Soc. Med.* **56,** 469.

FRAZER A.C., HOOD C., MONTGOMERY R.D., DAVIES A.G., SCHNEIDER R., CARTER P.A. & GOODHART J. (1966) Carbohydrate intolerance in ulcerative colitis. *Lancet* **i,** 503.

FRETER R. & GANGAROSA E.J. (1963) Oral immunization and production of coproantibody in human volunteers. *J. Immunol.* **91,** 724.

GITLIN D., GROSS P.A.M. & JANEWAY C.A. (1959) The gamma globulins and their clinical significance. II. Hypogammaglobulinaemia. *New Engl. J. Med.* **260,** 72.

GOUGH K.E., READ A.E. & NAISH J.M. (1962) Intestinal reticulosis as a complication of idiopathic steatorrhoea. *Gut* **3,** 232.

GOWANS J.L. (1962) The fate of parental strain small lymphocytes in F_1 hybrid rats. *Ann. N.Y. Acad. Sci.* **99,** 432.

GRAVES J.H. (1963) Transfer of neutralizing antibody by colostrum to calves born of foot-and-mouth disease vaccinated dams. *J. Immunol.* **91,** 251.

GUNTHER M., CHEEK R., MATTHEWS R.H. & COOMBS R.R.A. (1962) Immune responses in infants to cow's milk proteins taken by mouth. *Int. Arch. Allergy* **21,** 257.

HADFIELD G. (1939) The primary histological lesion of regional ileitis. *Lancet* **ii,** 773.

HAMMARSTRÖM S., LAGERCRANTZ R., PERLMANN P. & GUSTAFSSON B.E. (1965) Immunological studies in ulcerative colitis. II. 'Colon' antigen and human blood group A- and H-like antigens in germfree rats. *J. exp. Med.* **122,** 1075.

HARRIS O.D., COOKE W.T., THOMPSON H. & WATERHOUSE J.A.H. (1966) Malignancy in adult coeliac disease and idiopathic steatorrhoea. *Gut* **7,** 710.

HARRISON W.J. (1965a) Auto-antibodies against intestinal and gastric mucous cells in ulcerative colitis. *Lancet* **i,** 1346.

HARRISON W.J. (1965b) Thyroid, gastric (parietal-cell) and nuclear antibodies in ulcerative colitis. *Lancet* **i,** 1350.

HEINER D.C., LAHEY M.E., WILSON J.F., GERRARD H., SCHWACHMAN H. & SHAW K.-T. (1962) Precipitins to antigens of wheat and cow's milk in celiac disease. *J. Pediat.* **61,** 813.

HOBBS J.R. & HEPNER G.W. (1966) Deficiency of γ-macroglobulin in coeliac disease. *Gut* **7,** 711.

JUHLIN L. (1963) Basophil leukocytes in ulcerative colitis. *Acta med. scand.* **173,** 351.

KIRSNER J.B. (1961) Experimental 'colitis' with particular reference to hypersensitivity reactions in the colon. *Gastroenterology* **41,** 307.

KIRSNER J.B. (1965) The immunologic response of the colon. *J. Am. med. Ass.* **191,** 809.

KIVEL R.M., KEARNS D.H. & LIEBOWITZ D. (1964) Significance of antibodies to dietary proteins in the serum of patients with non-tropical sprue. *New Engl. J. Med.* **271,** 769.

KLAVINS J.V. (1962) Demonstration of antibody in serum of ulcerative colitis which combines with cytoplasm of colonic mucosal cells. *J. Am. med. Ass.* **180,** 759.

KOFFLER D., MINKOWITZ S., ROTHMAN W. & GARLOCK J. (1962) Immuno-cytochemical studies in ulcerative colitis and regional ileitis. *Am. J. Pathol.* **41,** 733.

KOLETSKY S. & STECHER R.M. (1939) Primary systemic amyloidosis. Involvement of cardiac valves, joints and bones, with pathologic fracture of the femur. *Arch. Path. (Chicago)* **27,** 267.

KURLANDER D.J. & KIRSNER J.B. (1964) The association of chronic 'non-specific' inflammatory bowel disease with lupus erythematosus. *Ann. int. Med.* **60,** 799.

KYLE J., BELL T.M., PORTEOUS I.B. & BLAIR D.W. (1963) Factors in the aetiology of regional enteritis. *Bull. Soc. Internat. Chir.* **22,** 575.

LAGERCRANTZ R., WINBERG J. & ZETTERSTRÖM R. (1958) Extra-colonic manifestations in chronic ulcerative colitis. *Acta Paediatr., stockh.* **47,** 675.

LAGERCRANTZ R., HAMMARSTRÖM S., PERLMANN P. & GUSTAFSSON B.E. (1966) Immunological studies in ulcerative colitis. III. Incidence of antibodies to colon-antigen in ulcerative colitis and other gastro-intestinal diseases. *Clin. exp. Immunol.* **i,** 263.

LIPPARD V.W., SCHLOSS O.M. & JOHNSON A.P. (1936) Immune reactions induced in infants by intestinal absorption of incompletely digested cow's milk protein. *Am. J. Dis. Child.* **51,** 562.

LOCKE R.F., SEGRE D. & MYERS W.L. (1964) The immunologic behaviour of baby pigs. IV. Intestinal absorption and persistence of 6.6 S and 18 S antibodies of ovine origin and their role in the immunologic competence of baby pigs. *J. Immunol.* **93,** 576.

LONG P.I. & UESU C.T. (1964) Pyoderma gangrenosum. *J. Am. med. Ass.* **187,** 336.

LONGCOPE W.T. & FREIMAN D.G. (1952) A study of sarcoidosis: based on combined investigation of 160 cases including 30 autopsies. *Medicine (Balt.)* **31,** 1.

MCAULEY R.L. & SOMMERS S.C. (1961) Mast cells in nonspecific ulcerative colitis. *Am. J. digest. Dis.* **6,** 233.

MCCARTHY C.F., FRASER I.D., EVANS K.T. & READ A.E. (1966) Lymphoreticular dysfunction in idiopathic steatorrhoea. *Gut* **7,** 140.

MCGOVERN V.J. & ARCHER G.T. (1957) The pathogenesis of ulcerative colitis. *Australasia Ann. Med.* **6,** 68.

MACKAY I.R., WEIDEN S. & HASKER J. (1965) Autoimmune hepatitis. *Ann. N.Y. Acad. Sci.* **124,** 767.

MALIK G.B., WATSON W.C., MURRAY D. & CRUICKSHANK B. (1964) Immunofluorescent antibody studies in idiopathic steatorrhoea. *Lancet* **i,** 1127.

MEYER P.C. (1960) The pathogenesis of segmental enteritis. *Brit. J. Surg.* **47,** 375.

MILLER M.M. & KEENEY E.L. (1965) Gastrointestinal allergy. In *Gastroenterology*, vol. III, p. 1131, ed. BOCKUS H.L. Philadelphia: Saunders.

MIRVISH L. (1960) The mucosa of the rectosigmoid in ulcerative colitis. *S. Afr. med. J.* **34,** 732.

MORRIS I.G. (1963) Interference with the uptake of guinea-pig agglutinins in mice due to fractions of papain hydrolyzed rabbit γ-globulin. *Proc. R. Soc., B.*, **157,** 160.

MORRIS I.G. (1965) The transmission of anti-*Brucella abortus* agglutinins across the gut in young rats. *Proc. R. Soc., B* **163,** 402.

NAIRN R.C., FOTHERGILL J.E., MCENTEGART M.G. & RICHMOND H.G. (1962) Loss of gastrointestinal-specific antigen in neoplasia. *Brit. med. J.* **i,** 1791.

PARKINS R.A. (1966) The metabolic activity of human small intestinal biopsies in health and coeliac sprue. The effect of wheat gliadin. *Gastroenterology* **51,** 345.

PAULLEY J.W. (1954) Observations on the aetiology of idiopathic steatorrhoea. Jejunal and lymph node biopsies. *Brit. med. J.* **ii,** 1318.

PERLMANN P. & BROBERGER O. (1960) Auto-antibodies against antigen derived from colon in the microsomes of regional colonic lymph glands in human ulcerative colitis. *Nature (Lond.)* **188,** 749.

PERLMANN P. & BROBERGER O. (1963) *In vitro* studies of ulcerative colitis. 2. Cytotoxic action of white blood cells from patients on human fetal colon cells. *J. exp. Med.* **117,** 717.

PERLMANN P., HAMMARSTRÖM S., LAGERCRANTZ R. & GUSTAFSSON B.E. (1965) Antigen from colon of germfree rats and antibodies in human ulcerative colitis. *Am. N.Y. Acad. Sci.* **124,** 377.

PRIEST R.J., REBUCK J.W. & HAVEY G.T. (1960) A new qualitative defect of leukocyte function in ulcerative colitis. *Gastroenterology* **38,** 715.

REICHERT F.L. & MATHES M.E. (1936) Experimental lymphedema of the intestinal tract and its relation to regional cicatrizing enteritis. *Ann. Surg.* **104,** 601.

RIDER J.A., MOELLER H.C., DEVEREAUX R.G. & WRIGHT R.R. (1960) The use of an intramucosal test to demonstrate food hypersensitivity in ulcerative colitis. *Acta allerg. (Kbh.)* **15,** suppl. 7, 486.

RIIS P. & ANTHONISEN P. (1964) Eosinophilia in peripheral blood and inflammatory exudate in non-specific proctocolitis. *Acta med. scand.* **175,** 85.

RIISAGER P.M. (1959) Eosinophil leucocytes in ulcerative colitis. *Lancet* **ii,** 1008.

ROWE A.H. (1942) Chronic ulcerative colitis—allergy in its etiology. *An intern. Med.* **17,** 83.

ROWE A.H., ROWE A. JR. & UYEYAMA K. (1955) Chronic ulcerative colitis due to pollen allergy, with six case reports. *Acta med. scand.* **152,** 139.

ROWE D.S. & FAHEY J.L. (1965) A new class of human immunoglobulins. II. Normal serum IgD. *J. exp. Med.* **121,** 185.

RUBIN C.E., BRANDBORG L.L., FLICK A.L., PHELPS P., PARMENTIER C. & VAN NIEL S. (1962) Studies of celiac sprue. III. The effect of repeated wheat instillation into the proximal ileum of patients on gluten-free diet. *Gastroenterology* **43,** 621.

RUBIN W., FAUCI A.S., SLEISENGER M.H. & JEFFRIES G.H. (1965) Immunofluorescent studies in adult celiac disease. *J. clin. Invest.* **44,** 475.

SABESIN S.M. (1963) A function of the eosinophil: phagocytosis of antigen–antibody complexes. *Proc. Soc. exp. Biol. (N.Y.)* **112,** 667.

SAKULA J. & SHINER M. (1957) Coeliac disease with atrophy of the small intestine mucosa. *Lancet* **ii,** 876.

SANDERS R.C. (1964) An attempt to devise a skin test for regional enteritis. *Gut* **5,** 194.

SAQUET E., VARGUES R. & CHARLIER H. (1961) Études sur l'hypo-γ-globulinémie des animaux sans germe (germ-free), *Ann. Inst. Pasteur.* **101,** 703.

SCHEPERS G.W.H. (1945) The pathology of regional ileitis. *Am. J. digest. Dis.* **12,** 97.

SELL S. & FAHEY J.L. (1964) Relationship between γ-globulin metabolism and low serum γ-globulin in germfree mice. *J. Immunol.* **93,** 81.

SOERGEL K.H. & INGELFINGER J.F. (1961) Proteins in serum and rectal mucus of patients with ulcerative colitis. *Gastroenterology* **40,** 37.

SOMMERS S.C. (1966) Mast cells and paneth cells in ulcerative colitis. *Gastroenterology* **51,** 841.

SPEIRS R.S. (1960) In *Reticuloendothelial Structure and Function*, p. 113. ed. HELLER J.H. New York: Ronald Press Co.

SPEIRS R.S. & SPEIRS E.E. (1964) Cellular reactions to reinjection of antigen. *J. Immunol.* **92,** 540.

SPRINZ H. (1962) Morphological response of intestinal mucosa to enteric bacteria and its implications for sprue and Asiatic cholera. *Fed. Proc.* **21,** 57.

STOEBNER R. & PATTERSON M. (1965) Lymphocytic skin testing in ulcerative colitis and regional enteritis; a preliminary report. *Clin. Res.* **13,** 32.

TAYLOR K.B. & TRUELOVE S.C. (1961) Circulating antibodies to milk proteins in ulcerative colitis. *Brit. med. J.* **ii,** 924.

TAYLOR K.B., TRUELOVE S.C., THOMSON D.L. & WRIGHT R. (1961) An immunological study of coeliac disease and idiopathic steatorrhoea: serological reactions to gluten and milk proteins. *Brit. med. J.* **ii,** 1727.

TAYLOR K.B., TRUELOVE S.C. & WRIGHT R. (1964) Serologic reactions to gluten and cow's milk proteins in gastrointestinal disease. *Gastroenterology* **46,** 99.

THORBECKE G.J. (1959) Some histological and functional aspects of lymphoid tissue in germ-free animals. I. Morphological studies. *Ann. N.Y. Acad. Sci.* **78,** 237.

TRUELOVE S.C. (1961) Ulcerative colitis provoked by milk. *Brit. med. J.* **i,** 154.

VICKERS H.R., BAGRATUNI L. & ALEXANDER S. (1958) Dermatitis caused by penicillin in milk. *Lancet* **i,** 351.

VOORHUIS F.J. & EIJLERS W. (1961) Een onstekingstumor van de morg, waarschijnlijk veroorzaakt door de haringworm. *Ned. T. Geneesk.* **105,** 2542.

WATSON D.W., STYLER H. & BOLT R.J. (1965) The autologous leucocyte skin test in patients with ulcerative colitis. *Gastroenterology* **48,** 856.

WATSON D.W., QUIGLEY A. & BOLT R.J. (1966) Effect of lymphocytes from patients with ulcerative colitis on human adult colon epithelial cells. *Gastroenterology* **51,** 985.

WEIJERS H.A. & VAN DE KAMER J.H. (1953) Coeliac disease; criticisms of various methods of investigation. *Acta paediat., Uppsala* **42,** 24.

WELSH R.A. & GREER J.C. (1959) Phagocytosis of mast cell granules by the eosinophil leukocyte in the rat. *Amer. J. Path.* **35,** 103.

WERTHER J.L., SCHAPIRA A., RUBINSTEIN C. & JANOWITZ H.D. (1960) Amyloidosis in regional enteritis. A report of five cases. *Am. J. Med.* **29,** 416.

WESER E., RUBIN W., ROSS L. & SLEISENGER M.H. (1965) Lactase deficiency in patients with the 'irritable-colon syndrome'. *New Engl. J. Med.* **273,** 1070.

WILLIAMS W.J. (1964) Histology of Crohn's syndrome. *Gut* **5,** 510.

WILLIAMS W.J. (1965) A study of Crohn's syndrome using tissue extracts and the Kveim and Mantoux tests. *Gut* **6,** 503.

WINKELMANN R.K. & DITTO W.B. (1964) Cutaneous and visceral syndromes of necrotizing or 'allergic' angiitis: a study of 38 cases. *Medicine (Balt.)* **43,** 59.

WOSTMANN B.S. (1961) Recent studies on the serum proteins of germfree animals. *Ann. N.Y. Acad. Sci.* **94,** 272.

WRIGHT R., TAYLOR K.B., TRUELOVE S.C. & ASCHAFFENBURG R. (1962) Circulating antibodies to cow's milk proteins and gluten in the newborn. *Brit. med. J.* **ii,** 513.

WRIGHT R. & TRUELOVE S.C. (1965a) A controlled therapeutic trial of various diets in ulcerative colitis. *Brit. med. J.* **ii,** 138.

WRIGHT R. & TRUELOVE S.C. (1965b) Circulating antibodies to dietary proteins in ulcerative colitis. *Brit. med. J.* **ii,** 142.

WRIGHT R., LUMSDEN K., LUNTZ M.H., SEVEL D. & TRUELOVE S.C. (1965) Abnormalities of the sacro-iliac joints and uveitis in ulcerative colitis. *Quart J. Med.* **34,** 229.

WRIGHT R.S. & TRUELOVE S.C. (1966a) Auto-immune reactions in ulcerative colitis. *Gut* **7,** 32.

WRIGHT R. & TRUELOVE S.C. (1966b) Circulating and tissue eosinophils in ulcerative colitis. *Am. J. digest. Dis.* **11,** 831.

WRIGHT R. & TRUELOVE S.C. (1966c) Serial rectal biopsy in ulcerative colitis during the course of a controlled therapeutic trial of various diets. *Am. J. digest. Dis.* **11,** 847.

WRIGHT R. (1968) Awaiting publication.

CHAPTER 40

THE BRAIN AND NERVOUS SYSTEM IN ALLERGIC DISEASE

E.J.Field

INTRODUCTION

When Pasteur in 1876 introduced the treatment of rabies since known by his name, he stimulated investigation of the effects of inoculation of normal brain as well as those made from nervous tissue containing attenuated rabies virus. Several early reports appeared even before the turn of the century (e.g. Centanni 1898) suggesting that animals did not tolerate very well the injection of normal brain material. Aujeszky (1900) whilst attempting to immunize dogs against rabies by the inoculation of normal nervous tissue (10 ml of 1 in 11 bovine cord daily over 18 days) found the animals became thin, lost weight and some developed convulsions and paralysis. Similar observations were made with rabbits (Harvey & Acton 1923; Koritschoner & Schweinburg 1924). Weston Hurst (1932) summarized this early work. He himself was unable to establish any morphological basis in the central or nervous system for the disturbances shown by his animals.

In 1933, however, Rivers *et al* observed an 'encephalomyelitis with myelin destruction' in two of eight monkeys that had received repeated intramuscular

injections of aqueous emulsions or alcohol-ether extracts of normal rabbit brain. These results were confirmed and extended by Rivers & Schwentker (1935) whose work constitutes the point of departure for all modern studies.

The introduction by Freund & McDermott (1942) of adjuvants as a means of increasing immune response to an antigen led Isabel Morgan (1946, 1947) and Kabat *et al* (1946)—apparently working independently—to the discovery that their use with an emulsion of nervous tissue as a combined inoculum reduced very markedly the number of injections needed to produce the experimental encephalitis so laboriously brought about by Rivers. Soon it was found that the two or three injections used by Morgan and Kabat could be reduced to a single inoculation and that small laboratory animals such as guinea-pigs were highly susceptible (Freund, Stern & Pisani 1947). The facility with which the disease could be produced in small laboratory animals (rats, guinea-pigs, rabbits) ushered in a period of widespread activity in many laboratories all over the world. Largely on account of the marked potentiation brought about by adjuvant, the encephalomyelitis came to be regarded as allergic, though even as late as 1959 this adjective appeared in quotation marks in the title of a monograph devoted to the subject (Kies & Alvord 1959) since many difficulties and discrepancies continued to stand in the way of its acceptance as a truly immunological phenomenon. In the last 5 years, many of these have been at least partially resolved and the immunological pathogenesis of the condition is now generally recognized.

A further powerful stimulus to the study of EAE was the possibility that the experimental disease might bear a pathogenetic relationship to human multiple sclerosis (MS). The experimental study of this condition, of which a strikingly modern clinical and pathological account had been set out by Charcot as long ago as 1868 and 1872, had not fallen into the common pattern of investigation since no animal model disease had been produced. The announcement by Rivers and his co-workers in the mid-thirties that it was possible to produce in monkeys an encephalomyelitis accompanied by myelin destruction was naturally greeted with considerable hope and expectancy. Some years before, Glanzmann (1927) had suggested that the post-infectious encephalitis which was a rare sequel to some common childhood virus infections, might be allergic in origin and in 1932 van Bogaert extended the idea to embrace multiple sclerosis, largely on the basis of supposed pathological similarities, and this suggestion has received majority support over the intervening years.

EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN THE GUINEA-PIG

The disease will be described in some detail in the guinea-pig and major variations in other species indicated. It was Freund *et al* (1947) who first showed how

eminently suitable this animal was for the ready production and study of EAE, being much more susceptible than the mouse. They also pointed out that 'the lesions found in the monkey and in our own guinea-pigs differ in one important respect; namely, in the guinea-pigs demyelinization did not occur'.

Dose of Antigen

Guinea-pigs and rats are today the most widely used small laboratory animals for the study of EAE. Whilst some of the earlier workers gave relatively large doses of brain-adjuvant suspension (e.g. 0.5 or even 1.0 ml inoculated at several sites) the disease can be produced with regularity by inoculation volumes as low as 0.1 ml. Inoculation is most easily made into the dorsum of a hind paw and 0.1 ml of the mixture is introduced intracutaneously as far as possible. This ensures rapid and ready access to the regional lymph nodes both in the groin and popliteal fossa.

The quantitative relationship between adjuvant and antigen to give optimal results has been studied by Shaw *et al* (1962). It will be recalled (see above) that in the early experiments of Rivers no adjuvant at all was used. Bell & Paterson (1960) were able to produce EAE in rats without the use of adjuvant, whereas in the mouse Lee & Schneider (1962) found that the proportions of antigen and adjuvant were critical for the production of the disease. A sure and ready way of producing the disease in the guinea-pig (Hartley or strain 13) is to inject 0.1 ml of an aqueous suspension of 20% (w/v) human white matter made up in Freund's complete adjuvant (Difco) in the ratio of one part of brain to two of adjuvant. This corresponds with a dose of about 7 mg of white matter in 0.1 ml of the final mixture. If white matter be assumed to have a 70% water content then about 2.0 mg of dry weight of brain is contained in the inoculum. Freund's adjuvant (Difco) comprises Arlacel A (mannide mono-oleate); an emulsifying agent; (1.5 ml) Bayol F (paraffin oil) (8.5 ml); mycobacterium butyricum (killed and dried) 5 mg.

A good suspension may be stored for weeks at +4°C.

Clinical Course of the Disease

When injected as indicated above, more than 90% of animals become ill after 11–16 days. The first sign of illness is almost always a sudden weight decrease, normally about 20 or 30 g for a 400–500 g guinea-pig, occurring 24 hr or so before detectable weakness. Often an animal feels 'floppy' on being lifted from its cage. Weakness of one or both hind legs is shown by the ease with which the animal may be thrown over and it will sometimes remain resting on its side. Urinary incontinence and faecal impaction commonly occur as the posterior weakness becomes more marked. An animal may become completely paralysed in its posterior half and yet remain lively so that it drags itself about the cage and

feeds normally. The posterior half of an affected animal always shows marked wasting. About one-third to one-half of the animals die, death being usually preceded by a period of coma during which there may be occasional convulsions. Of the remainder, half recover gradually and often dramatically after a more or less prolonged period of spasticity in the hind limbs. The remainder suffer only a mild though for the most part definite disease, from which they recover rapidly and completely. A few animals die suddenly without prodromal loss of weight or weakness and these may show very few if any lesions in the nervous system beyond generalized vascular engorgement.

Histological Examination

Apart from muscle wasting and occasional bladder infection changes are found only at the site of injection, the draining lymph nodes and spleen. The injected foot is swollen and reddened; sometimes it is ulcerated. The groin and popliteal nodes are markedly enlarged. Microscopically, architecture of the regional lymph node is to a great extent disorganized by large numbers of macrophages with pale vesicular nucleus and faintly staining eosinophilic cytoplasm. Accumulations of paraffin oil may leave spaces in paraffin sections, and there are commonly foci of pyknotic polymorphs. Plasma cells are present but not as a rule in great numbers. These also occur in distant nodes such as the cervical, but can be very numerous in uninoculated animals so that their pathogenetic significance is doubtful. In the spleen plasma cell cuffs around arterioles are common.

No macroscopic changes are visible in the nervous system apart from hyperaemia. Earliest and mildest microscopic changes are in infiltration of the tela choroidea of the lateral and third ventricles with focal collections of lymphocytes and a few plasma cells. There is usually also a patchy infiltration of the meninges, especially over the base of the brain and the spinal cord in the vicinity of the nerve roots. Mild cases may show no more than this. Usually, however, there is a widespread perivascular infiltration with lymphocytes amongst which there is only an occasional plasma cell. This infiltration is principally in the white matter, though cuffs also occur in the cortex and basal ganglia (Plate 40.1). Careful examination of Zenker-fixed material stained by Unna-Pappenheim's method for plasma cells bears out the impression gained from haematoxylin and eosin stained material. Sometimes the lymphocyte cuffs are limited by the *membrana limitans gliae* (i.e. they remain within the Virchow-Robin space), but sometimes they spread out into the adjacent nervous parenchyma (Plate 40.2). Occasionally, especially when an animal has been comatose for some time before examination, considerable numbers of polymorphs are also present in the perivenous exudate. (If an animal has been dead some time before the brain is fixed for histological examination, then fragmentation of lymphocytic nuclei may occur so that casual examination may give a false impression of polymorphs.) Staining with Hortega's silver carbonate method for micro-

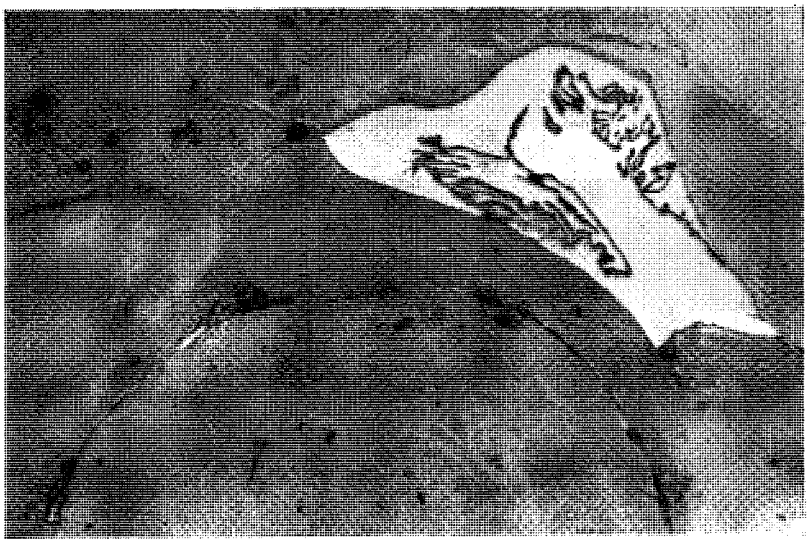


PLATE 40.1. Brain of guinea-pig with EAE showing widely disseminated perivascular lymphocytic accumulations. Closer examination shows these cuffs to be around small veins. Haematoxylin and eosin ×18.

facing p. 1066

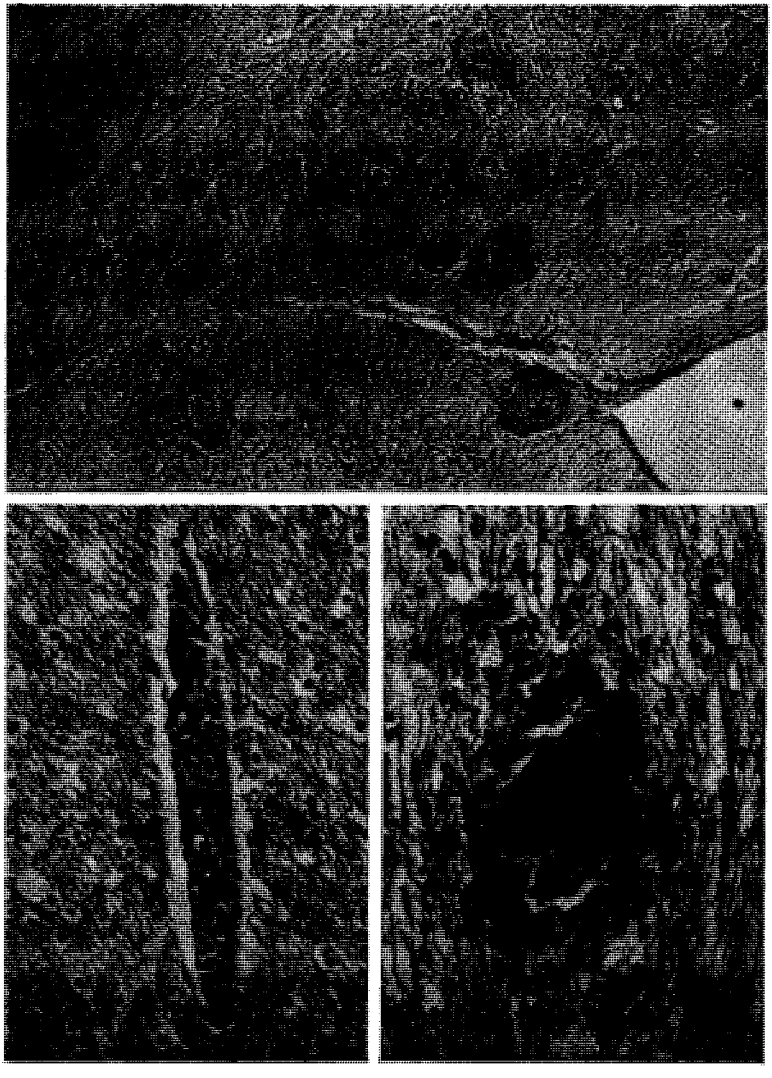


PLATE 40.2. (a) Inner angle of lateral ventricle shown in Plate 40.1. Note accumulations of lymphocytes limited to vicinity of some veins (lower right) but spreading out from others (above). Haematoxylin and eosin ×72. (b)

(b) Oblique section through small vein showing lymphocyte cuff strictly limited to the Virchow-Robin space. Haematoxylin and eosin ×350.

(c) Transverse section through small vein showing the cells of the perivascular cuff spreading out into the surrounding parenchyma ×350.

and oligo-dendroglia shows that even in the vicinity of small veins where no mononuclear cells have accumulated, microglia may be activated though oligodendrocytes remain normal. The accumulation of lymphocytes around small veins occurs chiefly in the white matter though they are by no means limited to it and such cuffing may accompany small veins inwards from the surface of the brain. The accumulated lymphocytes push aside the adjacent myelinated fibres so that in Weigert-Pal or Luxol Blue stained sections clear perivenous areas occur, though closer inspection shows them to be occupied by mononuclear cells and that very little if any actual myelin destruction has occurred. Whilst some of the mononuclear cells in the vicinity of a cuff are activated local microglia, some authors have thought them to be derived principally from the blood (Waksman 1959; Waksman & Adams 1962). Freund, Stern & Pisani (1947) stated that myelin destruction did not occur in EAE of the guinea-pig and certainly it is of great rarity and minimal in degree. Silver impregnation shows that whilst many axis cylinders remain intact, those which traverse a large lesion may show irregular swelling and beading. Here (as elsewhere in neuropathological changes) axis clyinders are relatively resistant (Plate 40.3).

A remarkable feature of EAE is the extraordinary way in which perivascular cell exudates may disappear completely if an animal which has recovered from severe paralysis is examined some weeks or months later.

Increased vascular permeability is an early event in the development of EAE (Barlow 1956; Vulpe, Hawkins & Rozdilsky 1960; Ridley 1963; Seitelberger & Jellinger 1963) and this has recently been confirmed by electron microscopic studies (Lampert & Carpenter 1965; Levine *et al* 1965; Field & Raine 1966). These latter studies have suggested to some authors (Lampert & Carpenter 1965) that sensitized lymphocytes attack myelin sheaths directly by insinuating themselves into the sheath along the line of the external mesaxon and gradually 'unwinding' the myelin spiral. Others (Condie & Good 1959; Bubis & Luse 1964; Field & Raine 1966) have found early changes in axis cylinders and the latter authors interpreted the appearances as best explained by the action of some diffusable agent rather than by direct attack by lymphocytes (Waksman's 'messengers of death') 'primed' to 'home upon the target organ' (Plate 40.4). The changes in axis cylinders so clearly antecede those in myelin that to speak of EAE as a 'demyelinating disease' is to emphasize differentially a feature which is apparently not primary. Anyone taking up a study of the disease without the years of conditioning imposed by the literature would have difficulty in recognizing it as a 'demyelinating condition' any more than JHM virus infection or visna. Indeed Adams & Kubik (1952) in a very careful survey of the morbid anatomy of the demyelinative disease wrote: 'Neuropathologists pretend to know what a demyelinative disease is,' and go on to give their opinion 'that there is probably no disease in which myelin destruction is the primary or exclusive pathologic change' and that 'the whole idea of a demyelinative disease

is more or less of an abstraction'. (Perhaps the nearest approximation is experimental diphtheritic neuritis.)

EAE IN OTHER SPECIES

In the *rat* (Lewis-Mai strain) EAE can readily be produced both with human brain (Field—personal observation) and with homologous material (Patterson). The ordinary laboratory white rat is susceptible to human brain but gives poor results with homologous material. Whilst the disease can be produced in *rabbits* it sometimes requires more than one inoculation. *Mice* are not so susceptible (Olitsky & Yager 1949) but are rendered more so by preliminary injection with *H. pertussis* vaccine (Lee & Olitsky 1955). Mice also show a very marked genetically determined susceptibility to the disease (Schneider 1959). *Dogs* are also susceptible (Thomas *et al* 1950; Shiraki & Otani 1959). Very severe lesions may readily be produced in the *rhesus monkey* by a single inoculation of human white matter in Freund's adjuvant. Lesions are often gross and confluent so as to be readily visible to the unaided eye (Plate 40.5). Haemorrhage (which is a rare occurrence in small rodents) is common and the condition is essentially a focal perivenous necrosis. Whilst all parts of the monkey nervous system may be affected, the cerebellum and mid-brain commonly show some of the most severe lesions (Plate 40.6). Animals which survive for some days may show many veins cuffed with sudanophilic compound granular corpuscles (Plate 40.7). In the more severe lesions axis cylinders as well as myelin sheaths are destroyed.

In the *human*, inoculation of foreign brain material may lead to an acute encephalomyelitis (Jellinger & Seitelberger 1958). When survival for some months has occurred, the lesions may heal with the production of areas closely resembling the plaques of multiple sclerosis (Uchimura & Shiraki 1957). These findings will be considered in more detail below.

ENCEPHALITOGENIC FACTOR(S) (EF)

Problem of Assay of Encephalitogenic Factor

The encephalitogenic activity of a given preparation derived from nervous tissue is estimated by the proportion of animals in which it produces EAE when injected (with Freund's adjuvant) at a given dosage. Strain, sex, weight, nutrition, etc., of animals should be standardized as far as possible. A further difficulty arises because the proportion of antigen and adjuvant may affect the apparent pathogenicity of the antigen. In order to introduce some uniformity of method Kies *et al* (1957) established a *disease index* based upon clinical and histological evidence of disease. In this simple system, a score of 0–5 is allotted

to an animal, the highest score going to an animal which dies or has to be killed with severe paralysis within 30 days of injection. This disease index is far from perfect and has not been widely adopted, though it does afford some quantitation of results within a given laboratory. It has been much criticized (e.g. for not taking account of the day upon which paralysis appears) and a more complicated index which seeks to incorporate the degree of lipaemia in EAE (a doubtful parameter of disease) has not been widely accepted. A full discussion of the quantitation of encephalitogenic activity is given in Chapter 7 of Kies & Alvord (1959).

Nature and Properties of Encephalitogenic Factor (EF)

Whilst there have been some suggestions that EAE may be produced by injection of tissues other than brain (Tal & Behar 1958) it is generally agreed that the antigen responsible for EAE is organ specific. In general, despite certain species differences, some of which have been noted above, nervous tissue from warm-blooded animals (but not from fishes or amphibians) is able to produce EAE in other species. Much work has gone into the isolation, purification and characterization of the 'encephalitogen'. Since poorly myelinated neonatal brain was deficient in encephalitogenic activity whilst white matter was much more potent than grey (Morgan 1947), the agent was believed to be associated with myelin. Recently more direct evidence of the association of the encephalitogenic factor (EF) with myelin has been brought forward by Laatsch *et al* (1962) who showed that activity was essentially associated with the greatly enriched 'myelin fraction' prepared by differential centrifugation of brain homogenate. Early studies of proteolipids from brain depended upon chloroform-methanol extraction procedures. Within the last decade much more potent encephalitogenic factors have been produced. It is now clear, however, that EF from whatever source it is prepared has the character of a strongly basic protein resembling a histone. Roboz-Einstein *et al* (1962) isolated an EF from lyophilized bovine spinal cord by extraction with water or sodium acetate solution of defatted bovine nervous tissue (acetone and petroleum-ether treated). After various purification procedures the EF obtained was active in doses of 8 μg (with Freund's adjuvant). Marion Kies about the same time developed a method of acid extraction of defatted nervous tissue (Kies & Alvord 1959; Kies *et al* 1961). Their most recent procedure, as applied to bovine cord or guinea-pig brain, has been described in detail by Kies *et al* 1965. Essentially the nervous tissue is defatted by prolonged extraction with chloroform-methanol mixture and the residue extracted with water and sodium chloride solution (when protein devoid of activity at the 0.1 mg level is removed). The leached residue is then extracted with 0.01 N HCl and as the pH falls to 2.5, encephalitogenic proteins are extracted. These are then precipitated with ammonium sulphate solution,

resuspended in a minimum volume of 0.1 N HCl and dialysed overnight in Visking tubing against a large volume of distilled water. Approximately 1% of the original dry weight is recovered in the water-soluble non-diffusible fraction. This material may be lyophilized and stored. Well-prepared material is capable of producing EAE in 50% of guinea-pigs when injected in doses of 1–2 μg (with Freund's adjuvant). In this method the encephalitogenic activity of the final product is nearly one hundred times that of the original dry nervous tissue on a weight basis.

EF, of whatever provenance, is strongly basic, though minor differences have been recorded (e.g. Caspary & Field 1965; Kies 1965; Wolfgram 1965). It has some remarkable properties. Thus it is unaffected when boiled for 1 hr or autoclaved for 15 min (15 lb sq. in.), either as lyophilized powder or made up in Freund's adjuvant (Caspary & Field 1965). Other experiments have shown it to withstand autoclaving for 6 hr. On standing at room temperature (especially at highly alkaline pH) it shows some loss of activity. It is susceptible to papain digestion (Kies *et al* 1965) and in some degree to tryptic digestion (Kies *et al* 1965; Caspary & Field 1965) though it is possible that EF made by different workers may have slightly different sensitivities.

The question of dialysability of EF is still unsettled. As long ago as 1949 Hottle *et al* reported that the encephalitogenic principle of nervous tissue was dialysable. Robertson *et al* (1962) reported that dialysate had high encephalitogenic activity even at 5 μg doses. More recently Lumsden *et al* (1966) and Carnegie & Lumsden (1966) have claimed that the active principle which passes the dialysis membrane is released by the spontaneous breakdown of larger basic polypeptides and may have a molecular weight as low as 4400–4800. Nakao & Roboz-Einstein (1965) have also isolated a dialysable encephalitogen from bovine spinal cord. However, the problem of dialysability is a complex one since the terms 'dialysable' and 'non-dialysable' are not absolute and the conditions of dialysis must be rigidly determined. Thus Thompson (1965) quotes Carr (1961): 'In dialysis with conventional membranes the non-diffusible substance has a particle weight of 40,000 or more. If the non-diffusible substance is smaller than this, then special effort must be made in selecting the proper membrane.' Kies *et al* (1965) agree that the basic encephalitogenic material obtained by acid extraction of defatted nervous tissue may be slowly dialysable under certain conditions but point out that dialysability is *per se* no indication of the peptide nature of EF. The question whether a small molecular weight peptide (about 4600) is indeed *the* encephalitogen (as claimed by Lumsden) and whether it exists as such or is merely an artefact of preparation (in the sense that it is broken off from a larger naturally occurring molecule) remains unsettled at the moment. What is clear, however, is that there appear to be several active encephalitogenic proteins obtained by various workers, amongst them a collagen-like material isolated by Kies *et al* (1958).

CIRCULATING ANTIBODIES

With the general acceptance of the notion that EAE is immunological in pathogenesis much attention had been devoted to studying antibody to brain in the blood of diseased animals. In studies carried out before purified encephalitogens were available, complement fixation tests using brain suspensions or extracts were used. As the result of many such investigations it became clear that whilst complement fixing (CF) antibodies were demonstrable, their presence had no fixed association with the development of EAE (Freund *et al* 1947; Hill 1949) and this conclusion has been sustained by later studies.

Since it has long been recognized that the use of Freund's adjuvant results in the development of delayed-type hypersensitivity (Dienes & Schoenheit 1929; Freund 1947), attempts were made to find out whether delayed (tuberculin-type) sensitivity was developed in EAE (Waksman & Morrison 1951). These authors concluded that intracutaneous testing with saline suspension of homologous spinal cord gave reactions with the gross and histologic characters of the tuberculin response.

The isolation of highly purified encephalitogens has enabled much more precise studies of circulating antibody and of delayed-type hypersensitivity to be carried out.

CIRCULATING ANTIBODY TO EF

Although a number of preparations of EF have now been made from human, guinea-pig and bovine nervous material, few studies of antibody to EF have so far been undertaken. With the tanned red cell method of Boyden, antibody to EF can be demonstrated in the blood of guinea-pigs immunized with brain and Freund's adjuvant, reaching its highest level at about 14 days, after which it tends to remain elevated for some time. Control animals injected with Freund's adjuvant alone likewise showed antibody production against EF, though in lesser degree (Caspary & Field 1965). This accords with the suggestion that EF and mycobacterium share some common antigen (Field, Caspary & Ball 1963). It is interesting in this connection to recall that Frick (1951) found that rabbits immunized with killed tubercle bacilli in lanolin and paraffin oil developed antibody both to tubercle antigen and to aqueous MS brain extracts.

Immunofluorescent studies of the ability of antisera prepared against nervous tissue or EF to combine specifically with myelin have been made. Staining of rabbit central nervous system myelin with conjugated serum from rabbits with EAE has been reported by Sherwin *et al* (1961) who found that myelinated fibres of human spinal cord were also stained. These workers produced their antiserum with rabbit spinal cord, thus confirming the non-specificity of antibrain serum. Earlier Beutner *et al* (1958) obtained positive staining of rabbit spinal cord with serum from one of four rabbits immunized with the same material. Both

groups of workers noted that there was no relationship between staining power of the antiserum and the onset of experimental disease in the animal from which the serum came. The same divorce was noted by Field, Ridley & Caspary (1963) who found serum to be active long before clinical illness was apparent and concluded that it 'seems unlikely, therefore, that the myelin fixing antibody can be of immediate pathogenetic significance in the production of EAE'. These workers also reported that whilst rabbit-antihuman brain serum attaches to guinea-pig myelin both central and peripheral, a similar serum made against human peripheral nerve combines only with peripheral nerve and nerve roots and is less avid than anti-brain serum. Recently Allerand & Yahr (1964) and Yahr & Allerand (1965) have reported upon the affinity of γ-globulin for human glia and myelin. They found that the 7S fraction of both normal and abnormal spinal fluid or serum has a specific affinity for myelin sheaths and for astroglial cells. This specific staining was not seen with other major fractions. Since no significant qualitative difference could be found between the γ-globulins from either spinal fluid or serum by this method, the authors do not feel that positive results can be taken as indicative of an auto-antibody. However, Rauch & Raffel (1964) using EF isolated by Roboz-Einstein and co-workers from bovine cord, prepared a rabbit antiserum to EF and found it to stain specifically myelin sheaths in sections of normal human, guinea-pig and bovine spinal cord. They concluded that EF is localized in myelin and that it lacks species specificity. Recently Kornguth *et al* (1966) have used an anti-EF serum to trace the appearance of EF in developing spinal cord. The discrepancy between these results and those of Allerand and Yahr remains unresolved. Pitfalls in the interpretation of immunofluorescent studies are well recognized and with EAE the method has on the whole given less clear-cut results than with experimental auto-immune thyroiditis (Balfour *et al* 1961; Beutner & Witebsky 1962; Mellors *et al* 1962).

Tissue Culture Studies

Following the basic work of Margaret Murray, Edith Petersen and Murray Bornstein in the United States it became possible to maintain explants of neonatal rat cerebellum for some weeks and observe the development of myelin sheaths *in vitro*. Using such cultures as test objects Bornstein & Appel (1961) demonstrated the presence in the serum of animals with EAE of some factor which was capable of causing myelin destruction (with little or no accompanying alteration in axis cylinders). Cultures of newborn rat cerebellum begin to show recognisable myelin sheaths after about 12 days *in vitro*, when they are most easily seen in bright field illumination. Only when they are relatively thick can they be recognized in polarized light. Many cultures show minor stigmata of degeneration in the form of blebs and beadings along the course of the myelin sheaths. Since these have a close resemblance to the changes brought about by a toxic

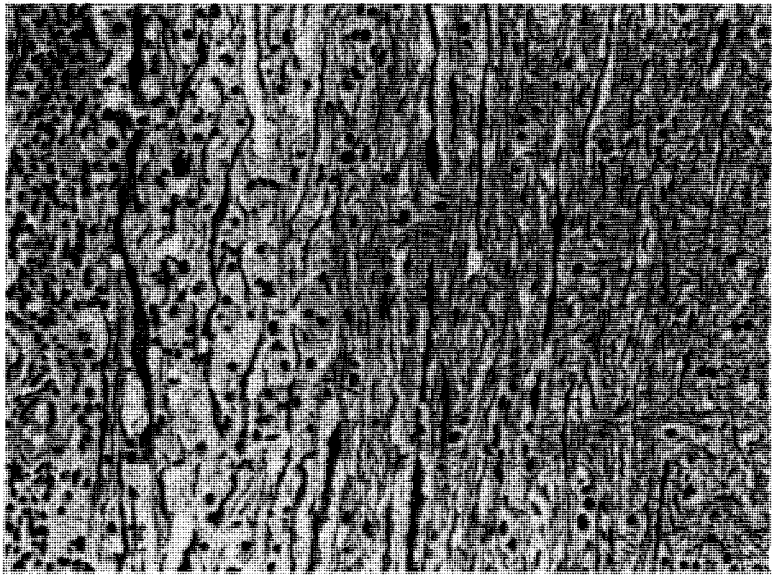
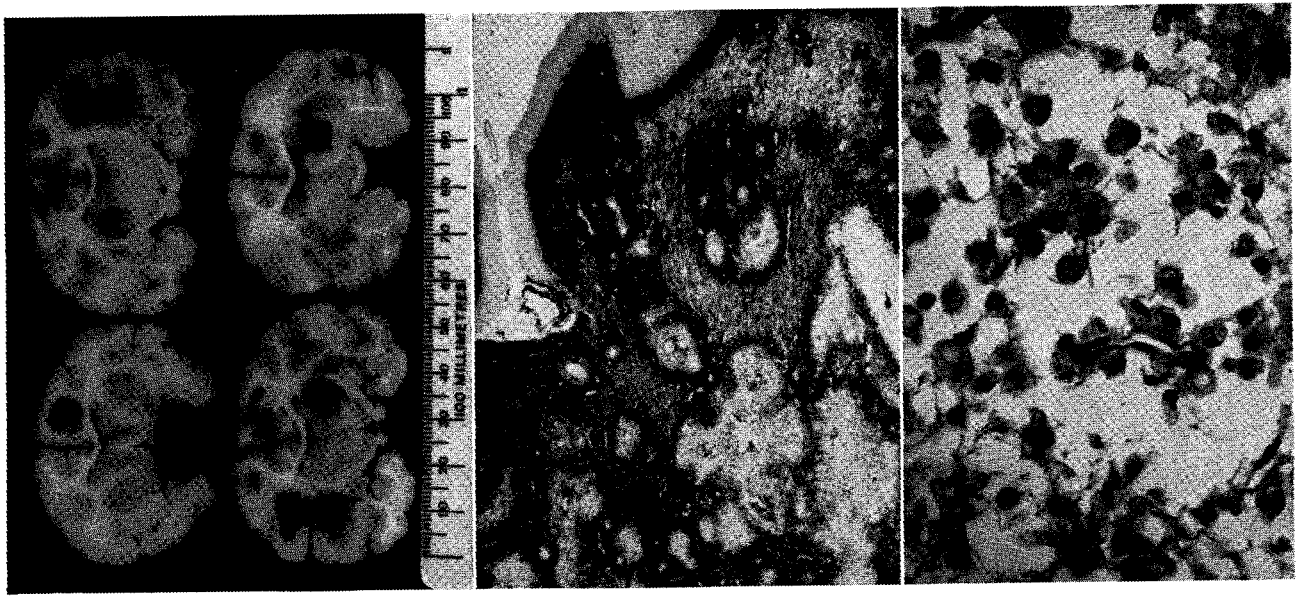


PLATE 40.3. Severe lesion in spinal cord of guinea pig with EAE. Normal axis cylinders to right. Those on the left are swollen, irregular and fragmented, and their myelin sheaths are destroyed. Bodian stain ×288.

facing p. 1072

PLATE 40.4. Electron micrograph of EAE lesion of guinea-pig, showing mononuclear cell infiltration round a small vein. Some are within the advential space around the vein (Lum = lumar) whilst others are present in the adjacent tissue. Early axonal changes in the form of an increased number of small dense mitochondria are present at A and B even though the myelin sheaths are here preserved. At C the sheath has disappeared leaving an axis cylinder crowded with degenerate mitochondria. There is no evidence of direct attack of sensitized lymphocytes upon myelin. Reynold's stain ×3600. (Preparation by C.S.Raine, B.SC.)



40.5 40.6 40.7

PLATE 40.5. Coronal sections through brain of monkey with EAE, killed *in extremis*. There are scattered necrotic haemorrhagic lesions which show no predilection for the angles of the ventricles.

PLATE 40.6. Cerebellum of monkey with severe EAE. Perivascular myelin destruction. Many lesions are confluent so that extensive areas of destruction are produced. Loyez stain ×14.

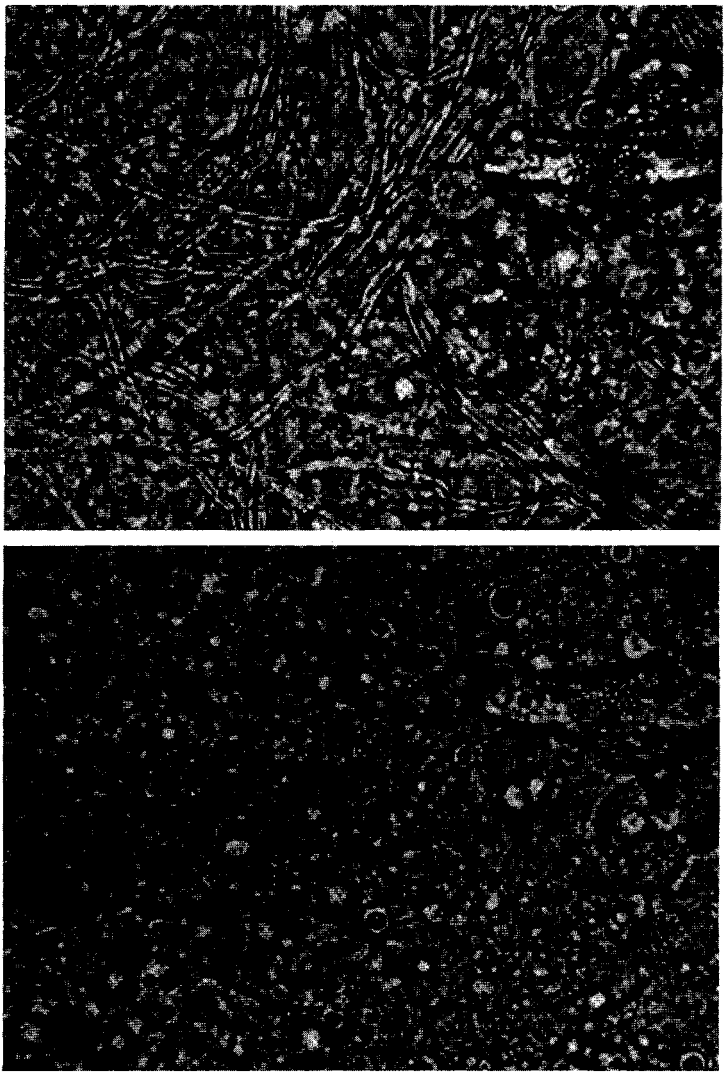


PLATE 40.8. (a) Culture of neonatal rat cerebellum 21 days *in vitro*. Myelin sheaths appear as parallel bright lines flanking axis cylinders. Bright field illumination.

(b) Same field 24 hr after application of 33% serum from a case of active MS. Note widespread myelin destruction leaving only scattered debris ×860. (Preparations by D. Hughes, B.SC.)

serum only perfect cultures can be used at the outset of a test. Application of a 20–33% myelotoxic serum in nutrient medium results in the myelin sheath becoming irregular and beaded within a few hours and they then break up into myelin ellipsoids. At the same time adjacent glial cells swell up and by pressure cause the nerve fibres to become devious in their course. Swollen glial cells contain granules in Brownian movement and if the reaction is severe the cells finally burst. A highly toxic serum may cause widespread devastation in a culture within 18 hr (Plate 40.8). Silver staining shows, however, that axis cylinders remain well preserved. According to Bornstein the early stages of demyelination are reversible in that if the toxic serum is removed and replaced by normal nutrient medium, the sheaths are gradually reconstituted over 2 or 3 days.

Bornstein's animal results have recently been confirmed by Lamoureux *et al* (1966), though their photographs are difficult to interpret. They moreover report toxicity for nerve cells and glia and this is a material departure from Bornstein's claim of clear-cut and specific myelotoxicity. Appel & Bornstein (1964) have found the demyelinating factor in EAE rabbit serum to be γ_2-globulin fraction requiring complement for its activity. The tissue-culture method is very sensitive, as little as 1 μg of γ-globulin being enough to produce demyelination. Their immunofluorescent study must, however, be viewed in the light of Allerand and Yahr's findings outlined above. From studies of human neurological disease, it is clear that myelotoxic factor is by no means limited to MS (for which EAE is commonly accepted as an experimental model —see below). Bornstein & Appel (1965), for example, found nine of fifteen cases of amyotrophic lateral sclerosis to have myelotoxic factor in the serum and this has been confirmed by Field & Hughes (1965). In a further critical study of the tissue-culture method, Hughes & Field (1967) have detailed some of the difficulties in assessing experimental findings. As with antibody to EF it seems very possible that circulating myelotoxic factor may be the result and not the cause of demyelination (such as it is) in experimental allergic encephalomyelitis. 'The failure to transfer EAE *in vitro* with large amounts of serum is a serious objection to the etiological significance of circulating demyelinating antibodies', write Appel & Bornstein (1964) and suggest that antibody may be only one of several factors in the pathogenesis of the disease.

Recently Bornstein & Crain (1965) have found serum from animals with EAE (at concentrations of 10–25%) to cause extensive alterations in the bioelectric properties of myelinated cultures long before morphological changes are demonstrable. Cerf & Carels (1966) have likewise shown a complement-dependent factor in the serum of acute multiple sclerosis patients capable of producing reversible alterations in the bioelectric response of isolated frog spinal cord. It is possible that access of such serum factors to nervous tissue around small vessels whose blood-brain barrier is lowered in the early stages of

EAE (see above) may be responsible for mild or transient disturbance both in animal and human disease.

It has been claimed that serum from EAE animals (and MS cases) is specifically gliotoxic. Unfortunately a high proportion of normal sera, especially when applied in high concentration to exposed glial cells with delicate branched processes *in vitro*, exhibit some degree of toxic activity. Berg & Kallen (1962) exposed glial cells to 100% serum. In our own experience results with serum more concentrated than 33% are to be treated with the greatest reserve. Actually whenever 'myelotoxic' activity is seen in a culture treated with a diluted serum, glial cells amongst the nerve fibres also show degenerative changes.

DELAYED HYPERSENSITIVITY

Cutaneous Hypersensitivity

The observations on delayed-type skin hypersensitivity made with crude brain extracts by Waksman & Morrison (1951) have been greatly extended since highly purified EF has become available. Much of this work has come from Marion Kies, E.C.Alvord, Jr. and their associates. Delayed-type hypersensitivity reactions may readily be elicited in rabbits with homologous or heterologous neural antigens (Waksman 1956; Hill 1949). However, in guinea-pigs only heterologous reactions were at first described (Waksman 1959; Chase 1959; Field 1961; Caspary & Field 1963) and this anomaly appeared especially puzzling since the guinea-pig is so sensitive in the production of EAE. Caspary & Field (1965) found no sensitivity to 1 μg of human EF introduced intradermally, though a markedly positive result was shown by non-encaphalitogenic aqueous extract of whole brain (1 μg of the EF with adjuvant was itself able to produce the disease). More recent work suggests that much higher intradermal doses of EF may be necessary to elicit a delayed-type response, though there is always the danger that such high doses (50 μg) may carry appreciable impurity with them. In a well-planned series of experiments designed to test the development of delayed-type hypersensitivity Shaw *et al* (1965) found it to be maximal about 11 days after challenge. In this work 15 μg of EF was used in 0.1 ml, though only 1 μ of PPD in simultaneous tests with purified protein derivative of *M tuberculosis*. With doses of 25 or 50 μg in this laboratory it has been confirmed that positive reactions (as measured by diameter of erythema around injection site) occur after about 9 days, though induration is relatively slight.

In general, then, delayed-type skin hypersensitivity can be demonstrated with purified antigen provided a large enough dose is injected into the skin and appears greatest shortly before the onset of neurological disturbance. Shaw *et al* (*loc. cit*) maintain that the reaction to a test carried out at 10 days is a good predictor of the onset of EAE within the next week, since all with a strongly

positive reaction (11 mm or more) developed EAE by 17 days, whilst none with negative reactions (4 mm or less) did so.

Transfer Studies with Sensitized Cells

Failure to induce EAE by intravenous or other administration of serum from animals in various stages of the disease has been referred to above. That transfer was indeed feasible was shown by the experiments of Freund & Lipton (1955) with parabiotic rats, when some success was achieved in establishing lesions of EAE in the parabiotic partner if the other was challenged with encephalitogenic mixture. These experiments could not by their nature distinguish between serum or cellular transfer; or indeed indicate whether both factors might be at work simultaneously. Moreover, EAE developed in the unsensitized partner 8 or 9 days after it was placed in parabiosis with the injected animal. However, although transplacental passage of antibody is known to occur in the guinea-pig, no lesions are found in the offspring of sows which come to term during the development of EAE. Failure to induce EAE by passive transfer of serum cannot be simply a failure of a circulating antibody to penetrate the blood-brain barrier since measures designed to breach it (e.g. production of a brain wound) do not facilitate development of the disease. On the other hand EAE localizes in and around lesions induced by cyanide (Levine 1960), reminiscent of the manner in which their location can be predetermined by setting up electrolytic damage before challenge with encephalitogenic mixture (Clark & Bogdanove 1955). Presumably in both Clark's and Levine's experiments, lowered blood-brain barrier was a factor in localizing the disease. The demonstration that 'myelotoxic antibody' is present in the serum of animals with EAE suggests that further attempts at transfer with serum should be made. It may be that massive doses over a narrow segment of time may be necessary (Chase 1959).

With the recognition that EAE was associated with a tuberculin-type delayed hypersensitivity to brain and that delayed-type sensitivities in general are referable to sensitized cells of the lymphocytic series (Chase 1945, 1954), many attempts have been made to transfer EAE by means of lymphocytes from a challenged animal. Early work was uniformly unsuccessful and contrasted sharply with the ready transfer of sensitivity in other cases (e.g. to tuberculin). Many difficulties stand in the way of a successful transfer by means of white cells (summarized by Chase 1959). Thus the lymphocytes must be viable; the donor must be highly sensitized and this will determine the number of cells necessary for the transfer; sometimes cells of sufficient number can only be obtained from several donors; and there may be a critical time at which cells are harvested.

The first success with sensitized lymphocytes was reported by Paterson (1960) using recipient rats which had been rendered tolerant of the donor lymph cells so that these might enjoy a prolonged survival time. This was achieved by pretreating recipient rats neonatally with normal rat spleen cells and resulted in

satisfactory transfer. Splenectomy of the donor (originally carried out in order to provide lymphocytes for inducing tolerance in the recipient) was at first believed to be a factor facilitating successful transfer, though later experiments showed it to be unnecessary (Paterson 1960). This manœuvre had been designed to induce homograft tolerance (Woodruff & Simpson 1955; Woodruff & Sparrow 1957; Ashley *et al* 1958). Some success was also achieved by Koprowski *et al* (1960) using highly inbred Lewis rats. They found histological evidence of EAE in four of forty-eight recipient rats, though none showed clinical signs. One of the four animals had received sensitized lymphocytes intracerebrally.

Later Koprowski (1962) used X-irradiated recipients for the injection of sensitized lymphocytes from splenectomized donor rats and noted that transfer results were in general better in females than in males. X-irradiation was likewise used by Astrom & Waksman (1962). It is interesting to note that Field (1961) found that X-irradiation increased rather than diminished (as expected) susceptibility of Hartley guinea-pigs to challenge with encephalitogenic mixture. Stone (1961) using highly inbred strain 13 guinea-pigs was able to achieve transfer by lymphocytes, the disease being described (on clinical grounds) as 'severe or lethal'. Unfortunately none of the accounts given by Paterson, Koprowski or Stone includes a full histological description with adequate photomicrographic records. When these do appear in the paper by Astrom & Waksman (1962), they show very mild lymphocytic infiltration of the meninges. Since the rabbits used in this work were known to harbour encephalitozoon cuniculi which can produce similar mild lesions, their significance is difficult to assess. In our own laboratory attempts to transfer EAE in strain 13 animals have not been successful. Even when 'primed' lymphocytes have been inoculated intracerebrally (60×10^6 cells) there has been no trace of EAE either near or away from the site of injection. It seems probable that Lewis rats are more amenable to this sort of experiment than guinea-pigs (Paterson 1966). Even in the Lewis rat, however, only 'a few recipients exhibited typical neurological signs' of EAE and these appear to have all been females. What success there has been in transferring EAE with lymphocytes seems dependent upon prolonged survival of the transferred cells by ensuring histocompatibility (i.e. using highly inbred animal strains) or immunological tolerance. The difficulties in the way of successful transfer of EAE contrast with the relative ease with which other delayed hypersensitivities may be transferred and the same applies to other 'experimental auto-immune diseases' involving peripheral nerve, thyroid or testis.

Some of the transmission experiments with lymph-node cells (LNC) have been carried out with the regional nodes draining the inoculation site. These nodes became markedly enlarged (see above) and must contain a certain amount of the original inoculum. The first-station draining nodes often show islands of necrotic polymorphs, very large numbers of macrophages and relatively few plasma cells. Second-station or more distant nodes are therefore to be preferred

for transfer attempts. However, there is good evidence that antigenic material soon becomes disseminated in the body, so that extirpation of the regional lymph node 1 hr after inoculation does not interfere with the production of disease (Freund & Lipton 1955). These authors also found oil droplets and the cellular reaction usually seen about mycobacteria in paraffin oil appear in other lymph nodes and in the lungs. Transfer experiments with regional lymph nodes are thus open to the objection that small amounts of antigen may have been carried across with cells (cf. Paterson 1966, p. 106). There is, however, evidence that circulating lymphocytes in the blood of sensitized animals are able to transfer the disease if given to recipients in sufficiently large numbers (Wenk, Levine & Warren 1967). This accords with the demonstration by Rauch & Raffel (1965) using an immunofluorescence method that lymphocytes (lymph node) from animals with EAE can take up EF. This is an important finding in implicating a cellular mechanism in the pathogenesis of the condition.

Tissue Culture Studies with Sensitized Cells

Koprowski & Fernandes (1962) described what they called 'contactual agglutination' of sensitized lymphocytes to glial cells in culture. Instead of the usual neonatal rat cerebellar cultures they used explants of newborn puppy brain and found that LNC from rats with EAE adhered to the surface of these cells in clumps. Later there was degeneration of the glial 'target' cells. Best results were obtained with LNC obtained about 9 days after the animal was injected. Using newborn rat cerebellar cultures in which glial cells are more clearly differentiated than appears from Koprowski's photographs, it has not been possible to obtain evidence of contactual agglutination in experiments set up in this laboratory. Further experiments by Koprowski and Fernandes raised the possibility that small amounts of 'circulating factor' might be located on lymphocytes and be responsible for the aggression of the lymphocytes towards glial cells ('lymphocyte cytophilic antibody'). Winkler (1965) has reported *in vitro* demyelination of peripheral nerve (trigeminal ganglion) with sensitized lymphocytes, though he was not able to see the 'contactual agglutination' described by Koprowski.

Macrophage Migration System

Macrophages play a prominent part in the skin-delayed hypersensitivity reaction (Dumonde & Glynn 1965) and appear to be derived largely from the monocytes of blood (Spector *et al* 1965). When sensitized animals (showing a delayed-type skin reaction) are injected intraperitoneally with the soluble antigen, adherence of macrophages to each other and to the peritonium is found, suggesting that these cells have been sensitized (Nelson & Boyden 1963). Techniques have now been evolved for studying quantitatively the migration of macrophages (George & Vaughan 1962; David *et al* 1964) and recently extended to the study

of EAE (David & Paterson 1965). They found that macrophages from guinea-pigs with delayed hypersensitivity were inhibited from migrating by adult rat nervous tissue (actively encephalitogenic), but not by neonatal rat brain (non-encephalitogenic). Work in this laboratory (Hughes & Field 1968) with purified EF (from human brain) has confirmed specific inhibition both with animals challenged with EF and with whole brain. Sensitivity of macrophages was first detected about 5 or 6 days (at about the time when appreciable skin hypersensitivity is demonstrable) and is maintained for 122 days after challenge, but appears weak after 5 months. There is some evidence, too, that kidney antigen may inhibit macrophage migration, though less strongly as a rule than brain. There is other evidence, too, that brain and kidney have a common antigen (Caspary & Field 1963; Caspary *et al* 1964; Winkler & Arnason 1966). It has recently been recognized that the apparent sensitivity of 'sensitized' macrophages to antigen may in fact result either from the presence of sensitized lymphocytes which are stimulated by presence of the antigen to a further production of antibody (Bloom & Bennett 1966) or to adsorption of 'cytophilic' antibody. The mechanism of the macrophage immobilization test is by no means settled. As with other 'delayed-hypersensitivity' tests there is some evidence indeed that it may involve a cytophilic antibody. The subject is discussed by Dumonde (1967).

Other 'Target System' Studies

Clarke & Geiger (1964) have found that the serum of guinea-pigs with EAE caused release of free fatty acid from slices of rat cerebrum whilst normal serum was without effect. Since this property of serum was found to reside in the globulin fraction the authors concluded that an antibody might be involved. It is interesting to recall that many EAE sera have a high fat content and this has been used in establishing a disease index (see above). Another system for detecting circulating antibody (Caspary & Comaish 1967; Caspary & Field 1967) has recently used the liberation of C^{14}-labelled serotonin from platelets as an index of antigen-antibody reaction (cf. Humphrey & Jaques 1955). By this means it is possible to demonstrate circulating antibody to EF in cases of MS and the method might well find application in the study of EAE.

Further evidence of sensitization of lymph-node cells in EAE comes from the demonstration by Kornguth & Thompson (1964) that EF will stimulate protein synthesis in LNC of animals injected with brain tissue or EF (in Freund's adjuvant).

PREVENTION OF EAE RESPONSE

Injection of Brain or Complete Adjuvant

Protection against the development of EAE on challenge with a potent encephalitogenic mixture was shown many years ago by Ferraro and his co-workers

(1949a, b, 1950) who found that prior injection of brain emulsions prevented an encephalitic reaction. They were inclined to believe that a deposit of injected nervous tissue might act as an auxiliary 'brain' which took up the antibodies responsible for EAE and so shielded the animal's own nervous system. It has long been recognized that guinea-pigs which recover from an attack of EAE are relatively immune to further challenge. Protection is also seen when guinea-pigs are given inoculations of Freund's adjuvant before challenge with a potent encephalitogenic mixture (Kies & Alvord 1958; Svet-Moldavsky and co-workers 1958, 1959, 1960). An analysis of the factors in tubercle bacilli responsible for protection was made by Cunningham & Field (1965) who found tubercle cell wall, intracellular tubercle material and PPD to be active, whilst mycolic acid, lipopolysaccharide and its derivatives were inactive. Encephalitogenic material extracted from nervous system is also protective (Shaw *et al* 1960) especially when injected in incomplete adjuvant.

EAE Convalescent Serum

In addition to their possible role in producing EAE, antibodies in the serum of animals which have developed disease can exert a protective action when administered to other animals challenged with encephalitogenic mixture (Paterson & Harwin 1963). These authors claim that the protectant is a 19S complement fixing (CF) antibody though there was no close relation between CF titre of serum and suppressive activity. This promising approach to the problem of protection against EAE deserves further study since unresolved difficulties remain and it is possible that a specific desensitization may be brought about (Uhr & Scharff 1960). Thus, it has, for example, been shown that guinea-pigs immunized with EF show high agglutination but low CF antibody titre, though they are resistant to challenge with encephalitogenic mixture (Caspary *et al* 1966). Species differences are undoubtedly of importance in this problem and Paterson (1966) has sought to establish an inverse ratio between CF antibody production and development of EAE. Whilst treatment of convalescent serum with 2-mercapto-ethanol, which destroys CF (19S) antibody, also abolishes protective activity against EAE, administration of the drug *in vivo* is without influence on the disease (Caspary & Simpson 1964).

X-irradiation

Since it is known that whole-body X-irradiation may suppress immunological reactivity if treatment is carried out before challenge (Uhr & Scharff 1960), the effect of similar treatment in suppressing EAE has been investigated. There are, of course, considerable species differences in tolerance of whole-body irradiation and correspondingly different effects on the development of the disease. Thus Field (1961) found that guinea-pigs given 150 r whole-body irradiation (the maximum dose tolerated without obvious ill effect) 18 hr before

challenge (i.e. at a time when maximal effect upon antibody formation might be expected (Taliaferro 1957)) did not exhibit suppression of the disease. Indeed, its severity was increased and a similar finding in rats was reported by Allegretti & Matosic (1961); Allegranza (1959) irradiated guinea-pigs 7 days before challenge without effect.

Paterson & Beisaw (1963), however, working with Wistar and Fisher rats (which tolerate doses of X-rays up to 600 r) reported that treatment 21 or 26 hr before challenge exerted a marked depressant effect. The difference in results between Paterson on the one hand and Allegretti and Matosic on the other is difficult to explain. Condie & Nicholas (1962) have reported suppression by preliminary X-irradiation in the rabbit.

Immunosuppressive Drugs

Salicylates

Salicylates were reported by Good *et al* (1949) to have some inhibiting effect upon the development of EAE if given early and in high doses (0.3 g/kg body weight) by subcutaneous injection. Such doses constitute an appreciable stress to the animal (Field & Miller 1961) and it may well be the action is mediated through ACTH release (see below). Field and Miller found salicylates effective if begun at the time of challenge or even a week later.

Adrenocorticotrophic hormone (ACTH) and Prednisolone

Corticotrophin was reported by Moyer *et al* (1950) to be effective in suppressing EAE especially if treatment was begun before challenge. Gammon & Dilworth (1953) claimed that intensive treatment with very high doses of corticotrophin (5 mg) 6 hourly resulted in a curative effect in animals with paralysis. Field & Miller (1961), however, found ACTH to have only a very limited therapeutic effect in established EAE, chiefly in eliminating residual neurological deficit in those which recovered. Cortisone has also been reported effective (Kabat *et al* 1952) and since there is a general impression amongst neurologists that the therapeutic response of acute episodes of multiple sclerosis to cortisone and prednisone is significantly inferior to that obtained with corticotrophin (Paulley 1957, 1959, 1961; Alexander *et al* 1961), Field & Miller (1962) compared the efficacy of prednisolone and corticotrophin in prevention of EAE in guinea-pigs and concluded that ACTH was the more effective. Ross *et al* (1958) claimed that prednisolone intensified EAE but this is contrary to the experience of all other workers. Whether these drugs act by an antiphlogistic effect (as seems probable) or by suppression of antibody production is uncertain.

6-Mercaptopurine

This drug was shown by Schwartz *et al* (1958) to suppress humoral antibody

formation in rabbits. Hoyer *et al* (1962) found that EAE could be prevented by administration of 6-MP. Large doses were given intravenously to rabbits (up to 12 mg/kg body weight per day) and though this resulted in 20% mortality from the drug alone, the authors reject failure to respond as due to non-specific toxicity, debility or anti-inflammatory activity. When the dose of 6-MP in guinea-pigs was so limited that animals appeared healthy and gained weight (5 mg/kg body weight per day), then a deleterious effect was observed in that the time of onset of disease in guinea-pigs was significantly reduced. Histological lesions were in general more severe and characterized by fragmentation of cell nuclei in the perivascular cuffs (Field & Miller 1961). Thomson & Austin (1962) found guinea-pigs treated with 6-MP to show minimally more severe urinary incontinence but otherwise there was no change in the EAE picture. Certainly the drug did not protect. Field (1967) has used Immuran by mouth in guinea-pigs and found no protection so that the drug (in the guinea-pig) is certainly less effective than cyclophosphamide.

Cyclophosphamide and Methotroxate

This drug is highly efficacious in suppressing EAE after potent challenge. Calne & Leibowitz (1963) found that cyclophosphamide in doses of 20 mg/kg/day and methotroxate in doses of 5 mg/kg/day intraperitoneally were highly suppressive. Unpublished work in this laboratory has confirmed these results. The suppression is accompanied by severe diminution in circulating leucocytes and by disappearance of delayed hypersensitivity to 50μg of EF inoculated intradermally.

Anti-lymphocyte Serum

It is possible to prepare an antiserum against lymphocytes and this can be used to suppress rejection of grafted tissue. Waksman *et al* (1961) used such rabbit serum prepared against guinea-pig lymph-node cells to lower the circulating level of blood lymphocytes of guinea-pigs and this resulted in a marked suppression of the tuberculin reaction, contact allergic reactions and the 'delayed' type of skin reaction to purified diphtheria toxoid. There was also a relative suppression of allergic encephalomyelitis.

Dietary Factors

Mueller *et al* (1962) found that guinea-pigs maintained on a vitamin C deficient diet failed to develop EAE when challenged with potent encephalitogenic mixture. At the same time tuberculin sensitivity, as measured by the PPD skin reaction was also abolished. Upon restoration of vitamin C the animals recovered their sensitivity to PPD but did not develop EAE. Szego (1965) has confirmed that scorbutic guinea-pigs fail to develop EAE and shown that the low copper concentration in blood found in such animals is not the responsible factor. The mechanism of vitamin C action in the development of EAE is unknown.

Thymectomy

Thymectomy in the neonatal rat was found by Arnason *et al* (1962a and b) to result in a marked reduction in incidence of EAE as compared with sham operated control animals, and also marked depression of delayed hypersensitivity to serum albumen. Newborn thymectomized rats, however, also show diminished ability to make circulating antibody, so that these experiments *per se* cannot distinguish between the mechanisms at work in the disease. However, in the chicken whilst thymectomy depresses the development of delayed-type hypersensitivity, it does not interfere with antibody production. On the other hand removal of the bursa of Fabricius does not interfere with the development of delayed hypersensitivity and such birds can develop EAE (Jankovic & Isvaneski 1963). Removal of the thymus on the day of birth in Hartley guinea-pigs does not appreciably alter the animals' ability to develop EAE when challenged after it has attained maturity (personal observation).

RELATIONSHIP OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS TO MULTIPLE SCLEROSIS AND POST-EXANTHEMATOUS ENCEPHALITIS

Although the lesions of multiple sclerosis were first illustrated more than a century ago (Carswell 1838) and a strikingly modern clinical and pathological account of the disease set out nearly a hundred years ago (Charcot 1868, 1872) virtually no progress in our understanding of the disease other than accumulation of descriptive detail was made until the announcement by Rivers in the mid-thirties (see above) that an experimental encephalitis accompanied by myelin destruction could be produced in laboratory animals. Against this sombre background the new discovery was naturally hailed with considerable hope and expectancy, for it could provide a laboratory model which would soon lead to clarification of the pathogenesis and therapy of multiple sclerosis. Thirty years of intensive work in many laboratories have not realized this hope and as a result there has been a certain rethinking of the possible relationship between EAE and human demyelinating conditions. The allergic nature of EAE can now be accepted as established, especially since transfer with sensitized lymphocytes has been achieved, though further experiment may be expected to clarify conditions for successful transfer and bring the condition more into line with other delayed hypersensitivities. On the other hand the status of multiple sclerosis as an autoallergic disease or even as due primarily to some immunological reaction is far from secure. Whilst abnormal immunological findings are certainly present in the condition, their primary nature is doubtful since many are shared with other neurological disturbances associated with destruction of nervous parenchyma.

Post-infectious encephalomyelitis (occurring 11–16 days or so after common infectious fevers or Jennerian vaccination) was first suggested by Glanzmann in 1927 to be immunological in nature and largely on the grounds of pathological affinity with MS (perivascular cuffing and demyelination) this inspired guess was taken up and extended to multiple sclerosis by van Bogaert (1932). The idea received powerful support from Pette and his colleagues of the Hamburg school, the publication of whose monograph in 1942 was a landmark in the evolution of our concept of auto-allergic disease. Pette introduced the term 'neurallergy' and developed an attractive unitarian hypothesis which assigns essentially the same pathogenesis to vaccinal encephalomyelitis, post-exanthematous encephalomyelitis and multiple sclerosis. This unifying and intellectually seductive hypothesis visualizes a whole 'spectrum of demyelinating disease ranging from the most acute cases of frankly post-infective or post-vaccinal encephalitis or myelitis at one end of the scale, to the insidious and remorselessly progressive chronic spinal form of multiple sclerosis . . . at the other' (Miller & Schapira 1959). These authors see 'good reasons to include in such a spectrum also a number of other syndromes such as acute haemorrhagic leucoencephalitis (almost certainly a manifestation of hypersensitivity) and Schilder's disease (now widely regarded as an analogue of multiple sclerosis) . . . '. Over the years the unitary hypothesis has been strongly supported by distinguished experimental neuropathologists such as Ferraro and Alvord, and that MS has an immunological pathogenesis is current majority view.

Only when observational evidence in support of this hypothesis is sought do difficulties arise. Miller & Schapira (1959) themselves point out that 'to have induced acute disseminated encephalomyelitis in animals is not . . . to have reproduced multiple sclerosis, and these important observations have thrown no new light on the crucial problem of the relationship between these acute self-limited clinical and experimental syndromes on the one hand and the relapsing self-perpetuating course of the chronic human disease on the other'. For it is a disconcerting fact that no one has succeeded in reproducing plaques resembling those of MS in experimental animals. Kersting & Pette (1957), in presenting their own production of EAE in monkeys, review previous efforts by Ferraro and others and urge caution in carrying over results from one species to another. It is surprising that the gross lesions readily produced in monkeys do not heal with macroscopic scars. At most, slight gliosis around blood vessels, but nothing approaching an MS plaque is found, though it may be that plaque production is something specific to the human nervous system. Certainly EAE can be produced in the human as the case reported by Jellinger & Seitelberger (1958) clearly shows, where the result of repeated injection of sterile bovine nervous tissue was a massive confluent disintegration of brain parenchyma chiefly at the angles of the lateral ventricles. This was an acutely produced disease and the extraordinary findings in certain cases of neuroparalytic accident occurring

during the Pasteur treatment of suspected rabies (Uchimura & Shiraki 1957) are even more striking. These subjects survived acute neurological illness for some months and at autopsy scattered lesions in the central nervous system very closely resembling those of MS were found. Whilst the individual lesions in Shiraki's preparations closely resemble multiple sclerosis plaques and would be accepted as such by most neuropathologists, some are disinclined to accept them as MS chiefly on the grounds that 'inflammation and oedema are disproportionately great to the demyelination and lipid breakdown, and that the lesions are evidently monophasic and non-progressive peripherally' (Lumsden 1961). Here again a fundamental difference between experimental disease (whether produced in animals or man) and multiple sclerosis is brought out—the chronic, progressive character of the latter in the majority of cases (though non-progressive cases—*formes frustes*—were described long ago by Charcot).

Attempts to produce chronic experimental allergic encephalomyelitis in monkeys (Ferraro & Cazzulo 1948) and guinea-pigs (Stone & Lerner 1965) have not yielded the pathological picture of MS though the former authors reported clinical remissions and exacerbations in their animals. The usual acute EAE is essentially a closed episode illness with considerable immunity developing against subsequent challenge (see above). Both clinically and pathologically it bears considerable resemblance to post-exanthematous encephalitis. Indeed EAE may fairly be regarded as the laboratory model for vaccinal or post-infectious encephalomyelitis, and the recently described hyperacute form of EAE (Levine & Wenk 1964) bears some resemblance to haemorrhagic leucencephalitis.

Few antibody studies in MS using modern highly active EF as antigen have so far been published. Field *et al* (1963) found antibody to EF present in the blood of MS patients with a significantly greater frequency than normal. Since the same was, however, true of other degenerative conditions of the nervous system (notably general paralysis of the insane), it was concluded that antibody to EF is probably an effect rather than a cause of myelin destruction. Skin tests with EF (at 1 μg) have been negative, a finding confirmed by Cendrowski (1966). This confirms earlier negative findings using brain suspension as test antigen carried out by Stauffer & Waksman 1954 (who also reported normal results with tuberculin) and Broman *et al* (1960), though Böhme *et al* (1963) reported equivocal results when they used a (poorly encephalitogenic) proteolipid-A as antigen. They concluded that their results 'do not favour the view which considers multiple sclerosis a delayed-type hypersensitivity reaction against myelin antigen' though 'some immunological process might very well be involved'. Johnson & Miller (1963) found no difference in skin reactivity to tuberculin in MS patients as compared with normal subjects, though Canetti & Lacaze (1940) had previously reported that Mantoux reactions were significantly greater in patients suffering from miscellaneous nervous diseases. Smith *et al* (1961) on the other hand found evidence of anergy to tuberculin in their female patients with

MS. There is some evidence, too, that MS patients have a lesser capacity to produce circulating antibody than normal people as shown by response to a booster inoculation of TABT (Wellcome) (Field *et al* 1961). The controversy as to skin sensitivity exists also in relation to abnormal reactivity of the lining of the subarachnoid space to purified protein derivative (PPD) of tubercle bacillus (Smith *et al* 1957; Marshall & O'Grady 1959; O'Grady *et al* 1962). Marshall and O'Grady maintained that the altered reactivity of the subarachnoid lining cells *vis-à-vis* PPD is not limited to multiple sclerosis but occurs also in a number of degenerative nervous diseases. It may be that these findings are explicable on the basis of antigen sharing between EF and tuberculin (Field *et al* 1963).

The same non-specificity exists in some measure for the myelotoxicity of serum from cases of multiple sclerosis for cultures of neonatal rat cerebellum since two-thirds of motor neurone disease sera are also toxic. Lamoureux & Borduas (1966) have recently claimed that these sera are toxic not only to myelin, but also to glial cells and neurones—a significant departure from the claim made by Bornstein and probably referable to technical differences. The problem has recently been discussed by Hughes & Field (1967). Lamoureux and Borduas, admitting that conditions other than MS may show cytotoxicity, suggest that 'these other neurological diseases could, of course, resemble multiple sclerosis in pathogenesis in being caused by an abnormal immune response in the central nervous system'. Antibodies to brain or EF were indeed found in amyotrophic lateral sclerosis (Roemer *et al* 1953; Field *et al* 1963) but the simplest explanation of their presence is to regard them as consequent upon myelin destruction rather than a cause of it.

All in all the evidence that MS is related to EAE is largely that from analogy, and this is much more cogent for the relation between EAE and post-infectious encephalomyelitis. The enigma of continued progression or recurrent episodic illness in MS apparently quite unrelated to an exciting agent remains. In animal experiment repeated exhibition to antigen cannot produce such a phenomenon. The place of MS amongst the auto-allergic diseases though one of the earliest is perhaps the least secure.

Failure to establish a secure common immunological basis for EAE and MS does not, however, rule out the participation of immunological processes in the evolution of the clinical picture of the disease. Evidence has recently been brought forward that MS may be another example of a 'slow' virus infection with pathogenetic resemblances to scrapie and kuru (Palsson *et al* 1965; Field 1966; Beck *et al* 1966). It may well be that whilst the primary mechanism in MS is that of a 'slow' virus infection many features of the condition may result from immunological phenomena, e.g. platelet clumping in small veins, as postulated by Field *et al* (1963).

REFERENCES

ADAMS R.D. & KUBIK C.S. (1952) The morbid anatomy of the demyelinative diseases. *Amer. J. Med.* **13,** 510.

ALEXANDER L., BERKELEY A.W. & ALEXANDER A.M. (1961) *Multiple Sclerosis: Prognosis and Treatment.* Springfield, Illinois: Thomas; Oxford: Blackwell.

ALLEGRANZA A. (1959) In *'Allergic' Encephalomyelitis,* p. 498, ed. KIES M.W. & ALVORD E.C. JR. Springfield, Illinois: Thomas

ALLEGRETTI N. & MATOSIC M. (1961) Experimental allergic encephalomyelitis in irradiated rats. *Nature* **189,** 500.

ALLERAND C.D. & YAHR M.D. (1964) Gamma-globulin affinity for normal human tissue of the central nervous system. *Science* **144,** 1141.

APPEL S.H. & BORNSTEIN M.B. (1964) The application of tissue culture to the study of experimental allergic encephalomyelitis. II. Serum factors responsible for demyelination. *J. exp. Med.* **119,** 303.

ARNASON B.G., JANKOVIC B.D. & WAKSMAN B.H. (1962) Effect of thymectomy on 'delayed' hypersensitivity reactions. *Nature* **194,** 99.

ARNASON B.G., JANKOVIC B.D., WAKSMAN B.H. & WENNERSTEN C. (1962) Role of the thymus in immune reactions in rats. II. Suppressive effects of thymectomy at birth on reactions of delayed (cellular) hypersensitivity and the circulating small lymphocyte. *J. exp. Med.* **116,** 177.

ASHLEY F.L., SLOAN R.F., SCHWARTZ A.N., JOSEPH G., STEIN H.& LONGMIRE W.P. JR. (1958) Studies on mammalian homotransplants of skin. I. Tolerance induced with a pooled antigen in rat. *Plastic and Reconstruct. Surg.* **22,** 462.

ASTROM K. & WAKSMAN B.H. (1962) The passive transfer of experimental allergic encephalomyelitis and neuritis with living lymphoid cells. *J. Path. Bact.* **83,** 89.

AUJESZKY A. (1900) Ueber Immunizierung gegen Wut mit normaler Nervensubstanz. *Centralb. f. Bakt.* **27,** 5.

BALFOUR B.H., DONIACH D., ROITT I.M. & COUCHMAN K.G. (1961) Fluorescent antibody studies in human thyroiditis: auto-antibodies to an antigen of the thyroid colloid distinct from thyroglobulin. *Brit. J. exp. Path.* **42,** 307.

BARLOW C.F. (1956) A study of abnormal blood-brain permeability in experimental allergic encephalomyelitis. *J. Neur. Exp. Neurol.* **15,** 196.

BECK E., DANIEL P.M., ALPERS M., GAJDUSEK D.C. & GIBBS C.J. JR. (1966) Experimental 'Kuru' in chimpanzees: a pathological report. *Lancet* **ii,** 1056.

BELL J. & PATERSON P.Y. (1960) Rapid induction of allergic encephalomyelitis in rats without the use of mycobacteria. *Science* **131,** 1448.

BERG O. & KALLEN B. (1962) An *in vitro* gliotoxic effect of serum from animals with experimental allergic encephalomyelitis. *Acta Path. Microbiol. Scan.* **54,** Fasc. 4, 425.

BEUTNER E.H. & WITEBSKY E. (1962) Studies on organ specificity. XIV. Immunofluorescent studies of thyroid reactive autoantibodies in human sera. *J. Immunol.* **88,** 462.

BEUTNER E.H., WITEBSKY E., ROSE N.R. & GERBASI J.R. (1958) Localization of thyroid and spinal cord autoantibodies by fluorescent antibody technic. *Proc. Soc. exp. Biol. Med.* **97,** 712.

BLOOM B.R. & BENNETT B. (1966) Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science* **153,** 80.

BÖHME D., PAAL G., KERSTEN W. & KERSTEN H. (1963) Skin reactions in neurological patients after intracutaneous administration of proteolipid A extracted from human brain. *Nature* **197,** 609.

BORNSTEIN M.B. & APPEL S.H. (1961) The application of tissue culture to the study of experimental 'allergic' encephalomyelitis. I. Patterns of demyelination. *J. Neuropath.* **20,** 141.

BORNSTEIN M.B. & APPEL S.H. (1965) Tissue culture studies of demyelination. *Ann. N.Y. Acad. Sci.* **122,** 280.

BORNSTEIN M.B. & CRAIN S.M. (1965) Functional studies of cultured brain tissue as related to 'demyelinative disorders'. *Science* **148,** 1242.

BOYDEN S.V. (1964) Cytophilic antibody in guinea-pigs with delayed-type hypersensitivity. *Immunology* **7,** 474.

BROMAN T., LIDVALL H., LIND A. & MEYER P. (1960) Investigations of multiple sclerosis patients concerning skin and serological reactions to injection with brain tissue. *Acta psychiat. scand.* **35,** 403.

BUBIS J.J. & LUSE S.A. (1964) An electron-microscopic study of experimental allergic encephalomyelitis in the rat. *Amer. J. Path.* **44,** 299.

CALNE D.B. & LEIBOWITZ S. (1963) Suppression of experimental allergic encephalomyelitis by cytotoxic drugs. *Nature* **197,** 1309.

CANETTI G. & LACAZE H. (1940) Donnes nouvelles sur l'evolution et la signification de l'allergie tuberculonique des grandes tendances evolutives de l'allergie tuberculinque chez les sujets non tuberculeaux allergie et veinfection. *Ann. Inst. Pasteur* **65,** 435.

CARNEGIE P.R. & LUMSDEN C.E. (1966) Encephalitogenic peptides from spinal cord. *Nature* **209,** 1354.

CARR C.W. (1961) Dialysis. In *Physical Methods in Chemical Analysis,* **4,** 1, ed. BERL W.G. New York: Academic Press.

CARSWELL R. (1838) *Pathological Anatomy; Illustrations of the Elementary Forms of Disease.* London: Longman, Orme, Brown, Green & Longman.

CASPARY E.A. & COMAISH S. (1967) Release of serotonin from human platelets in hypersensitivity states. *Nature* (In the press).

CASPARY E.A. & FIELD E.J. (1963) Immunological examination of the soluble antigens of human brain and their modification by proteolytic enzymes. *Deutsch. Z. Nervenheilk.* **184,** 478.

CASPARY E.A. & FIELD E.J. (1965) An encephalitogenic protein of human origin; some chemical and biological properties. *Ann. N.Y. Acad. Sci.* **122,** 182.

CASPARY E.A. & FIELD E.J. (1967) Serotonin release from blood platelets in multiple sclerosis; the effect of encephalitogenic factor and ovalbumin *in vitro. Deuts. Z. Nervenheilk* (in the press).

CASPARY E.A., FIELD E.J. & BALL E.J. (1964) Antibody studies multiple sclerosis and experimental 'allergic' encephalomyelitis. *J. Neurol.* **27,** 25.

CASPARY E.A. & SIMPSON C.A. (1964) Dimercaprol in multiple sclerosis and allergic encephalomyelitis. *Lancet* **2,** 909.

CASPARY E.A., SINDEN R.E. & FIELD E.J. (1966) Some observations on the role of circulating antibody in protection against experimental allergic encephalomyelitis. *Z. Immunitatsf.* **130,** 454.

CENDROWSKI W. (1966) Concerning the reaction to encephalitogenic factor in familial multiple sclerosis. *Arch. immunol. therapeut. exper.* **14,** 491.

CENTANNI (1898) *La Rif Med.* **3** (cited by Hurst E.W. 1932).

CERF J.A. & CARELS G. (1966) Multiple sclerosis: serum factor producing reversible alterations in bioelectric responses. *Science* **152,** 1066.

CHARCOT J.M. (1868) Histologie de la sclerose en plaques. *Gaz. Hôp., Paris* **41,** 554, 557, 566.

CHARCOT J.M. (1872) *Maladies du Systeme Nerveu. I. De la sclerose en plaques disseminées—Anatomie pathologique,* p. 168.

CHASE M.W. (1945) The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc. Soc. exp. Biol. Med.* **59,** 134.

CHASE M.W. (1954) Experimental sensitization with particular reference to picryl chloride. *Internat. Arch. Allergy App. Immunol.* **5,** 163.

CHASE M.W. (1959) Discussion. In *Allergic Encephalomyelitis,* p. 441, eds. KIES M.W. & ALVORD E.C. JR. Springfield, Illinois: Thomas.

CHASE M.W. (1959) A critique of attempts at passive transfer of sensitivity to nervous tissue. In *Allergic Encephalomyelitis,* eds. KIES M.W & ALVORD E.C. JR. Springfield, Illinois: Thomas.

CLARK G. & BOGDANOVE L.H. (1955) The induction of the lesions of allergic meningo-encephalomyelitis in a predetermined location. *J. Neuropath.* **14,** 433.

CLARKE D.W. & GEIGER L. (1964) Effect of experimental allergic encephalomyelitis serum on fatty acid output of brain slices. *Nature* **201,** 401.

CONDIE R.M. & GOOD R.A. (1959) Experimental allergic encephalomyelitis: its production, prevention and pathology as studied by light and electron microscopy. In *Biology of Myelin* ed. Korey, Saul R. London: Cassell.

CONDIE R.M. & NICHOLAS T. (1962) Effect of total body irradiation (TBR) on the development of experimental allergic encephalomyelitis. *Fed. Proc.* **21,** 43.

CUNNINGHAM V.R. & FIELD E.J. (1965) Experimental allergic encephalomyelitis: protection experiments with encephalitogenic factor and tubercle fractions. *Ann. N.Y. Acad. Sci.* **122,** 346.

DAVID J.R., AL-ASKARI S., LAWRENCE H.S. & THOMAS L. (1964) Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93,** 264.

DAVID J.R. & PATERSON P.Y. (1965) *In vitro* demonstration of cellular sensitivity in allergic encephalomyelitis. *J. exp. Med.* **122,** 1161.

DIENES L. & SCHOENHEIT E.W. (1929) The production of tuberculin hypersensitiveness in guinea-pigs with various protein substances. *Am. Rev. Tuberc.* **20,** 92.

DUMONDE D.C. (1967) The role of the macrophage in delayed hypersensitivity. *Brit. med. Bull.* **23,** 9.

DUMONDE D.C. & GLYNN L.E. (1965) The reaction of guinea-pigs to autologous and heterologous fibrin implants. *J. Path. Bact.* **90,** 649.

FERRARO A. & CAZZULLO C.L. (1948) Chronic experimental allergic encephalomyelitis in monkeys. *J. Neuropath.* **7,** 235.

FERRARO A. & CAZZULLO C.L. (1949a) Prevention of experimental allergic encephalomyelitis in guinea-pigs. *J. Neuropath.* **8,** 61.

FERRARO A. & CAZZULLO C.L. (1949b) Prevention of experimental allergic encephalomyelitis in guinea-pigs (note II). *J. Neuropath.* **8,** 226.

FERRARO A., ROIZIN L. & CAZZULLO C.L. (1950) Experimental studies in allergic encephalomyelitis; prevention and production —note III. *J. Neuropath.* **9,** 18.

FIELD E.J. (1961) Effect of X-irradiation upon the development of experimental allergic encephalomyelitis in guinea-pigs. *Brit. J. exp. Path.* **42,** 303.

FIELD E.J. (1966) Transmission experiments with multiple sclerosis: an interim report. *Brit. med. J.* **2,** 564.

FIELD E.J. (1967) The effect of immuran on the development of experimental allergic encephalomyelitis in the guinea-pig. *Arch. Int. Pharmac.* **165,** 138.

FIELD E.J., CASPARY E.A. & BALL E.J. (1963) Some biological properties of a highly encephalitogenic factor isolated from human brain. *Lancet* **2,** 11.

FIELD E.J., GREEN C.A. & MILLER H.G. (1961) Response of normal and multiple sclerotic subjects to typhoid-paratyphoid vaccine injections. *J. Neurol.* **24,** 78.

FIELD E.J. & HUGHES D. (1965) Toxicity of motor neurone disease serum for myelin in tissue culture. *Brit. med. J.* **2,** 1399.

FIELD E.J. & MILLER H.G. (1961) Studies on the inhibition of experimental allergic encephalomyelitis. *Arch. int. Pharmacodyn.* **134,** 76.

FIELD E.J. & MILLER H.G. (1962) Experimental allergic encephalomyelitis; comparison of protective effects of prednisolone and corticotrophin *Brit. med. J.* **1,** 843

FIELD E.J. & RAINE C.S. (1966) Experimental allergic encephalomyelitis: an electron microscopic study. *Amer. J. Path.* **49,** 537.

FIELD E.J., RIDLEY A.R. & CASPARY E.A. (1963) Specificity of human brain and nerve antibody as shown by immunofluorescence microscopy. *Brit. J. exp. Path.* **44,** 631.

FREUND J. (1947) Some aspects of active immunization. *Ann. Rev. Microbiol.* **1,** 291.

FREUND J. & LIPTON M.M. (1955) Experimental allergic encephalomyelitis after the excision of the injection site of antigen-adjuvant emulsion. *J. Immunol.* **75,** 454.

FREUND J. & MCDERMOTT K. (1942) Sensitization to horse serum by means of adjuvants. *P.S.E.B.M.* **49,** 548.

FREUND J., STERN E.R. & PISANI T.M. (1947) Isoallergic encephalomyelitis and radiculitis in guinea-pigs after one injection of brain and mycobacteria in water, in oil emulsion. *J. Immunol.* **57,** 179.

FRICK E. (1951) Serologische Untersuchungen bei multipler sklerose. *Deutsch. Z. Nervenheilk.* **166,** 54.

GAMMON G.A. & DILWORTH M.J. (1953) Effect of corticotropia on paralysis of experimental allergic encephalomyelitis. *Arch. Neurol.* **69,** 649.

GEORGE M. & VAUGHAN J.H. (1962) *In vitro* cell migration as a model for delayed hypersensitivity. *Proc. Soc. exp. Biol. Med.* **111,** 514.

GLANZMANN E. (1927) Die nervosen Komplikationen der Varizellen, Variola und Vakzine. *Schweiz. med. Wschr.* **57,** 145.

GOOD R.A., CAMPBELL B. & GOOD T.A. (1949) Prophylactic and therapeutic effect of para-aminabenzoic acid and sodium salicylate on experimental allergic encephalomyelitis. *Proc. Soc. exp. Biol. Med.* **72,** 341.

HARVEY W.F. & ACTON H.W. (1923) An examination into the degree of efficacy of antirabic treatment. *Ind. J. med. Res.* 10, 1020.

HILL K.R. (1949) An investigation into the presence of antibodies and hypersensitivity in the encephalitis produced experimentally by the injection of homologous brain suspension. *Bull. Johns Hopkins Hosp.* **84,** 302.

HOTTLE G.A., NEDZEL G.A., WRIGHT J.T. & BELL J.F. (1949) Allergic encephalitis following injection of dialysate of brain tissue. *Proc. Soc. exp. Biol. Med.* **72,** 289.

HOYER L.W., GOOD R.A. & CONDIE R.M. (1962) Experimental allergic encephalomyelitis: the effect of 6-mercaptopurine. *J. exp. Med.* **116,** 311.

HUGHES D. & FIELD E.J. (1967) Myelotoxicity of serum and spinal fluid in multiple sclerosis; a critical assessment. *Clin. exp. Immunol.* **2,** 295

HUGHES D & FIELD E.J. (1968) Inhibition of macrophage migration *in vitro* by brain and encephalitogenic factor in allergic encephalomyelitis. *Int. Arch. Allergy* **33,** 45

HUMPHREY J.H. & JAQUES R. (1955) The release of histamine and 5-hydroxytryptamine from platelets by antigen-antibody reactions (in vitro). *J. Physiol.* **128,** 9.

HURST E.W. (1932) The effects of the injection of normal brain emulsion into rabbits, with special reference to the aetiology of the paralytic accidents of antirabic treatment. *J. Hygiene* **32,** 33.

JANKOVIC B.D. & ISVANESKI M. (1963) Experimental allergic encephalomyelitis in thymectomized, bursectomized and normal chickens. *Int. Arch. All.* **23,** 188.

JELLINGER K. & SEITELBERGER F. (1958) Akute todliche entmarkungs-encephalitis nach wiederholten hirntrockenzellen-injektionen. *Klin. Wschr.* **36,** 437.

JOHNSON R.T. & MILLER H. (1963) Dermal sensitivity tests in multiple sclerosis. *J. Neurol.* **26,** 151.

KABAT E.A., WOLF A. & BEZER A.E. (1946) The rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants. *J. exp. Med.* **85,** 117.

KABAT E.A., WOLF A. & BEZER A.E. (1952) Studies on acute disseminated E.M. produced experimentally in rhesus monkeys. VII. The effect of cortisone. *J. Immunol.* **68,** 265.

KERSTING G. & PETTE E. (1957) Zur Pathohistologic und pathogenese der experimentellen 'allergischen' encephalomyelitis des affen. *Deutsch Z. Nervenheilk.* **176,** 387.

KIES M.W. (1965) *Ann. N.Y. Acad. Sci.* **122,** 194 (discussion).

KIES M.W., GOLDSTEIN N.P., MURPHY J.B., ROBOZ E. & ALVORD E.C. JR. (1957) Occurrence of lipemia in experimental allergic encephalomyelitis. *Neurology* **7,** 175.

KIES M.W. & ALVORD E.C. JR. (1958) Prevention of allergic encephalomyelitis by prior injection of adjuvants. *Nature* **182,** 1106.

KIES M.W., ALVORD E.C. JR. & ROBOZ E. (1958) The allergic encephalomyelitis activity of a collagen-like compound from bovine spinal cord—II. *J. Neurochem.* **2,** 261.

KIES M.W. & ALVORD E.C. JR. (1959) Encephalitogenic activity in guinea-pigs of water soluble protein fractions of nervous tissue. In *Allergic Encephalomylitis,* pp. 293–9, eds. KIES M.W. & ALVORD E.C. JR. Springfield, Illinois: Thomas.

KIES M.W. & ALVORD E.C. JR. (1959) *Allergic Encephalomyelitis.* Springfield, Illinois: Thomas.

KIES M.W., MURPHY J.B. & ALVORD E.C. JR. (1961) Studies of the encephalitogenic factor in guinea-pig central nervous system. In *Chemical Pathology of the Nervous System,* pp. 197–204. ed. FOLCH-PI J. Oxford: Pergamon Press.

KIES M.W., THOMPSON E.B. & ALVORD E.C. JR. (1965) The relationship of myelin proteins to experimental allergic encephalomyelitis. *Ann. N.Y. Acad. Sci.* **122,** 148.

KOPROWSKI H. (1962) The role of hyperergy in measles encephalitis. *Amer. J. Dis. Children* **103,** 273.

KOPROWSKI H. & FERNANDES M.V. (1962) Autosensitization reaction *in vitro.* Contactual agglutination of sensitized lymph node cells in brain tissue culture accompanied by destruction of glial elements. *J. exp. Med.* **116,** 467.

KOPROWSKI H., PARAF A., BILLINGHAM R. & JERVIS G. (1960) Etudes sur le mechanisme de l'encephalite allergique chez le rat. *Comp. Rendus hebd. de l'Acad. Sci. (Paris)* **250,** 2956.

KORITSCHONER R. & SCHWEINBURG F. (1924) Klinische exp. Beobachtunger über Lähmungen nach Wutschutzimpfung. *Z. Immunitätsforsch.* **42,** 217.

KORNGUTH S.E., ANDERSON J.W. & SCOTT G. (1966) Temporal relationship between myelinogenesis and the appearance of basic protein in the spinal cord of a white rat. *J. comp. Neurol.* **127,** 1.

KORNGUTH S.E. & THOMPSON H.G. JR. (1964) Stimulation of L.N. protein synthesis by a basic protein from brain. *Arch. Biochem. Biophys.* **105,** 308.

LAATSCH R.H., KIES M.W., GORDON S. & ALVORD E.C. JR. (1962) The encephalomyelitic activity of myelin isolated by ultracentrifugation. *J. exp. Med.* **115,** 777.

LAMOUREUX G., BOULAY G. & BORDUAS A.G. (1966) A cytotoxic antibody for cultured nervous tissue in serum and cerebrospinal fluid from rhesus monkeys with allergic encephalomyelitis. *Clin. exp. Immunol.* **1,** 307.

LAMPERT P. & CARPENTER S. (1965) Electron microscopic studies on the vascular permeability and the mechanism of demyelination in experimental allergic encephalomyelitis. *J. Neuropath,* **24,** 11.

LEE J.M. & OLITSKY P. (1955) Simple method for enhancing development of acute disseminated encephalomyelitis in mice. *Proc. Soc. exp. Biol. Med.* **89,** 610.

LEE J.M. & SCHNEIDER H.A. (1962) Critical relationships between constituents of the antigen-adjuvant emulsion affecting allergic encephalomyelitis in a completely susceptible mouse genotype. *J. exp. Med.* **115,** 157.

LEVINE S. (1960) Localization of allergic encephalomyelitis in lesions of cyanide encephalopathy. *J. Neuropath.* **19,** 238.

LEVINE S., HIRANO A. & ZIMMERMAN H.M. (1965) Hyperacute allergic encephalomyelitis; electron microscopic observations. *Amer. J. Path.* **47,** 209.

LEVINE S. & WENK E.J. (1964) Allergic encephalomyelitis: a hyperacute form. *Science* **146,** 1681.

LUMSDEN C.F. (1961) Consideration of multiple sclerosis in relation to the auto-immunity process. *Proc. Roy. Soc. Med.* **54,** 11.

LUMSDEN C.F., ROBERTSON D.M. & BLIGHT R. (1966) Chemical studies on experimental allergic encephalomyelitis; peptide as the common denominator in all encephalitogenic 'antigens'. *J. Neurochem.* **13,** 127.

MARSHALL J. & O'GRADY F. (1959) The non-specificity of the intrathecal reaction to tuberculin in multiple sclerosis. *J. Neurol.* **22,** 277.

MELLORS R.C., BRZOSKO W.J. & SONKIN L.S. (1962) Immuno-pathology of chronic non-specific thyroiditis (autoimmune thyroiditis). *Amer. J. Path.* **41,** 425.

MILLER H.G. & SCHAPIRA K. (1959) Actiological aspects of multiple sclerosis. *Brit. med. J.* **1,** 737, 811.

MOEN J.K. (1936) Tissue culture studies on bacterial hypersensitivity. III. The persistence *in vitro* of the inherent sensitivity to tuberculin of cells from tuberculous animals. *J. exp. Med.* **64,** 943.

MORGAN I.M. (1946) Allergic encephalomyelitis in monkeys in response to injection of normal monkey cord. *J. Bact.* **51,** 53.

MORGAN I.M. (1947) Allergic encephalomyelitis in monkeys in response to injection of normal monkey nervous tissue. *J. exp. Med.* **85,** 131.

MOYER A.W., JERVIS G.A., BLAKE J., KOPROWSKI H. & COX H.R. (1950) Action of adrenocorticotropic hormone (ACTH) in experimental allergic encephalomyelitis of the guinea-pig. *Proc. Soc. exp. Biol. Med.* **75,** 387.

MUELLER P.S., KIES M.W., ALVORD E.C. JR. & SHAW C.M. (1962) Prevention of experimental allergic encephalomyelitis (EAE) by vitamin C deprivation. *J. exp. Med.* **115,** 329.

NAKAO A. & ROBOZ-EINSTEIN E. (1965) Chemical and immunological studies with a dialysable encephalitogenic compound from bovine spinal cord. *Ann. N.Y. Acad. Sci.* **122,** 171.

NELSON D.S. & BOYDEN S.V. (1963) The loss of macrophages from peritoneal exudates following the injection of antigens into guinea-pigs with delayed-type hypersensitivity. *Immunology* **6,** 264.

O'GRADY F., MARSHALL J. & SHAW D.A. (1962) Variability of the intrathecal response to antigens. *J. Path. Bact.* **83,** 276.

OLITSKY P. & YAGER R.H. (1949) Experimental disseminated encephalomyelitis in white mice. *J. exp. Med.* **90,** 213.

PALSSON P.A., PATTISON I.H. & FIELD E.J. (1965) Transmission experiments with multiple sclerosis. In NINDB Monographs No. 2, *Slow, Latent and Temperate Virus Infections,* ed. GAJDUSECK D.C., GIBBS C.J. JR. & ALPERS M. U.S. Dept of Health, Education and Welfare.

PATERSON P.Y. (1960) Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J. exp. Med.* **111,** 119.

PATERSON P.Y. (1966) Experimental allergic encephalomyelitis and auto-immune disease. In *Advances in Immunology*, vol. 5, ed. DIXON F.J. JR. & HUMPHREY J.H. New York and London: Academic Press.

PATERSON P.Y. & BEISAW N.E. (1963) Effect of whole body X-irradiation on induction of allergic encephalomyelitis in rats. *J. Immunol.* **90,** 532.

PATERSON P.Y. & HARWIN S.M. (1963) Suppression of allergic encephalomyelitis in rats by means of antibrain serum. *J. exp. Med.* **117,** 755.

PAULLEY J.W. (1957) ACTH in multiple sclerosis. *Brit. med. J.* **1,** 760.

PAULLEY J.W. (1959) Aetiology of disseminated sclerosis. *Brit. med. J.* **2,** 99.

PAULLEY J.W. (1961) Treatment of multiple sclerosis with corticotrophin. *Lancet* **2,** 1257.

PETTE H. (1942) *Die akut entzundlichen erkrankungen des nervensystems.* Stuttgart: Thieme.

RAUCH H.C. & RAFFEL S. (1964) Immunofluorescent localization of encephalitogenic protein in myelin. *J. Immunol.* **92,** 452.

RAUCH H.C. & RAFFEL S. (1965) Antigen uptake by specifically reactive cells in experimental allergic encephalomyelitis. *Ann. N.Y. Acad. Sci.* **122,** 297.

RIDLEY A.R. (1963) Localization of gamma-globulin in experimental encephalomyelitis by the fluorescent antibody technique. *Z. Immunitatsf.* **125,** 173.

RIVERS T.M. & SCHWENTKER F.F. (1935) Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. *J. exp. Med.* **61,** 689.

ROBERTSON D.M., BLIGHT R. & LUMSDEN C.F. (1962) Dialysable peptide as the causative factor in experimental 'allergic' encephalomyelitis. *Nature* **196,** 1005.

ROBOZ-EINSTEIN E., ROBERTSON D.M., DICAPRIO J.M. & MOORE W. (1962) The isolation from bovine spinal cord of a homogeneous protein with encephalitogenic activity. *J. Neurochem.* **9,** 353.

ROEMER G.B., SCHRADER A. & SCHILD W. (1953) Ergebnisse serologischer untersuchungen bei der multiplen sklerose. *Klin. Wschr.* **31,** 946.

ROSS J., COLMANT H.J. & BOHM P. (1958) Über die experimentelle encephalomyelitis des meerschweinchens. *Deutsch Z. Nervenheilk.* **177,** 475.

SCHNEIDER H.A. (1959) Genetic and nutritional aspects of experimental 'allergic' encephalomyelitis. In *Allergic Encephalomyelitis*, ed. KIES M.W. & ALVORD E.C. JR. Springfield, Illinois: Thomas.

SCHWARTZ R., STACK J. & DAMESHEK R. (1958) Effect of 6-mercaptopurine on antibody production. *Proc. Soc. exp. Biol. Med.* **99,** 164.

SEITELBERGER F. & JELLINGER K. (1963) Die pathologische anatomie der allergischen erkrankungen des nervensystems. *Wien. Klin. Wschrf.* **75,** 475.

SHAW C.H., FAHLBERG W.J., KIES M.W. & ALVORD E.C. JR. (1960) Suppression of experimental 'allergic' encephalomyelitis in guinea-pigs by encephalitogenic protein extracted from homologous brain. *J. exp. Med.* **111,** 171.

SHAW C.M., ALVORD E.C. JR., FAHLBERG W.J. & KIES M.W. (1962) Adjuvant-antigen relationships in the production of experimental 'allergic' encephalomyelitis in the guinea-pig. *J. exp. Med.* **115,** 169.

SHAW C.M., ALVORD E.C. JR., KAKU J. & KIES M.W. (1965) Correlation of experimental allergic encephalomyelitis with delayed type sensitivity to specific homologous encephalitogen. *Ann. N.Y. Acad. Sci.* **122,** 318.

SHERWIN A.L., RICHTER M., COSGROVE J.B.R. & ROSE B. (1961) Myelin-binding antibodies in experimental allergic encephalomyelitis. *Science* **134,** 1370.

SHIRAKI H. & OTANI S. (1959) Clinical and pathological features of rabies post-vaccinal encephalomyelitis in man. In *Allergic Encephalomyelitis*, eds. KIES M.W. & ALVORD E.C. JR. Springfield, Illinois: Thomas.

SMITH H.V., ESPIRE M.L.E., WHITTY C.W.M. & RUSSELL W.R. (1957) Abnormal immunological reactions in disseminated sclerosis. *J. Neurol.* **20,** 1.

SMITH H.V., HUGHES I.E. & HUNTER G. (1961) Intrathecal tuberculin in disseminated sclerosis: the immunological aspects. *J. Neurol.* **24,** 101.

SPECTOR W.G., WALTERS M.N.I. & WILLOUGHBY D.A. (1965) The origin of the mononuclear cells in inflammatory exudates induced by fibrinogen. *J. Path. Bact.* **90,** 181.

STAUFFER R.E. & WAKSMAN B.H. (1954) Dermal and serologic reactions to nervous tissue antigens in multiple sclerosis. *Ann. N.Y. Acad. Sci.* **58,** 570.

STONE S.H. (1961) Transfer of allergic encephalomyelitis by lymph node cells in inbred guinea-pigs. *Science* **134,** 619.

STONE S.H. & LERNER E.M. (1965) Chronic disseminated encephalomyelitis in guinea-pigs. *Ann. N.Y. Acad. Sci.* **122,** 227.

SVET-MOLDAVSKY G.J., SVET-MOLDAVSKY I.A. & RAFFKINA L.I. (1959) Various types of acquired resistance to experimental 'allergic' encephalomyelitis. *Nature* **184,** 1552.

SVET-MOLDAVSKY G.J., SVET-MOLDAVSKY I.A. & RAFFKINA L. (1960) Further studies of acquired resistance to experimental allergic encephalomyelitis. *Acta virol.* **4,** 94.

SVET-MOLDAVSKY I.A. & SVET-MOLDAVSKY G.J. (1958) Acquired resistance to experimental allergic encephalomyelitis. *Nature* **181,** 1536.

SZEGO G. (1965) Dietary deficiency and experimental allergic encephalomyelitis. *Proc. Aust. Ass. Neurol.* **3,** 127.

TAL C. & BEHAR A.J. (1958) A demyelinative disease of the brain produced by injections of liver or liver proteolipid. *J. Path. Bact.* **76,** 483.

TALIAFERRO W.H. (1957) Modification of the immune response by radiation and cortisone. *Ann. N.Y. Acad. Sci.* **69,** 745.

THOMAS L., PATERSON P.Y. & SMITHWICK B. (1950) Acute disseminated encephalomyelitis following immunization with homologous brain extracts. *J. exp. Med.* **92,** 133.

THOMPSON E.B. (1965) Discussion. *Ann. N.Y. Acad. Sci.* **122,** 179.

THOMSON J.D. & AUSTIN R.W. (1962) Effects of 6-mercaptopurine on susceptibility of guinea-pigs to experimental allergic encephalomyelitis. *Proc. Soc. exp. Biol. Med.* **111,** 121.

UCHIMURA I. & SHIRAKI H. (1957) A contribution to the classification and the pathogenesis of demyelinating encephalomyelitis. *J. Neuropath.* **16,** 139.

UHR J.W. & PAPPENHEIMER A.M. JR. (1958) Delayed hypersensitivity. III. Specific desensitization of guinea-pigs sensitized to protein antigens. *J. exp. Med.* **108,** 891.

UHR J.W. & SCHARFF M. (1960) Delayed hypersensitivity. V. The effect of X-irradiation on the development of delayed hypersensitivity and antibody formation. *J. exp. Med.* **112,** 65.

VAN BOGAERT L. (1932) Memoires originaux. Essai d'interpretation des manifestations nerveuses observées au cours de la vaccination, de la maladie serique et des maladies éruptives. *Rev. Neurol.* **2,** 1.

VULPE M., HAWKINS A. & ROZDILSKY B. (1960) Permeability of cerebral blood vessels in experimental allergic encephalomyelitis studied by radioactive iodinated bovine albumin. *Neurology (Minneap).* **10,** 171.

WAKSMAN B.H. (1956) Further study of skin reactions in rabbits with experimental allergic encephalomyelitis. Reactions to tests with purified white matter fractions. Use of a quantitative technique for evaluating production of sensitization and disease by intradermal injection of antigen without adjuvant. *J. infect. Dis.* **99,** 258.

WAKSMAN B.H. (1959) Evidence favouring delayed sensitization as the mechanism underlying experimental allergic encephalomyelitis. In *Allergic Encephalomyelitis* eds. KIES M.W. & ALVORD E.C. JR. Springfield, Illinois: Thomas.

WAKSMAN B.H. (1959) Experimental allergic encephalomyelitis and the 'auto-allergic' diseases. *Internat. Arch. Allergy* **14,** Suppl. 1.

WAKSMAN B.H. & ADAMS R.D. (1962) A histologic study of the early lesions in experimental allergic encephalomyelitis in the guinea-pig and rabbit. *Amer. J. Path.* **41,** 135.

WAKSMAN B.H., ARBOUYS S. & ARNASON B.G. (1961) The use of specific 'lymphocyte' antisera to inhibit hypersensitive reactions of the 'delayed' type. *J. exp. Med.* **114,** 997.

WAKSMAN B.H. & MORRISON R.L. (1951) Tuberculin type sensitivity to spinal cord antigen in rabbits with isoallergic encephalomyelitis. *J. Immunol.* **66,** 421.

WENK E.J., LEVINE S. & WARREN B. (1967) Transfer of allergic encephalomyelitis with blood leucocytes. *Nature* **214,** 803.

WINKLER G.F. (1965) *In vitro* demyelinization of peripheral nerve induced with sensitized cells. *Ann. N.Y. Acad. Sci.* **122,** 287.

WINKLER G.F. & ARNASON B.G. (1966) Antiserum to immunoglobulin A: inhibition of cell mediated demyelination in tissue culture. *Science* **153,** 75.

WOLFGRAM F. (1965) Macromolecular constituents of myelin. *Ann. N.Y. Acad. Sci.* **122,** 104.

WOODRUFF M.F.A. & SIMPSON L.I. (1955) Induction of tolerance to skin homografts in rats by injection of cells from prospective donor soon after birth. *Brit. J. exp. Path.* **36,** 494.

WOODRUFF M.F.A. & SPARROW M. (1957) Further observations on the induction of tolerance of skin homografts in rats. *Transpl. Bull.* **4,** 157.

YAHR M.D. & ALLERAND (1965) Brief report; affinity of gamma globulin for human glia and myelin. In *Contemporary Neurology Symposia. Vol. I. The Remote Effects of Cancer on the Nervous System*, eds. BRAIN, LORD & NORRIS F.H. JR. New York: Grune & Stratton.

CHAPTER 41

AUTO-ALLERGY IN DISEASES OF THE LIVER, ADRENAL, PANCREAS AND PROSTATE

J. R. ANDERSON

LIVER

INTRODUCTION

This account is concerned almost entirely with active chronic hepatitis and primary biliary cirrhosis. Because of the confused state of the nomenclature of chronic liver disease, it is necessary to state at the outset that the term *cirrhosis* is used here, as by most writers on the subject, to indicate the late stage of various types of liver injury, in which there is fibrosis throughout the liver, the parenchyma being divided by fibrous tissue into nodules which exhibit loss of the normal 'lobular' architecture and evidence of continued liver cell destruction and attempts at regeneration. The term *active chronic hepatitis* is applied to all stages of a chronic liver disease which cannot be defined briefly or precisely, but which is described below. This condition has also been called plasma cell

hepatitis, lupoid hepatitis, auto-immune hepatitis, post-necrotic cirrhosis and juvenile cirrhosis. Active chronic hepatitis, as used here, includes all these variants, and also the pre-cirrhotic and cirrhotic stages of the condition.

The term *primary biliary cirrhosis* is a misnomer, because true cirrhosis develops late in this condition, but its application to all stages if the disease is widespread, and has been adopted here.

The term *cryptogenic cirrhosis* is useful, for in many cases of cirrhosis the pathogenic agent is not apparent. It is likely that large groups of cases of this sort will be of heterogeneous pathogenesis, and will include advanced cases of nutritional (alcoholic) liver injury, active chronic hepatitis, and possibly primary biliary cirrhosis and other less common, unknown or complex pathogeneses.

Although active chronic hepatitis and primary biliary cirrhosis are of particular immunological interest, it has been suggested also that homologous serum hepatitis may have an allergic pathogenesis. Hotchin (1962, 1966) and Hotchin & Collins (1964) have compared it with lymphocytic choriomeningitis of mice and with kuru, and have suggested that, in these diseases, infection with the causative viruses may be asymptomatic and associated with a state of immunological tolerance. Hotchin postulates that homologous serum hepatitis results from breakdown of tolerance to the virus, and is due to a Type IV reaction resulting in injury to the liver cells containing the virus. This raises the further possibility that infective hepatitis also results from an allergic Type IV reaction and is not due to a direct cytotoxic effect of the virus. Although of considerable interest, these possibilities will not be discussed further, because both these virus diseases are typically acute and self-limiting, and if pathogenic factors of an allergic nature are concerned, they are most likely to result from an immune response against the hepatitic viruses and not from an auto-allergic response.

Active Chronic Hepatitis

General Features

A detailed study of patients with this condition was reported by Bearn, Kunkel & Slater (1956), who described the illness in a group of 26 young patients, mostly females, with chronic liver disease and a high incidence of certain other features not often encountered in patients with nutritional cirrhosis. These features include early and pronounced splenomegaly, recurrent febrile attacks with exacerbation of the hepatic and other changes, polyarthralgia or chronic polyarthritis, and pleural effusions. Several other reports have since appeared in which groups of patients with this condition have been observed over a number of years (Mackay & Wood 1962; Mackay & Burnet 1962; Read, Sherlock & Harrison 1963; Maclachan, Rodnan, Cooper & Fennell 1965; Mackay, Weiden & Hasker 1965). In all of these series, females have predominated and although the age-incidence is wide, many of the patients have been young.

Hyperglobulinaemia is commonly present, but like most other abnormalities, tends to be more pronounced during exacerbations and to subside during remissions. Hypergammaglobulinaemia was reported by Bearn *et al* (1956) in all their cases, while Read *et al* (1963) noted that the total serum globulin was 5% or more in 43 of their 81 cases, and that hypergammaglobulinaemia was a prominent feature. Feizi (personal communication, 1966) has estimated the three serum immunoglobulins IgG, IgM and IgA in groups of patients with various chronic liver diseases, and concluded that changes in the levels are not of much differential diagnostic value. However, IgG levels of over 4% were observed in 35% of cases of active chronic hepatitis, in 4 of 19 cases of cryptogenic cirrhosis, and in 2 of 14 cases of alcoholic cirrhosis: IgG levels around 5% or higher were observed in 25% of the cases of active chronic hepatitis, in 1 case of cryptogenic cirrhosis, and not in other liver diseases. It is apparent that high levels of IgG occur especially in active chronic hepatitis, and very high levels occur in some cases, particularly during exacerbations.

Liver biopsy has revealed infiltration, particularly in the portal areas, mainly with lymphocytes and plasma cells in various proportions, eosinophil and neutrophil polymorphs being relatively scanty. Degeneration and 'piecemeal necrosis' of liver cells is seen at the margins of the cellular infiltrates, and as the condition progresses lobular collapse and fibrosis become apparent: the fibrous tissue becomes septal, dividing the parenchyma into irregularly sized islands, and these changes, together with continued focal loss and proliferation of liver cells, give rise to post-necrotic cirrhosis. Frequently, active chronic hepatitis has progressed to this stage when diagnosed, and accordingly the latter term, and also 'juvenile cirrhosis' have sometimes been applied to all stages of the disease. In groups of cases followed over a number of years, mortality has been approximately 50%, and is attributable, as in other types of cirrhosis, to hepatocellular failure, the effects of portal hypertension, or a combination of the two. In a significant proportion of cases, the chronic hepatitis has progressed very slowly or has remained static over the period of observation, and occasionally the inflammatory changes have subsided completely. In most cases, the disease activity tends to fluctuate, bouts of fever and jaundice punctuating the chronic course.

In addition to the extra-hepatic lesions referred to above, there is evidence that the disease is associated, more often than would be expected by chance, with certain other conditions, namely ulcerative colitis, thrombocytopenia, leucopenia, haemolytic anaemia and membranous glomerulonephritis. Some of the extra-hepatic lesions and associated conditions resemble features of systemic lupus erythematosus, while the chronic polyarthritis which occurs in some cases may be indistinguishable from rheumatoid arthritis. For these reasons, a relationship with the connective tissue diseases was suggested by Bearn *et al* (1956), and this has been supported by subsequent observations. In the past few years, the

observed relationship with the connective tissue diseases has been strengthened by the demonstration, in the serum of patients with active chronic hepatitis, of auto-antibodies of the types associated with the connective tissue diseases. There is also some evidence suggesting that chronic thyroiditis (Hashimoto's disease and primary myxoedema) and thyrotoxicosis are unduly common in patients with active chronic hepatitis, and that there is an increased incidence of thyroid auto-antibodies in the serum of patients with this condition. These immunological considerations are of both practical and theoretical interest and are described in the following account. It has been considered necessary to provide some background information on some of the immunological features discussed, e.g. the 'auto-immune complement fixation reaction', and to refer also to the overlap with immunological features observed in other liver diseases, particularly primary biliary cirrhosis. In spite of this overlap, it seems appropriate to provide separate accounts of these two liver diseases.

Immunological Features

Antinuclear antibodies

There is an undoubted increase in the incidence of antinuclear antibodies in active chronic hepatitis, and in some cases the LE cell test is positive. The incidence of this latter finding varies considerably in different series, and obviously depends on case selection and upon the number and thoroughness of LE cell tests performed. In the cases reported by Read *et al* (1963) diagnosis was based on the occurrence of jaundice of hepatocellular type, lasting for 3 months or more, in the absence of excessive alcohol consumption. The LE cell test was usually performed three times, and was found positive, on one or more occasions, in 10 of their 81 cases. In the 20 cases described by Maclachlan *et al* (1965), the diagnosis had often been suspected because of hypergammaglobulinaemia or a positive LE cell test, and this presumably accounts for the test being positive in 14 cases.

Mackay and his colleagues (Mackay, Taft & Cowling 1956, 1959; Mackay & Burnet 1962; Mackay & Wood 1962; Mackay, Weiden & Hasker 1965; Whittingham, Mackay & Irwin 1966) have made a long study of patients with active chronic hepatitis, which they refer to as 'auto-immune hepatitis', and have introduced the term 'lupoid hepatitis' for those patients with a positive LE cell test. In this latter subgroup, the average age was lower, there was a higher proportion of females, a higher incidence of splenic enlargement and of pleurisy, arthritis, skin rashes, etc., and higher levels of serum IgG, and of serum transaminases. There was little difference in the mortality over a period of years, and in the duration of illness in fatal cases. This division of cases into two categories on the basis of the LE cell test is somewhat arbitrary, particularly as Mackay and his colleagues and other workers have commented on the occurrence of LE cells

in small numbers in tests upon some patients, and also on the tendency for the LE cell test to be positive during exacerbations and to become weaker or negative during periods of reduced disease activity.

The LE cell test is a rather insensitive method of demonstrating antibody to deoxyribonucleoprotein. Application of the more sensitive indirect immunofluorescence technique has demonstrated the occurrence of this antibody in many patients with active chronic hepatitis and negative LE cell tests. The low incidence (10%) reported by Mackay *et al* (1965) is presumably influenced by steroid therapy, because the incidence was only 32% in their cases of lupoid hepatitis, all of whom must, by definition, have had a positive LE cell test on at least one occasion. In the 43 cases investigated by Doniach, Roitt, Walker & Sherlock (1966), approximately 60% gave a positive immunofluorescence test for deoxyribonucleoprotein. In both of these investigations, antibodies to other nuclear constituents were detected, the incidence of antibody to saline-soluble nuclear protein (Beck 1963) being approximately 16% in the report of Doniach *et al* (1966). In age- and sex-matched healthy individuals, and in patients with alcoholic cirrhosis, the incidence of antinuclear antibodies was very much lower.

The auto-immune complement fixation reaction

It has long been known that the serum of certain individuals is capable of reacting, in complement fixation tests, with crude extracts of human and other mammalian liver and other organs. Recent interest in this phenomenon was aroused by Gajdusek (1957, 1958) and Mackay & Gajdusek (1958) who tested the serum of a large number of individuals with extracts of various organs, and applied the term 'auto-immune complement fixation (AICF) reaction'. They encountered positive results, usually of low serum titre, in a small proportion of healthy individuals and patients with various diseases, and at this level the test appears to have little clinical significance. Titres of 1 in 32 or more, however, were especially common in patients with systemic lupus erythematosus, macroglobulinaemia, lupoid hepatitis and primary biliary cirrhosis. These findings have been confirmed and extended by Mackay and his colleagues and by other workers. Positive results have been shown to be common also in syphilis (Hackett, Beech & Forbes 1960) and to occur in various connective tissue diseases, including rheumatoid arthritis and dermatomyositis (Pasnick, Beall & van Arsdel 1962), and in Sjögren's syndrome (Deicher, Holman & Kunkel 1960; Anderson, Gray, Beck & Kinnear 1961).

Doubts about the immunological nature of the AICF reaction have been expressed by several authors, and other possible interpretations were suggested by Hackett *et al* (1960) and by Beall (1963). However, the serum factors responsible for the reaction have been shown to reside in the immunoglobulin fractions, IgG and IgM participating in various proportions in different sera (Deicher *et al* 1960), and the immunological nature of the AICF reaction has now received

support from recent reports describing positive antibody tests by techniques other than complement fixation see page page 1102

Doubt about the *auto*-immunological nature of some AICF reactions was raised by Mackay & Larkin (1959), who tested positive sera with tissue extracts from the same individuals: in some instances this autologous test was positive, but in others it was negative or weak, although both the serum and the tissue extract reacted strongly in homologous tests (i.e. with tissue extracts or positive serum respectively from other individuals). A possible explanation was provided by Anderson, Goudie & Gray (1960) on the basis that multiple antigenic determinants and their corresponding auto-antibodies could participate in AICF reactions, negative or weak autologous tests being attributed to blocking of one or more antigenic determinants by the individual's own auto-antibody during preparation of the tissue homogenate: this would leave other antigenic determinants free to react with any serum containing the corresponding antibodies. This explanation would account for the negative autologous AICF reactions being observed particularly in cases with a high serum titre.

Evidence that multiple antigen-antibody systems can give rise to a positive AICF test was provided by Asherson (1959) and Deicher *et al* (1960), who showed, by tests upon centrifugal fractions of tissue homogenates, that some positive sera reacted predominantly with one or more of the fractions, e.g. nuclear, mitochondrial or microsomal preparations, or with the supernatant fluid. Deicher *et al* (1960) showed also that multiple antigens capable of participating in the AICF reaction were present in the microsomal fraction, while Beall (1963) showed a major antigen, which closely resembled IgG in many of its properties, in the soluble protein fraction, and Sleeman (1963) demonstrated that different positive sera reacted with different antigenic constituents of the supernatant fluid. It follows that the results of AICF tests will depend upon the amounts of various cellular constituents in the tissue preparation used as antigen, and thus on the method and tissue used to prepare the antigen. Variations in technique of antigen preparation probably account for some of the differences between the AICF results of different workers.

Although organ-specific auto-antibodies to the microsomal antigens of thyroid, gastric mucosa, and adrenocortical cells are capable of fixing complement, the extensive studies of Gajdusek (1958) provided no evidence that multiple organ-specific antigen-antibody systems were responsible for the AICF reaction, nor has subsequent work provided such evidence, the antigens concerned in the reaction being present in extracts of various organs. Marked differences have been encountered in the titres of individual sera reacting with various organs, and are probably due to differences in the amounts of various cellular constituents in the various organs.

In active chronic hepatitis the AICF reaction is positive in moderate or high serum titre in approximately one-third of cases (Hackett *et al* 1960; Pasnick *et al*

1962; Mackay & Wood 1962; Mackay & Burnet 1965; Doniach, Roitt, Walker & Sherlock 1966). The findings of Mackay & Wood (1962) and Mackay, Weiden & Haskar (1965) suggest a higher incidence of positive results in cases of lupoid hepatitis and they observed also that the serum titres were, in general, higher in cases of lupoid hepatitis.

In some instances, a positive AICF test of high serum titre is of value in the diagnosis of active chronic hepatitis, but the test is positive also in most cases of primary biliary cirrhosis (Walker *et al* 1965) and in some cases of cryptogenic cirrhosis. Positive tests, usually of low serum titre, occur in some cases of acute virus hepatitis (Gajdusek 1958; Hackett *et al* 1960), and were observed by Gajdusek to become negative following subsidence of the disease process. As already mentioned, positive AICF tests occur in diseases other than those involving mainly liver, and it is apparent that a positive test indicates no more than the presence of one of many antibodies to nuclear or cytoplasmic cellular constituents. In this situation, the AICF test is obviously of limited diagnostic value. Evidence that different antigen-antibody systems are involved in the AICF reaction in different liver diseases was provided by Gökcen (1962) who performed tests with human liver from adults, foetuses, and patients with extra-hepatic biliary obstruction. Positive sera from patients with post-necrotic cirrhosis reacted with the liver extracts of 'normal' adults and of patients with bile duct obstruction, whereas in primary biliary cirrhosis, tests were positive with 'normal' adult and foetal liver but not with the liver of patients with bile duct obstruction: sera from patients with systemic lupus reacted with all three liver preparations. These results suggested the possibility that different antigen-antibody systems might be involved in the AICF reactions in primary biliary cirrhosis and post-necrotic cirrhosis. However, it has now been established by Doniach *et al* (1966) and Goudie, MacSween & Goldberg (1966) that antibody to mitochondrial antigen is mainly responsible for the positive AICF reactions in primary biliary cirrhosis, and accounts also for most of those encountered in cases of active chronic hepatitis. As it concerns especially primary biliary cirrhosis, this work is described in the section on that disease (see p. 1111).

Antibody to smooth muscle

Antibody reacting with the sarcoplasm of smooth muscle fibres was described by Johnson, Holborow & Glynn (1965), who observed staining of the muscle layers, and media of small vessels, in immunofluorescence tests with gastric tissue. At a serum dilution of 1 in 10, they detected the antibody in 8 of 10 cases of 'probable' and 3 of 6 cases of 'possible' lupoid hepatitis. Diagnostic criteria did not include a positive LE cell test, and active chronic hepatitis therefore seems a more appropriate diagnosis for these cases. In the extensive investigation of Doniach *et al* (1966), tests were performed upon undiluted sera and antibody to smooth muscle was observed in 67% of cases of active chronic hepatitis, 50%

of cases of primary biliary cirrhosis and 28% of cases of cryptogenic cirrhosis: the test was negative in 45 cases of other liver diseases (alcoholic cirrhosis, extra-hepatic biliary obstruction and infective hepatitis). The occurrence of antibody to smooth muscle was confirmed also by Whittingham *et al* (1966), who detected it, at a 1 in 10 serum dilution, in 26 of 34 cases of lupoid hepatitis and 10 of 21 cases of active chronic hepatitis: results were consistently negative in 43 patients with other liver diseases, in 42 patients with systemic lupus erythematosus, in 132 patients with various other diseases, and in 172 healthy individuals. From their results, Whittingham *et al* assume that there must be a hepatic antigen which reacts with this antibody, and that this reaction must be injurious to the liver. However, the occurrence of smooth muscle antibody does not appear to be confined to patients with clinically overt diseases of the liver, for its presence was reported by Johnson *et al* (1965) in 3 of 47 cases of rheumatoid arthritis, while Doniach *et al* detected it in 5 of 32 cases of this disease. It is of interest that the three reports describing this antibody include tests upon the serum of a total of 46 patients with systemic lupus erythematosus, all of which were negative, whereas, as stated above, the test was positive in 8 of 79 cases of rheumatoid arthritis.

Antibody to smooth muscle was reported also by Ironside, de Boer & Nairn (1966) in 4 cases of lupoid hepatitis and was shown to have the properties of IgG. Negative tests were observed with sera from patients with other diseases and from healthy individuals.

Other antibodies

In the investigation of Whittingham *et al* (1966), the serum of some cases of lupoid hepatitis and of active chronic hepatitis was observed to react with the cytoplasm of cells of the renal glomerulus (human and rat), and of human thymus, lymph nodes, liver and spleen: these reactions were weak, and were not observed in tests upon the serum of 20 patients with systemic lupus erythematosus. Immunofluorescent staining of glomerular cells was also reported by Williamson, Housley, Hulme & Wall (1966) with serum from 3 of 5 cases of 'auto-immune' (? active chronic) hepatitis. Thirty-five sera from patients with other types of liver disease were negative, but the test was positive for 68 of 100 sera from patients with rheumatoid arthritis, and the positive rheumatoid sera reacted also with other tissues, fluorescent staining of reticulin, synovial membrane, and sarcolemma of smooth and cardiac muscle being observed. Immunofluorescent staining of glomeruli by serum from 4 patients with lupoid hepatitis was reported also by Ironside *et al* (1966), but these workers had the impression that the reaction was with stromal elements (presumably basement membrane) of capillary walls, and was not confined to glomeruli.

None of the antibodies referred to above reacts solely with liver tissue. The possibility of a liver-specific human antibody was, however, raised by Johnson,

Holborow & Glynn (1966) who described immunofluorescent staining of the bile canaliculi of rat liver. These structures are merely spaces between adjacent liver cells, and presumably the immune reaction was with cytoplasmic elements aggregated at this site. This staining reaction was observed with serum from 5 of their 16 cases of 'probable' or 'possible' active chronic hepatitis (see p. 1101). The positive sera all reacted also with smooth muscle and glomeruli, but both of these latter reactions were observed with some sera which failed to stain bile canaliculi: this could mean that different antibodies are involved, or that the antibodies are the same and the differences are quantitative. Immunofluorescence tests with parenchymal cells of certain glandular organs other than liver are not described in this report, and the liver-specific nature of the reaction thus appears uncertain. Bright immunofluorescent staining of the secretory margin of glandular epithelial cells is observed with the thyroid-specific 'microsomal' antibody (Roitt *et al* 1964), although less bright staining of the remainder of the cytoplasm is apparent in this reaction: judging by their illustration, the reaction described by Johnson *et al* (1966) appears to be sharply localized to the canaliculi. Immunofluorescence studies have been described also by Paronetto and Popper and their co-workers (Paronetto, Schaffner & Popper 1961; Paronetto *et al* 1964; Popper, Paronetto & Schaffner 1965) who provided evidence of an antibody reacting with the cytoplasm of ductular epithelium. This reaction was observed particularly with serum from patients with virus hepatitis, primary biliary cirrhosis and post-necrotic cirrhosis, but a minority of sera from patients with various diseases accompanied by raised level of IgG, and even some normal sera, gave positive results. This reaction differs in two important respects from those described by other workers. Firstly, it was observed in tests with acetone-fixed sections of liver; secondly, tests with normal liver were negative, the positive findings being with the tall columnar ductular epithelium in conditions exhibiting proliferation and destruction of the bile ductules, namely 'active post-necrotic cirrhosis' (active chronic hepatitis) and primary biliary cirrhosis. Paronetto *et al* (1964) consider that the incidence of the antibody reflects the extent of proliferation and destruction of bile ductules. These findings resemble in some respects those of Gökcen, who described variations in the reactivity of selected livers in complement fixation tests (see p. 1101).

The above findings do not establish the occurrence of organ-specific antibodies to liver tissue, and other attempts to demonstrate such antibodies in man have been negative or inconclusive.

Rheumatoid factor has been detected in the serum of an appreciable proportion of patients with active chronic hepatitis, in some instances in high titre, and this finding is not confined to those patients with polyarthritis. In the cases investigated by Read *et al* (1963), the sheep cell agglutination test was positive at titres of 1 in 64 or more in 8 of 35 patients, while Maclachlan *et al* (1965)

reported positive latex fixation (F11) tests, of titres 1 in 20 to over 1 in 160, in 11 of their 19 cases.

An increased incidence of thyroid antibodies has also been reported in active chronic hepatitis, but some of the tests are subject to interference by the presence of non-organ-specific antibodies to various tissue constituents. Care must be taken to avoid misinterpretation of the effects of AICF antibodies, and particularly mitochondrial antibody, in complement fixation and immunofluorescence tests for the thyroid 'microsomal' antibody (see also p. 1113). However, these difficulties do not apply to tests for antibodies to thyroid colloid antigens, which were found positive by Doniach *et al* (1966) in 12 of 43 cases of active chronic hepatitis, as compared with 3 positive results in 43 age- and sex-matched control subjects. Positive direct Coombs' tests have also been reported in occasional patients with active chronic hepatitis. The clinical applications of tests for the various antibodies described above are discussed on page 1114.

Effects of immunosuppressive therapy

Several groups of workers have reported beneficial effects of corticosteroid therapy in patients with active chronic hepatitis (e.g. Mackay *et al* 1959; Page & Good 1962; Read *et al* 1963; Maclachlan *et al* 1965). These effects include clinical improvement and fall in the levels of serum bilirubin, transaminases, IgG and various auto-antibodies. The fall in transaminase levels indicates diminished hepatocellular injury, and yet there is some doubt about the long-term effects of steroid therapy. Mackay & Burnet (1962) consider that it may prolong life, but this was not confirmed by Read *et al* (1963) who consider that steroids should be reserved for those patients who feel ill. There is evidence also that other immuno-suppressants may be of benefit in active chronic hepatitis. Mackay & Wood (1963) reported suppression of lupoid hepatitis by short courses of 6-mercaptopurine, but relapse occurred after stopping the drug. In a subsequent report, Mackay, Weiden & Ungar (1964) described the success of prolonged treatment of cases of active chronic hepatitis and lupoid hepatitis with 6-mercaptopurine and also azothiaprine ('Imuran'). Page, Condie & Good (1964) also reported suppression of the hepatitis in four cases treated with 6-mercaptopurine. Although of great practical importance, these findings contribute little to the understanding of the disease, for both corticosteroids and 6-mercaptopurine could suppress the lesion in various ways. Page *et al* (1964) point out that the effective dose of 6-mercaptopurine was less than that required to suppress antibody production or delayed hyposensitivity in man: they quote evidence on the suppression of virus replication by 6-mercaptopurine, and consider that this may explain its effect in patients with active chronic hepatitis.

Aetiological Considerations

The essential pathological feature of active chronic hepatitis is a progressive

'piecemeal' necrosis of hepatic parenchymal cells in the vicinity of lymphocytic and plasma-cell in filtrates which are concentrated in the portal areas. As in other diseases of the liver, the development of cirrhosis can be regarded as the result of chronic liver cell destruction. The aggregation of lymphocytes and plasma cells in grossly abnormal numbers in any tissue may be regarded as indication of an immunological response or reaction: these cells have no known function other than their participation in the development and expression of actively acquired immunity. In the case of active chronic hepatitis, the fact that liver cell destruction occurs in close proximity to the cellular infiltrates suggests that the latter are responsible for the destructive process, and that liver cell injury is mediated by an allergic process. The type of allergic process concerned, and the nature of the antigen(s) are still unknown. The most likely possibilities are, firstly, that the reaction is with an abnormal (foreign) antigen within the liver, or, secondly, that an auto-allergic reaction, involving auto-antigen(s), is concerned. As regards the first possibility, the most likely foreign antigens are the viruses of infective or homologous serum hepatitis. There are, however, no generally applicable methods of detecting these viruses, nor of demonstrating antibodies to them, and until such methods become available, the possibility that active chronic hepatitis is a virus infection cannot very well be substantiated or refuted. The fact that the onset is often acute and indistinguishable clinically from acute virus hepatitis is of little aetiological significance, for the chronic illness is characterized by repeated exacerbations, and the acute onset might simply represent the first of these. Moreover, most cases of acute virus hepatitis recover completely, and even if it is assumed that active chronic hepatitis is initiated by an acute attack of virus hepatitis, it does not follow that the chronic course is attributable to persistence of the infection.

The second main possibility, that the lesion is auto-allergic, has been championed by Mackay and his colleagues, whose views have been summarized in a recent publication (Mackay *et al* 1965). They postulate that active chronic hepatitis is initiated by hepatocellular injury, most often due to acute virus hepatitis, and that this triggers off an auto-allergic reaction to liver cells, which is responsible for the progressive lesions. This view does not explain the variety of the auto-antibodies which may be encountered in active chronic hepatitis. None of the antibodies so far described has been detected in the serum of all active cases, and most of them occur also in association with other diseases of the liver, particularly primary biliary cirrhosis, and in rheumatoid arthritis and the other connective tissue diseases, in which liver cell injury is usually slight or absent. On present evidence, the occurrence of organ-specific auto-antibodies to liver tissue has not been established in active chronic hepatitis or other diseases of the liver. Many workers have sought for such antibodies, but with negative or inconclusive results. As already stated, the antibody to liver cell canaliculi described by Johnson *et al* (1966) requires further investigation before it can be

regarded as organ-specific, and the antibody to the cytoplasm of proliferating bile ductular epithelium detected by Paronetto *et al* (1961, 1964) in the serum of patients with various conditions does not appear to have been observed by most other workers.

The findings outlined above make it unlikely that the hepatocellular injury in active chronic hepatitis is due to the reaction of circulating auto-antibodies with liver cells, although sinusoids of the liver are somewhat unusual in having a discontinuous basement membrane (Bennett, Luft & Hampton 1959) which might render them more permeable to circulating antibodies than the capillaries of most other organs. It remains possible that plasma cells in the hepatic lesion either produce organ-specific antibody to liver which reacts with, and is largely absorbed by, adjacent liver cells, or that they produce the various non-organ-specific auto-antibodies detectable in the serum, and that, again because of their intimate contact, these bring about injury mainly in the adjacent liver cells.

Another possibility, which is supported by experimental work on animals, is that liver cell injury is mediated by antigen-antibody complexes (i.e. a Type III reaction). This has been suggested by Steiner, Carruthers, Baumal & Kalifat (1961) and by Popper, Paronetto & Schaffner (1965) as a possible pathogenic mechanism in active chronic hepatitis. However, many investigations, including the extensive work of Dixon, Feldman & Vazquez (1961) have demonstrated experimentally that circulating antigen-antibody complexes produce more severe lesions in the glomeruli than in the liver, and the same is probably true of the lesions of serum sickness in man. It was suggested by Popper *et al* (1965) that circulating complexes might, by encountering and combining with free antigen within the liver, enhance their toxicity within this organ, but the hepatic lesions produced by Steiner (1961) in the liver of rabbits, by injecting complexes into the portal vein, were not obviously influenced by the ratio of antigen to antibody in the complexes.

Whenever tissue destruction is accompanied by a cellular infiltrate which includes lymphocytes, a possible explanation is an auto-allergic Type IV reaction. In active chronic hepatitis there is no direct evidence to support this possibility, and arguments advanced in its favour are usually based upon the similarity of the lesion to that in homograft rejection or in auto-allergic thyroiditis, both of which are widely regarded as being mediated largely by Type IV reactions. The speculative nature of such arguments is obvious.

The various auto-antibodies and extra-hepatic manifestations of active chronic hepatitis suggest that it is associated in some way with systemic lupus erythematosus and rheumatoid arthritis. It must be emphasized that the hepatic lesion usually dominates the course of active chronic hepatitis, and that the various features suggesting a relationship to systemic lupus are usually mild, whereas clinically overt chronic hepatitis is unusual in systemic lupus or rheumatoid arthritis. Moreover, since the pathogenic agents responsible for the lesions

of the connective tissue diseases remain unknown, their association, whatever its nature, with active chronic hepatitis, does not at present throw much light on the aetiology of this condition. Several authors have suggested that active chronic hepatitis may result from hepatocellular injury in individuals with a basic defect which carries a predisposition to systemic lupus, but Popper *et al* (1965) have pointed out that the clinical course of acute virus hepatitis is very much the same in patients with systemic lupus as in otherwise healthy individuals.

Popper *et al* (1965) refer also to non-specific activation of the mesenchymal tissue resulting from various types of liver injury, and provide evidence that mice and rats with liver injury caused by allyl alcohol or carbon tetrachloride respond to injections of horse or bovine serum by much higher antibody titres than normal control mice. Enhanced immune responses to foreign antigens have also been reported in patients with 'chronic viral hepatitis' (? active chronic hepatitis) and cirrhosis (Havens, Shaffer & Hopke 1951; Havens, Myerson & Klatchko 1957) but has not been confirmed by other workers (Cherrick, Pothier, Dufour & Sherlock 1959; Barr, Buchanan, Doniach & Roitt 1964). The hyperplasia of the spleen and lymphoid tissues in active chronic hepatitis is in accord with the raised levels of serum immunoglobulins encountered in many cases, but the aetiological significance of these features remains obscure.

Investigations on experimental animals

Much of the experimental work relating to allergic injury of the liver was reviewed and discussed by Steiner *et al* in 1961. The present account is limited to a brief review of recent investigations on the development of auto-antibodies reactive with liver tissue and of attempts to produce auto-allergic liver injury in experimental animals.

Auto-antibodies following liver injury. In a series of investigations, Weir (1961, 1963a and b, 1964) has shown that injection of carbon tetrachloride into rats is followed not only by hepatic necrosis but also by the appearance of a serum factor which reacts with rat liver in complement fixation tests. The factor was shown to be of IgM nature, and reacted with liver tissue of rat and other species (including autologous liver tissue) and with certain other tissues. Before injection, many of the rats' sera gave complement fixation reactions of low titre with liver tissue, and the titre rose 1–2 days following the injection and fell a few days later: a similar but more prolonged response was observed following an injection of rat liver homogenate incorporated in Freund's adjuvant, but repeated injections of carbon tetrachloride did not result in enhanced responses. The immunological nature of this apparent antibody response was supported by the demonstration that its development was inhibited by whole body X-irradiation some hours before the injection of carbon tetrachloride or by splenectomy. In subsequent work by Pinckard & Weir (1966) it was shown that a major antigenic component in the complement fixation reaction was associated

with the mitochondrial fraction of tissue homogenates. The antibodies demonstrated in these experiments appear to be without a pathogenic role, for neither the degree nor the duration of liver injury resulting from injection of carbon tetrachloride were influenced by the titre of antibody, nor were the lesions altered by suppression of antibody production by X-irradiation. The production of antibodies reactive with liver tissue constituents provides a possible explanation for the development of similar antibodies in some cases of acute virus hepatitis and in the more chronic destructive processes of active chronic hepatitis and primary biliary cirrhosis. The effect of chronic liver injury resulting from infection of rabbits with *Eimeria steidae* was studied by Asherson & Rose (1963) who detected a rise in titre of the naturally occurring non-organ-specific IgM antibodies described by Kidd & Friedewald (1942).

Auto-antibody production following liver-adjuvant injections. Several groups of workers have described the development of antibodies in rabbits following injections of rat liver homogenate, or centrifuged fractions thereof, incorporated in Freund's adjuvant. D'Amelio & Perlmann (1960), using immunodiffusion precipitin tests, demonstrated the development of antibodies to multiple antigens of liver microsomal and soluble protein fractions. Asherson & Dumonde (1962, 1963) demonstrated complement fixing antibodies of both IgG and IgM types: they reacted with homogenates of various rat and rabbit organs, including those of the immunized animals, and may thus be regarded as auto-antibodies. The results of absorption studies suggested that both non-organ-specific and organ-specific antibodies had developed, the latter reacting in complement fixation and precipitin tests with rabbit liver but not with other organs. Similar findings were reported by Bollag (1960) and by Vogt (1960), who reported the demonstration, by quantitative precipitation techniques, of antibody reacting with 'microsomal' membrane (endoplasmic reticulum) of liver tissue, but not with other tissues. This last finding appears to correspond to the well-established localization of organ-specific auto-antigens in the microsomal fractions of thyroid epithelial and gastric parietal cells. The *in vivo* localization in rat tissues of rabbit antibodies to rat liver was investigated by Sulitzeanu, Yagi & Pressman (1963). Using radioiodine to label the antibodies, they found localization in both the liver and kidney, but provided evidence that antibody which had been absorbed by, and elicited from, rat liver, tended to localize preferentially in rat liver *in vivo*.

In the investigations described above, the development of auto-antibodies reactive with liver tissue has required immunization with heterologous liver tissue, homologous liver tissue usually being ineffective. However, the development in rats of auto-antibodies following injections of homologous liver homogenate was reported recently by Richter, Sargent, Myers & Rose (1966). Six injections of liver homogenate incorporated in Freund's adjuvant were administered to each rat, and antibodies were detected by agglutination of

tannic-acid-treated red cells sensitized with tissue homogenate. The antibodies were observed to react with liver tissue of rat and various other species, but not with other rat tissues. In immunofluorescence tests, the antibodies reacted with autologous liver tissue, giving diffuse staining of the liver cell cytoplasm.

Attempts to produce auto-allergic hepatitis. No evidence of auto-allergic hepatic lesions was provided by any of the investigations outlined above, and attempts to produce such lesions have usually been unsuccessful or inconclusive. Behar & Tal (1959) reported extensive liver necrosis in guinea-pigs and hamsters following injection of homologous liver homogenates emulsified in Freund's adjuvant. Although the lesions differed from those observed in animals receiving injections of liver homogenate alone, or brain-adjuvant emulsion, they are unlikely to be of an auto-allergic nature, for in some instances they developed within 72–96 hr following the injection. Other workers have not observed specific hepatic lesions in guinea-pigs (Jahiel & Koffler 1961; Norkin, Gottlieb & Zamcheck 1962) or mice (Tal & Laufer 1960) injected with homologous liver homogenate incorporated in Freund's adjuvant. In contrast to these negative findings are the interesting reports of Dodd *et al* (1962) and Bigley, Dodd & Geyer (1963). These workers described the development of various auto-antibodies and pathological changes in rabbits injected with rat or rabbit liver ribosomes in Freund's adjuvant. Auto-antibodies to red cells, to nucleotides, nucleosides and bases of RNA, and to ribosomal protein were described. The pathological changes, which were observed approximately 10 weeks after injection, included haemolytic anaemia, leucopenia, focal lymphocytic and plasma cell infiltration of the liver with associated liver cell injury, and changes in the heart, brain and kidneys. Injection of the serum of affected rabbits into normal rabbits was reported to result in the development of similar pathological changes. Although of considerable interest, further reports on this work have not appeared. The length of the 'incubation period' of the experimentally produced lesions may explain why they have not been described in the similar investigations of other workers.

Conclusions

Once the auto-allergic features of chronic thyroiditis had become well established, many workers interested in the subject were stimulated to review the features of various other diseases, with the possibility of auto-allergic aetiological factors in mind. Active chronic hepatitis seemed a particularly good candidate. The features of this disease, which suggest the possibility of an auto-allergic pathogenesis, include the histological changes, usually progressive course, raised levels of serum IgG, sex incidence, occurrence of various auto-antibodies, favourable response of some cases to immunosuppressive therapy, associated features suggestive of the connective tissue diseases, and apparent association with chronic thyroiditis. However, organ-specific auto-antibodies to liver

tissue have not been demonstrated with any degree of certainty, there is no evidence of an auto-allergic Type IV lesion, and no comparable disease of an auto-allergic nature has been produced experimentally. The possibility that the disease is due to a chronic infection with a hepatitis virus has, because of technical difficulties, remained largely unexplored, and preferment for an auto-allergic pathogenesis is, at present, largely intuitive.

Primary Biliary Cirrhosis

General Features

This is a chronic liver disease in which the development of a true cirrhosis, usually of portal type, is a late manifestation, and is preceded by a chronic inflammatory destruction of the small intra-hepatic biliary ducts and ductules, usually of several years' duration. Because of the long pre-cirrhotic stage, other names have been proposed, including cholangiolitic hepatitis, chronic intra-hepatic obstructive jaundice, and chronic non-suppurative destructive cholangitis. It may be that the cases described by Hanot included examples of this condition, but the term 'Hanot's cirrhosis' has often been applied without any accurate definition. Because of its wide usage, the term 'primary biliary cirrhosis' will be applied in this account to all stages of the disease.

Accounts of the clinical and other features of primary biliary cirrhosis have been provided by Ahrens, Payne, Kunkel, Eisenmenger & Blondheim (1950), Sherlock (1959) and Longmire, Joseph & Mellinkoff (1965). Accounts dealing especially with the pathological changes in the liver include those of MacMahon (1948), Baggenstoss, Foulk, Butt & Bahn (1964), Rubin, Schaffner & Popper (1965), Greville Williams (1965) and Goudie, MacSween & Goldberg (1966). Middle-aged and elderly women are affected more often than men. The onset is insidious, the first complaint often being pruritis, which in some cases persists for years before jaundice or other clinical features of the disease become apparent. In addition to jaundice of various degrees, there is pigmentation of the exposed skin surfaces. Serum bilirubin of all levels up to 20 mg per 100 ml are encountered in different cases, the average being 4–5 mg: the level fluctuates in individual cases, and in general a high level is associated with a short fatal course (Sherlock 1959). Serum levels of alkaline phosphatase and lipids, including cholesterol, are usually markedly raised, and multiple xanthomatous deposits in the skin are common.

It is apparent that the above features are those of biliary obstruction, but retention of bilirubin is disproportionately low as compared with cholesterol and alkaline phosphatase. Additional features include a palpably enlarged smooth firm liver, and usually palpable splenomegaly. Flocculation tests are usually normal, or only slightly abnormal, and serum transaminase levels only slightly raised. The disease activity tends to fluctuate, and some patients have periods of remission, when the biochemical changes may approach or revert to

normal. Corticosteroid therapy appears to be without beneficial effect, and eventually many patients progress to true cirrhosis and die from hepatocellular failure or portal hypertension. Of the 42 patients investigated by Sherlock (1959), 23 had died at the time of reporting, with an average duration of illness of 5 years 5 months.

The early pathological changes in the liver include heavy infiltration of the portal areas with lymphocytes and plasma cells, sometimes with formation of germinal centres, and frequently the infiltration can be seen to be most marked in relation to the smaller interlobular biliary ducts. In a minority of cases, tubercle-like granulomata composed of epithelioid and giant cells are to be seen in the portal areas, particularly when the disease is florid at the time of biopsy (Rubin *et al* 1965). The affected biliary ducts show heaping-up and degenerative changes of the lining epithelium. In some cases, biopsy reveals also inflammatory infiltration around the smallest epithelial-lined biliary passages, or ductules, which show necrosis and proliferative changes, while in others the small biliary ducts have largely disappeared and the inflammatory and necrotic lesions of the ductules are still conspicuous. These variations probably represent progressive stages of the lesion. At an early stage, there is little evidence of hepatocellular injury of the parenchyma, and the biliary canaliculi are not distended. Changes in the ductules are usually accompanied by distension of the canaliculi with inspissated bile (intra-hepatic cholestasis), and hepatocellular injury is more conspicuous. In addition to these changes, fibrous tissue in the portal areas increases and extends into the parenchyma, but true cirrhosis develops late and resembles portal (nutritional) cirrhosis apart from a striking reduction in the number of biliary ducts, and the prominence of intra-hepatic cholestasis.

Immunological Features

In contrast to active chronic hepatitis, the level of serum IgG is usually normal or only slightly raised, and increases in other serum globulins are probably related in part to the high levels of lipoproteins.

The heavy lymphocyte and plasma cell infiltration of the portal areas in primary biliary cirrhosis suggests an immunological reaction, and its relationship to the smaller biliary ducts and ductules suggests the further possibility that the destruction of these is of an allergic nature.

The occurrence of non-organ-specific antibodies to various cellular and tissue constituents has been observed in primary biliary cirrhosis by a number of workers. In fact, as already stated in the section on active chronic hepatitis, most of the antibodies which occur in that condition have been detected also in some patients with primary biliary cirrhosis. However, outstanding differences are the high incidence and high titres of AICF antibodies in primary biliary cirrhosis. In their recent report, Doniach *et al* (1966) found this test positive in 35 of 41 cases, and in 27 of these the titre was 1 in 64 or more. Application of immuno-

fluorescence and absorption techniques indicated that antibody to a mitochondrial antigen was present in the serum of nearly all (40 of the 41) cases. In tests upon various human and other mammalian tissues, the mitochondrial antibody was found to give bright immunofluorescent staining of the epithelium of the distal renal convoluted tubule, eosinophil ('Askanazy') cells of the human thyroid, and gastric parietal cells. The intensity of staining presumably depends on the number, size, and composition of mitochondria in the cells, and in certain cell types, e.g. the hypertrophic epithelial cells of the thyrotoxic thyroid gland, the staining can sometimes be seen to be of granular nature. Very recently, Berg, Roitt & Doniach (personal communication) have provided evidence that the antigen concerned in this reaction resides in the mitochondrial membrane material, and is abundant in mitochondria with prominent cristae. By contrast, mitochondria rich in soluble protein are stained less brightly by the mitochondrial antibody, although the presence of antigen in them was demonstrated by absorption tests. The immunofluorescence test for mitochondrial antibody was reported as positive for all six cases tested by Whittingham *et al* (1966), while Goudie *et al* (1966) obtained positive AICF tests of titre 1 in 128 or more in 23 of 27 cases of primary biliary cirrhosis, and a positive immunofluorescence test for mitochondrial antibody of titre 1 in 128 or more in 26 of 30 cases.

It is clear from these investigations that the mitochondrial antibody is responsible for most of the positive AICF tests in primary biliary cirrhosis and other liver diseases. In the report of Goudie *et al* (1966), the AICF test was positive in all cases of primary biliary cirrhosis with a positive mitochondrial antibody test, and only one patient had a positive AICF test (titre 1 in 16) and a negative immunofluorescence test: the only other positive results were in 3 of 42 patients with post-necrotic or portal cirrhosis, in 1 of 184 patients with miscellaneous diseases, and in 1 of 22 patients with rheumatoid arthritis. Negative results were obtained with 72 cases of extra-hepatic biliary obstruction, 5 cases of secondary biliary cirrhosis, and in infective hepatitis and drug-induced jaundice: tests were also uniformly negative in chronic thyroiditis, pernicious anaemia, and systemic lupus erythematosus. In the report of Doniach *et al* (1966), correlation between AICF and immunofluorescence tests was not so close, and mitochondrial antibody was detected more often in patients without primary biliary cirrhosis: in particular, it was found in 12 of 43 cases of active chronic hepatitis, 10 of 32 cases of cryptogenic cirrhosis, in 2 (both weak results) of 28 cases of extra-hepatic bile duct obstruction, and in 10–29% of cases of rheumatoid arthritis and other connective tissue diseases. Review of the clinical, biochemical and histological features of the cases of cryptogenic cirrhosis with a positive test for mitochondrial antibody suggested strongly that some of these were advanced cases of primary biliary cirrhosis, but there was no evidence of features suggestive of primary biliary cirrhosis among the cases of active chronic hepatitis with a positive test for mitochondrial antibody. The differences in the results of

Doniach *et al* (1966) and Goudie *et al* (1966) are probably attributable, at least in part, to the antigenic preparations used for AICF tests, in which Doniach *et al* used liver tissue homogenate, whereas Goudie *et al* used a centrifugal deposit likely to be rich in microsomes, mitochondria and lysosomes, but poor in soluble protein and nuclei. Doniach *et al* preferred the immunofluorescence test to detect mitochondrial antibody, but in the results of Goudie *et al* the two tests are closely comparable. The multiplicity of antigen-antibody systems capable of giving a positive AICF test has already been discussed (see p. 1100) and may account for the positive results reported by Doniach *et al* in some cases with a negative immunofluorescence test for mitochondrial antibody.

The diagnostic value of testing for mitochondrial antibody is discussed on pp. 1114-16.

Antibodies to nuclei and to smooth muscle both occur in the serum of some patients with primary biliary cirrhosis, but the incidences reported by Doniach *et al* (1966) tend to be lower than in active chronic hepatitis (see pp. 1099, 1101). Occurrence of organ-specific antibodies to thyroid epithelial and gastric parietal antigens is not easy to determine in patients with primary biliary cirrhosis, for the presence of mitochondrial antibody obscures the results of immunofluorescence and complement fixation tests for these antibodies. However, the investigations of Doniach *et al* (1966) included tests for antibodies to colloid antigens (CA2 and thyroglobulin) of the thyroid, and these were found in 28% of cases, as compared with 7% in age- and sex-matched control subjects: this difference is not significant at the 1 in 20 level, but thyroid antibodies were more commonly present also in cases of active chronic hepatitis and cryptogenic cirrhosis than in control subjects, and among the 106 patients with one of these three diseases, there were 11 who had also Hashimoto's disease, primary myxoedema, or treated Graves' disease, and 9 with non-toxic goitre. It is considered by the authors that some of the patients with non-toxic goitre may have had unrecognized chronic thyroiditis, and although no firm conclusions can be drawn without careful consideration of the factors operating in case selection (Berkson 1946), it seems highly probable that these chronic liver diseases are associated, more often than would be expected, with thyrotoxicosis and auto-allergic thyroiditis.

The immunofluorescent staining of proliferating biliary ductule epithelial cytoplasm by the serum of patients with several liver diseases, including primary biliary cirrhosis (Paronetto *et al* 1961, 1964) and the evidence provided by Gökcen (1962) of differences in the AICF antibodies in primary biliary cirrhosis and other conditions, have already been discussed in the section on active chronic hepatitis (pp. 1101, 1103). There is, however, no good evidence of the occurrence of organ-specific antibodies to liver tissue in the serum of patients with primary biliary cirrhosis, and attempts to detect such antibodies, such as those reported by Doniach *et al* (1966), have been negative or inconclusive.

Conclusions

The histological changes in the liver in primary biliary cirrhosis are consistent with destruction of the epithelium of the smaller biliary ducts and ductules by an allergic reaction. The features of the disease do not resemble those of virus hepatitis, and no evidence of an infective pathogenic agent has been provided. Accordingly, the possibility of an auto-allergic pathogenesis must be considered.

The demonstration of mitochondrial antibody in the serum of nearly all patients with primary biliary cirrhosis so far tested provides the closest association of a particular antibody with a particular liver disease, but mitochondrial antibody has also been detected in a proportion of cases of active chronic hepatitis, and also in the connective tissue diseases.

The difficulties of ascribing the lesions of active chronic hepatitis to an auto-allergic reaction have already been discussed. Evidence of an auto-allergic pathogenesis of primary biliary cirrhosis is equally unsatisfactory, for there is no indication that mitochondrial antibody is cytotoxic to bile duct epithelium, antibodies to other tissue constituents have, in general, a lower incidence than in active chronic hepatitis, and extra-hepatic lesions similar to those found in the connective tissue diseases are absent. There is no evidence, other than aggregation of lymphocytes in the portal areas, of a Type IV auto-allergic reaction, and no disease resembling primary biliary cirrhosis has been produced experimentally.

Clinical use of Antibody Tests in Liver Disease

Active chronic hepatitis

The diagnosis of active chronic hepatitis can very often be suspected strongly from the information provided by the clinical features and biochemical investigagations. Because of its serious nature, however, it is appropriate that confirmation should be sought by histological examination of the liver. The demonstration of raised levels of serum immunoglobulins, particularly IgG, and positive tests for AICF, nuclear, smooth muscle, or mitochondrial antibodies, or rheumatoid factor, provide information which, taken together with the clinical features and tests for hepatocellular injury, provide the evidence on which the decision to perform liver biopsy is based. In considering the results of the immunological tests, it is obviously important to realize that none of the antibodies listed above is present in all cases, and that each of them may be found in cases of primary biliary cirrhosis, and in various other diseases, particularly the connective tissue diseases. These associations have already been discussed. A positive LE cell test is also of diagnostic value, but is less sensitive than the indirect immunofluorescence test for nuclear antibodies, and the detection of LE cells may require prolonged examination of multiple tests. As already stated, the sub-classification of those cases with a positive LE cell test as lupoid hepatitis appears to have little clinical advantage: the clinical course and the response to

corticosteroid therapy do not appear to be related to the results of LE cell tests (Read *et al* 1963), nor do the mortality and survival time (Read *et al* 1963; Mackay *et al* 1965). The diagnostic value of the detection of certain antibodies which have been described recently, including antibodies to glomeruli and to biliary canaliculi, can only be assessed on the results of further experience.

Once the diagnosis of active chronic hepatitis has been made, the main hope of arresting or slowing down the destructive lesion lies in the administration of corticosteroids or immunosuppressive drugs. There is no doubt that corticosteroid therapy improves the clinical condition of some patients and lowers the levels of serum transaminases, but the long-term effect remains in doubt: Read *et al* (1963) were not convinced that survival was prolonged, but Mackay *et al* (1965) consider that the evidence supports this possibility. Evidence that immuno-suppressive drugs may be of therapeutic value in some cases has been provided by Page & Good (1962), Page *et al* (1964), Mackay & Wood (1963) and Mackay *et al* (1964). In view of these therapeutic possibilities, it is important that the diagnosis should be made at an early stage. Once the condition has progressed to fully developed post-necrosis cirrhosis, it is unlikely that any known therapeutic measure can arrest the cirrhotic process. In many cases of active chronic hepatitis, the onset is indistinguishable clinically from acute virus hepatitis. The AICF test is positive in a significant proportion of cases of acute virus hepatitis (Gajdusek 1958; Mackay & Gajdusek 1958; Hackett *et al* 1960; Pasnick *et al* 1962; Polish & Muschel 1962; Holborow *et al* 1963), but according to Mackay & Gajdusek (1958), the test reverts to negative with recovery, usually within 8 weeks of onset. Accordingly, persistence of a positive test beyond this time might raise or support the possibility that the case is one of active chronic hepatitis. The other antibody tests do not appear to have been applied to the early diagnosis of active chronic hepatitis simulating acute virus hepatitis.

Primary biliary cirrhosis

Nearly all patients with primary biliary cirrhosis present clinically with features of biliary obstruction, and the main diagnostic problem lies in distinguishing the condition from obstruction of the common or major bile ducts, and from drug-induced cholestasis. The immunological feature of greatest value in making the distinction is the occurrence of mitochondrial antibody in primary biliary cirrhosis. The test for this antibody is best done by the indirect immunofluorescence technique, which is more specific than the AICF test. The test has now been positive, often in high titre, in 72 of 77 cases of primary biliary cirrhosis included in the recent reports, whereas it has been found, and then in low titre, in only 2 of 105 cases of bile duct obstruction, and positive results have not been reported among approximately 12 cases of drug-induced cholestasis (Walker *et al* 1965; Doniach *et al* 1966; Goudie *et al* 1966).

The importance of distinguishing with certainty between primary biliary cirrhosis and mechanical bile duct obstruction is so great that at present it is usually considered necessary to perform exploratory laparotomy to exclude the latter condition. It seems likely, however, that the test for mitochondrial antibody, together with percutaneous liver biopsy, will greatly reduce the need for laparotomy, with its deleterious effect, in patients with primary biliary cirrhosis.

The mitochondrial antibody test is also likely to be of diagnostic help in those patients presenting with pruritis which may persist for months or years before jaundice becomes apparent (Sherlock 1959), and also in patients first seen in a period of remission.

In some cases of active chronic hepatitis, cholestasis is a distinct feature. The clinical picture may resemble that of primary biliary cirrhosis, and differential diagnosis between the two conditions may occasionally be difficult (Datta, Sherlock & Scheuer 1963). Mitochondrial antibody does not appear to develop more often in cholestatic than in more typical cases of active chronic hepatitis (Doniach *et al* 1966), but as the incidence in this condition is approximately 30% the finding of the antibody is of no real help in the differential diagnosis. On the other hand, absence of the antibody is likely to provide a good indication that the case is not one of primary biliary cirrhosis.

Cryptogenic cirrhosis

If it is accepted that the features of cirrhosis include diffuse loss of lobular architecture, nodule formation and fibrosis, then the condition must be regarded as irreversible, and liable to progress and cause death from hepatocellular failure or portal hypertension. It is particularly in cases of fully developed cirrhosis that difficulty is encountered in determining the nature of the pre-cirrhotic lesion, for the distinguishing features of active chronic hepatitis, nutritional deficiencies and alcoholism, and of primary biliary 'cirrhosis', tend to subside in the later stages of these diseases and to be obscured by true cirrhosis. Accordingly, the term cryptogenic cirrhosis is commonly applied to fully developed cirrhosis without features indicative of any of the known pathogenic processes. In such cases, immunologocal investigations may suggest the pathogenesis. For example, a high level of serum IgG or positive tests for nuclear, AICF, or smooth muscle antibodies, or for rheumatoid factor, raise the possibility that the condition may have resulted from active chronic hepatitis, whereas the demonstration of mitochondrial antibody is unduly common in patients with a clinical history suggestive of primary biliary cirrhosis (Doniach *et al* 1966). Such findings are not of much importance in the handling of individual patients, for the main clinical problems here are those resulting from cirrhosis, regardless of its pathogenesis. The investigation of the causal factor in cirrhosis is, however, of considerable importance in relation to world-wide studies of nutritional deficiency and of chronic liver disease.

ADRENAL CORTEX

Idiopathic Addison's Disease

The features of idiopathic Addison's disease (primary adrenal atrophy) which suggest that it is of auto-allergic nature include the histological changes in the adrenal glands, the occurrence of organ-specific antibodies to adrenocortical cells in the serum of some patients, and the occurrence of adrenalitis and of antibodies to adrenal cortex in animals receiving immunizing injections of preparations of adrenal tissue. In these respects, the condition parallels chronic thyroiditis, and the high incidence of chronic thyroiditis, and probably also of chronic gastritis, in patients with idiopathic Addison's disease, provides further support for inclusion of this condition among the organ-specific auto-allergic diseases. These features are discussed below in more detail.

Pathological Changes

Both adrenal glands are very much smaller than normally, and may indeed be difficult to find at post-mortem examination. Microscopy shows loss of most of the cortical cells, and infiltration of any surviving cortical tissue with lymphocytes and plasma cells. The changes which have led up to this extreme degree of atrophy are apparently progressive and irreversible.

Antibodies to Adrenocortical Cells

The occurrence of adrenocortical antibodies in the serum of a proportion of patients with idiopathic Addison's disease has now been reported by several groups of workers (Anderson *et al* 1957; Mead 1962; Blizzard, Kyle, Chandler & Hung 1962; Blizzard & Kyle 1963; Irvine 1963; Goudie, Anderson, Gray & Whyte 1966). The antibodies are demonstrable by complement fixation and indirect immunofluorescence techniques, and it has been the general experience that the latter technique provides the more sensitive method: by its use, antibody was detected in approximately 50% of cases in the two relatively large series of Blizzard & Kyle (1963) and Goudie *et al* (1966).

Nature of the antigens

Immunofluorescence tests demonstrate that the adrenal antibodies react diffusely with the cytoplasm of cells of the whole thickness of the adrenal cortex. The cells of the innermost part frequently stain particularly brightly, and the staining of small groups of cortical cells lying in the medulla is specially prominent. The antigen is destroyed by treating the tissue or sections with various fixations, and positive results have been reported only with unfixed adrenal tissue. In most cases, the antibody reacts only with adrenal tissue; tests with the other endocrine glands, and with liver, kidney, etc., are negative. These conclusions are based on the results of immunofluorescence and complement fixation tests,

and upon absorption studies. Tests with adrenal tissue of various mammalian species have, however, given positive results, and the antigen is thus adrenal-specific but not species-specific (Blizzard & Kyle 1963). Absorption studies with centrifugal fractions of adrenal tissue were also reported by Blizzard and Kyle, who concluded that the antigen was present in microsomal and mitochondrial fractions, but it appears from recent unpublished investigations, referred to by Goudie *et al* (1966), that the antigen is confined to the microsomal fraction, and is chemically similar to the thyroid and gastric microsomal antigens.

Blizzard & Kyle (1963) concluded, from the pattern of complement fixation observed in 'checkerboard' tests of positive sera with adrenal extract, that there were two distinct types of adrenal antibody, and further evidence of a second antigen-antibody system, based on tests with tissues other than adrenal, is provided on p. 1120).

Clinical associations of adrenal antibodies

As already mentioned, adrenal antibody has been detected in the serum of approximately 50% of patients diagnosed as idiopathic Addison's disease. However, it is not usually possible to ascertain the cause of primary adrenocortical insufficiency during life. In the absence of any evidence of tuberculosis, by far the most likely cause is primary adrenal atrophy, but tuberculous adrenalitis (or other pathological agents) cannot be ruled out, since radiologically visible adrenal calcification of the adrenals is present in only a proportion, probably less than 50%, of cases of tuberculous Addison's disease (Dunlop 1963). Conversely, adrenal calcification almost certainly indicates an infective granuloma, usually tuberculosis, but evidence of healed or active tuberculosis elsewhere in the body in a patient with Addison's disease is only of presumptive aetiological significance. With these reservations, adrenal antibody has been detected almost exclusively in cases of Addison's disease of idiopathic type. Blizzard & Kyle (1963) reported positive complement fixation tests for adrenal antibody with the serum of two patients with Addison's disease considered to be of tuberculous type. Although the two sera did not react to fix complement with kidney tissue, their titres with adrenal tissue are not stated, and antibody of the 'AICF' type (see p. 1099) could be responsible. The immunofluorescence test for adrenal antibody, which in this investigation was more sensitive than the complement fixation test, was negative with these two sera. In the investigation reported by Goudie *et al* (1966), 27 patients with Addison's disease had evidence of healed or active tuberculosis: of 7 with radiological evidence of adrenal calcification, the serum of 1 gave a positive immunofluorescence test for adrenal antibody: in 9 cases with evidence of active tuberculosis, the test was negative, while 1 of 11 cases with radiological evidence of healed tuberculosis was positive. It is of interest that 1 of these 2 patients with a positive test developed thyrotoxicosis after the onset of Addison's disease, and the other had diabetes mellitus. Both of these

conditions have been reported to have an association with the organ-specific auto-allergic group of diseases, and the nature of the lesion causing adrenal insufficiency in these 2 cases must be regarded as uncertain. The possible association of adrenal tuberculosis and idiopathic adrenocortical atrophy seems most unlikely, but Barker (1929) described a case which was apparently of this nature, and stated without giving references that several similar cases had been reported.

Adrenal antibodies are rarely found in patients without Addison's disease. Blizzard & Kyle (1963) reported negative results in other diseases of the adrenals, and in 68 subjects without adrenal disease. Goudie *et al* (1966) obtained negative results in tests on 89 patients with various auto-allergic diseases, including chronic thyroiditis and thyrotoxicosis. However, adrenal antibodies were detected by Blizzard & Kyle (1963) in 4 of 27 patients with idiopathic hypoparathyroidism, but without Addison's disease. The recent demonstration of antibodies to parathyroid tissue in the serum of some patients with idiopathic hypoparathyroidism (Blizzard, Chee & Davis 1966) suggests that it is of an auto-allergic nature, and the common association of idiopathic Addison's disease suggests that the 4 cases with adrenal antibodies reported by Blizzard and Kyle may have had subclinical chronic adrenalitis. It is not known whether such lesions can develop, and remain at subclinical levels, as occurs in chronic focal thyroiditis, or whether they invariably progress to idiopathic Addison's disease.

Adrenal antibody has been reported also in an unusual case of Cushing's syndrome: only one adrenal was hyperplastic, but both were infiltrated with lymphocytes and plasma cells, particularly in the medulla and cortico-medullary junction. Lymphocytic infiltration was found in the hyperplastic adrenals of 19 of 20 other cases of Cushing's syndrome, but 9 other cases were tested for adrenal antibodies with negative results. The possibility of a globulin factor stimulating the adrenal cortex, comparable to LATS in thyrotoxicosis, was raised in this report (Wegienka *et al* 1966).

It is apparent from the above findings that adrenal antibodies occur in the serum of approximately 50% of patients with idiopathic Addison's disease, and are rarely found apart from this condition. The test is therefore of some diagnostic value in patients in whom the evidence of adrenal insufficiency is equivocal, and in patients placed on steroid replacement therapy without proof of adrenal insufficiency, where it may be considered inadvisable to interrupt the therapy for diagnostic purposes.

The diagnostic value of antibody tests in Addison's disease obviously depends on the development of the antibody at an early stage of the pathological process. In this respect, the incidence of adrenal antibody did not appear to be influenced by age, sex or duration of disease in the cases investigated by Goudie *et al* (1966), and it is thus apparent that most or all of those patients who develop the antibody do so at an early stage. We have encountered a patient with Hashimoto's

disease who subsequently developed Addison's disease: a specimen of serum obtained 6 months before the clinical onset of Addison's disease was found to contain adrenal antibody.

The detection of adrenal antibodies in a patient with Addison's disease is strong evidence that the adrenal destruction is 'idiopathic,' and not tuberculous, but there is evidence, stated above, to indicate that occasional patients with tuberculosis and Addison's disease have adrenal antibodies in their serum. Obviously, the finding of antibody in such cases should not influence the assessment of the need for antituberculosis therapy, but the detection of antibody in cases with no evidence of tuberculosis, or with evidence of healed pulmonary tuberculosis, may be of value as an indication that such therapy is unnecessary.

Idiopathic hypoparathyroidism is such a rare disease that the occurrence of adrenal antibodies in some cases is of little practical importance. It will be of interest, however, to ascertain whether the presence of adrenal antibody in such cases presages the development of idiopathic Addison's disease.

Antibody to steroid-producing cells

In testing adrenal antibodies for organ-specificity by the immunofluorescence technique, we have encountered a serum which reacts not only with adrenocortical cells, but also with the cytoplasm of the interstitial cells of the testis, theca interna and luteal cells of the ovary, hilus cells of the ovary and testis, and placental syncytiotrophoblast. Tests with a wide range of other tissues were negative, and the reactivity of the serum was removed by absorption with adrenal or corpus luteal tissue, but not with thyroid, liver or kidney. The patient providing this serum was a young man with idiopathic hypoparathyroidism, malabsorption syndrome, and presumptive Addison's disease. He was on corticosteroid therapy when first seen, and in view of the complex nature of his illness, it was not considered justifiable to interrupt his therapy for the purpose of confirming the diagnosis of Addison's disease. This patient showed no evidence of hypogonadism, and the blood levels of gonadotrophins were not unusually high. Weak antibody of the same type has since been detected in a second patient, a man with apparently uncomplicated idiopathic Addison's disease. No more examples of this unusual antibody have been found among 30 sera containing adrenal antibodies.

There is no evidence that the development of antibody to steroid-producing cells in these patients was associated with lesions of the gonads, nor is there much evidence to suggest gonadal lesions in other cases of idiopathic Addison's disease. Lyman Duff & Bernstein (1933) described small shrunken ovaries with few or no graffian follicles in 2 patients dying at ages 36 and 37, and interstitial fibrosis of the testes with absence of interstitial cells was reported in a boy of 16 by Simpson (1932), but no lesions of the gonads were observed in the post-mortem study of Sloper (1953). An interesting case of idiopathic Addison's

disease with primary ovarian deficiency, primary myxoedema and diabetes mellitus was described by Christy, Holub & Tomasi (1962), but although adrenocortical antibody was detected, it was not found to react with ovarian tissue (Blizzard, Tomasi & Christy 1963).

Associated Diseases

Thyroid disorders

It has long been known that chronic thyroiditis, both clinically apparent and latent, is a common accompaniment of Addison's disease. The association of primary myxoedema was described by Schmidt (1926) and many cases have since been reported. The combination with Hashimoto's disease appears to be less common. Investigations on a total of 119 patients with idiopathic Addison's disease have been described by Blizzard & Kyle (1963), Irvine (1963) and Goudie *et al* (1966). The associated diseases which were observed in more than one of these 119 patients are listed in Table 41. 1, from which it is seen that 10 cases had

Table 41.1

Associated diseases in 119 patients with idiopathic Addison's disease

Disease	No. of cases
Primary myxoedema	7
Hashimoto's disease	3
Thyrotoxicosis	6
Pernicious anaemia	5
Diabetes mellitus	9
Primary hypoparathyroidism	15

clinically apparent chronic thyroiditis. The incidence of subclinical degrees of chronic thyroiditis has been shown in post mortem studies to be very high in patients with Addison's disease (Walls 1930; Sloper 1953; Bloodworth, Kirkendall & Carr 1954), and review of these reports indicates that the association with idiopathic Addison's disease is particularly strong: it remains uncertain whether the incidence is increased in tuberculous Addison's disease, but if so, the increase is less striking. In view of the common accompaniment of chronic thyroiditis, a high incidence of thyroid auto-antibodies might be expected in idiopathic Addison's disease, and this expectation appears to be fulfilled by the detection of thyroid microsomal antibody in 38 of the 119 cases referred to in Table 41. 1. It is, however, important to emphasize that the known immunological interests of the authors of the reports on these 119 patients are likely to have influenced the selection of cases encountered by them. This point has been made by Goudie *et al* (1966) in relation to their cases, and almost certainly applies

to the two other reports. For this reason, the high incidences of associated diseases listed in Table 41.1 must be regarded with suspicion, particularly as all of them have auto-allergic features. The high incidence of thyroid antibody in these cases may also not be representative for idiopathic Addison's disease. However, Goudie *et al* (1966) reported that the evidence of thyroid microsomal and/or gastric parietal cell antibody in their female idiopathic cases of Addison's disease was significantly greater than in their female tuberculous cases: this difference was not attributable to age or sex distributions and provides strong evidence for a raised incidence of these auto-antibodies in the idiopathic form of the disease.

It seems likely that there is an increased incidence of thyrotoxicosis in patients with Addison's disease. The subject was reviewed by Stewart, Green & Lowe (1962), who concluded that the association might be merely fortuitous. In reviewing the records of 538 patients with Addison's disease seen at the Mayo Clinic between 1913 and 1958, Gastineau, Myers, Arnold & McConahey (1964) report a 3% incidence (16 cases) of thyrotoxicosis. Many of the cases were seen at a time when the life expectancy of Addison's disease was short, and these 16 patients mostly developed Addison's disease after, or at the same time as, the onset of thyrotoxicosis: accordingly, the figure of 3% may be too low. On the other hand, as the authors point out, the apparent association may have been exaggerated by unintentional case selection.

Chronic gastritis

There is no statistical evidence of an increased incidence of pernicious anaemia in patients with Addison's disease, but cases of the two conditions are not rare (Table 41.1), and there is both direct and indirect evidence that chronic gastritis of the acid-secreting mucosa is common in Addison's disease. The direct evidence is provided by gastric biopsy studies which suggest that chronic gastritis is particularly common in idiopathic Addison's disease (Feyrter & Klima 1952; Smith, Delamore & Wynn Williams 1961). The indirect evidence includes the demonstration of a high incidence of gastric parietal cell antibody in idiopathic Addison's disease (Irvine 1963; Goudie *et al* 1966), which suggests that chronic gastritis is commonly present. As already stated, case selection may have influenced this finding. The common occurrence of histamine-fast achlorhydria or hypochlorhydria in Addison's disease cannot be regarded as evidence of chronic gastritis, for in many cases the acid secretion reappears or increases following steroid replacement therapy (Gray, Ramsay & Thorn 1956; Soffer, Dorfman & Gabrilove 1961).

Diabetes mellitus

The association of diabetes with Addison's disease was reviewed in 1954 by Stanton, Jones and Marble, who found reports on 46 cases. More recently,

Csapó, Dávid & Kovács (1963) referred to approximately 80 cases in the literature. As diabetes is a common disease, it is not possible to determine whether these figures indicate a true association. Theoretically, an association between the diseases might arise in two ways. Firstly, by diabetes predisposing to tuberculosis, and so possibly to tuberculous Addison's disease. Secondly, there is evidence that diabetes has organ-specific auto-allergic features (see Moore & Neilson 1963 and Mancini *et al* 1965) and it might therefore be expected to have associations with other diseases with organ-specific auto-allergic features, including idiopathic Addison's disease. Soffer *et al* (1961) appear to regard the association as fortuitous, but point out that of the 28 cases in which post-mortem examination of the adrenals was performed, the ratio of idiopathic to tuberculous cases was 3:1. They consider that this ratio is higher than in Addison's disease in general. The possibility of a true relationship between diabetes and idiopathic Addison's disease finds support also, with the reservations already stated, from the incidence shown in Table 41.1.

Idiopathic hypoparathyroidism

Idiopathic Addison's disease, idiopathic hypoparathyroidism and superficial moniliasis have been reported to be associated in all four possible combinations (see Soffer *et al* 1961; Blizzard & Kyle 1963; Blizzard *et al* 1966). Because idiopathic hypoparathyroidism is a rare condition, the number of cases in which it has accompanied idiopathic Addison's disease almost certainly indicates a true association. Very strong evidence in support of this is provided also by the finding that adrenocortical antibody rarely occurs in individuals without idiopathic Addison's disease, and yet is to be found in some cases of uncomplicated idiopathic hypoparathyroidism (Blizzard & Kyle 1963). Recently, Blizzard *et al* (1966) have reported the detection of antibody reacting in immunofluorescence tests with the cytoplasm of parathyroid cells. The antibody was organ specific and was detected in the serum of 38% of cases of idiopathic hypoparathyroidism, 26% of cases of idiopathic Addison's disease, 12% of cases of Hashimoto's disease and 6% of 'control' hospital patients. An increased incidence of thyroid and gastric auto-antibodies was also detected in patients with idiopathic hypoparathyroidism. If confirmed, these findings indicate that idiopathic hypoparathyroidism is one of the group of so-called organ-specific auto-allergic diseases, and its associations with idiopathic Addison's disease and pernicious anaemia (Hung, Migeon & Parrott 1963; Morse, Cochrane & Landrigan 1963; Blizzard & Kyle 1963; Irvine 1963; Goudie *et al* 1966) are not surprising. The significance of the association with superficial moniliasis remains unexplained.

EXPERIMENTAL AUTO-ALLERGIC ADRENALITIS

The development of adrenal lesions and auto-antibodies in guinea-pigs and rabbits, following injection of adrenal tissue homogenate plus Freund's adjuvant,

has been reported by several groups of workers. Results of such experiments have, however, been rather conflicting and are complicated by the occurrence of what may be termed 'non-specific' lesions in various organs, including the adrenals, following injection of adjuvant emulsion containing saline or various tissue extracts. Distinctive or 'specific' lesions, affecting only the adrenals and developing in animals injected with adrenal tissue homogenate in adjuvant emulsion, were first reported by Colover & Glynn (1958) in guinea-pigs, and similar findings were described by Steiner, Langer, Schatz & Volpe (1960) and by Barnett, Dumonde & Glynn (1963). In similar experiments, Witebsky & Milgrom (1962) and Terplan, Witebsky & Milgrom (1963) also described lesions, but they did not consider that there was any good evidence in support of an auto-allergic pathogenesis. In all these experiments, homologous or autologous adrenal tissue (the latter obtained by unilateral adrenalectomy) was used for injection. Antibody reacting specifically with guinea-pig adrenal tissue, including autologous adrenal, was demonstrated by Witebsky and his co-workers, in complement fixation, tanned red cell agglutination, and precipitation reactions. By contrast, Barnett *et al* demonstrated adrenal lesions, but not antibodies, in guinea-pigs receiving injections of homologous adrenal: they observed more extensive adrenal lesions, and also antibodies reacting with autologous adrenal tissue, in animals injected with heterologous adrenal tissue. The adrenal lesions observed in these experiments consisted of foci of lymphocytes and histiocytes, with smaller numbers of plasma cells and eosinophil leucocytes, and with degenerative changes of the adrenocortical cells in the foci. Usually, the lesions have been most numerous and extensive in the inner part of the cortex, but similar infiltrates occur in the medulla, and may be related to isolated adrenocortical cells which lie there (Steiner, Langer & Schatz 1960).

Lesions of the adrenals have also been described in rabbits injected with autologous or homologous adrenal tissue homogenate plus Freund's adjuvant (Milcou *et al* 1959; Witebsky & Milgrom 1962). Negative results were reported in similar experiments by Steiner *et al* (1960b) and Barnett *et al* (1963), although the latter workers observed lesions in rabbits injected with heterologous (guinea-pig or rat) adrenal tissue. Antibodies to adrenocortical tissue were detected by Witebsky & Milgrom (1962), by complement fixation, tanned red cell agglutination and precipitation tests, in rabbits injected with homologous or autologous adrenal tissue: the antibodies reacted with autologous adrenal tissue and not with various other rabbit tissues, including testis and ovary. Barnett *et al* (1963) also demonstrated auto-antibodies to adrenal tissue, which were of low titre in rabbits receiving injections of homologous adrenal tissue and of high titre in those injected with guinea-pig adrenal tissue. The antibodies were of IgG type, and reacted with certain other rabbit tissues in addition to adrenal: heating at 65°C to inactivate the 'natural' non-tissue-specific auto-

antibodies (Kidd & Friedewald 1942) did not render the sera adrenal-specific, but complement fixation, immunofluorescence and absorption studies demonstrated an antibody which reacted with the cytoplasm of adrenocortical cells, of interstitial cells of the ovary and testis, and in some instances with spermatozoa and their precursors. This antibody appears to behave similarly to that occurring rarely in patients with Addison's disease, and reacting with steroid-producing cells (see p. 1120).

It is well established, from the investigations described above, that auto-antibody to adrenal tissue develops in some guinea-pigs and rabbits injected with adrenal tissue and Freund's adjuvant. The antigenic adrenal constituent is heat-labile (Witebsky & Milgrom 1962; Barnett *et al* 1963) and evidence of its protein nature has been provided by Shulman, Centeno, Milgrom & Witebsky (1965). By use of Ouchterlony precipitation techniques upon concentrated rabbit adrenal auto-antibody, Centeno, Shulman, Milgrom & Witebsky (1965) have demonstrated four adrenal antigens, three of which were not detected in other rabbit tissues.

The auto-allergic nature of the experimentally produced adrenal lesions remains in doubt. Terplan *et al* (1963) pointed out that the development of lesions did not correspond to the number of immunizing injections, nor to the duration of the experiment. In a histological study of the lesions, they found no obvious injury to adrenocortical cells, and although the lesions were most pronounced in animals injected with adrenal tissue plus Freund's adjuvant, occasional lesions were observed following injection with other tissues. In spite of these observations, and taking into account the complicating 'non-specific' adrenal lesions in animals injected with adjuvant emulsion, an auto-allergic pathogenesis offers the best explanation which has been suggested for the experimental lesion. The type of the postulated auto-allergic injury is unknown. It has been the general finding (as in auto-allergic encephalomyelitis and thyroiditis) that circulating antibody does not parallel the incidence and severity of the lesions, and skin tests for delayed hypersensitivity to adrenal tissue were reported as negative by Barnett *et al* (1963). As in experimental auto-allergic thyroiditis, the lesions subside without progressing to extensive destruction of the gland, and functional deficiency has not been demonstrated. The production of lesions and of adrenal antibodies more readily by injection of heterologous, as compared with injections of homologous or autologous, adrenal tissue (Barnett *et al* 1963) is reminiscent of the antibody-stimulating effects of injections of heterologous liver (see p. 1108). The greater effectiveness of heterologous adrenal tissue cannot be regarded as evidence against the auto-allergic nature of the experimentally produced lesions: it is better explained on the established basis that cross-reactive adrenal antigens are common to various species, and that the auto-antigenic stimulus provided by heterologous adrenal tissue is greater than that of autologous or homologous adrenal.

Conclusions

There seems no point in discussing at length the case for an auto-allergic pathogenesis of idiopathic Addison's disease. The evidence is similar to that relating to chronic thyroiditis, but is less strong, firstly because opportunity has not arisen to investigate the *auto*-allergic nature of adrenal antibodies, and secondly because the development of experimental 'auto-allergic' adrenalitis has not been shown to be associated with delayed hyposensitivity, as has experimental auto-allergic thyroiditis, nor has the experimental condition been induced by transfer of 'sensitized' lymphoid cells.

Present evidence suggests a particular association between idiopathic Addison's disease and idiopathic hypoparathyroidism in addition to associations of both conditions with chronic thyroiditis and chronic gastritis. It thus appears that the two former conditions should be placed together, as having common predisposing factors, as a sub-group within the group of organ-specific auto-allergic diseases.

The demonstration, in two cases of idiopathic Addison's disease, of antibody reacting not only with adrenocortical cells but also with other cells which produce steroid hormones (see p. 1120), is of interest in relation to pathogenesis. In chronic thyroiditis and chronic gastritis, the antibodies reacting with the thyroid and gastric mucosa respectively are, like the lesion, organ-specific. There is good evidence that these auto-antibodies are not the major pathogenic agents, and the lesions are best explained on the assumption of delayed hypersensitivity to the same organ-specific antigenic constituents, resulting in injury by a Type IV auto-allergic reaction. If the adrenocortical destruction of idiopathic Addison's disease is attributable to a Type IV reaction, and if its specificity parallels that of the circulating antibody, then destruction of the testicular interstitial cells might have been expected to accompany the adrenocortical lesion in the two cases with antibody to steroid-producing cells. Testicular lesions in these cases can be excluded only by biopsy, and this was not considered to be justified. The absence of clinical evidence, and in one case the normal blood levels of pituitary gonadotrophins, indicate that testicular interstitial cell function was not seriously depressed, and it must be concluded either that a type IV reaction of the same specificity as the circulating antibody is not a major pathogenic factor in these two cases, or that the adrenal cortex is more susceptible than the testicular interstititial cells to injury by such a reaction.

PANCREAS

Clinical and experimental investigations have provided no good evidence that pancreatic disease can result from auto-allergic reactions. The occurrence of pancreatic antibodies has, however, been reported in patients with pancreatic lesions, and antibodies reacting specifically with pancreas have been demonstrated to develop in animals following the administration of pancreatic tissue extract

incorporated in Freund's adjuvant: it has also been reported that pancreatic antibodies develop following experimentally induced pancreatic injury. Curiously, in both man and animals, the pancreatic antibodies have reacted with homologous, but usually not with autologous pancreatic tissue: i.e. they are iso-antibodies. The occurrence of organ-specific iso-antigens, and the reported development of organ-specific pancreatic iso-antibodies in patients with lesions of the pancreas, provide intriguing problems and may have far-reaching significance.

Pancreatic Antibodies in Man

Most of the observations on pancreatic antibodies in man are those reported by Thal and his co-workers (Thal, Egner & Murray 1959; Thal, Murray & Egner 1959; Thal 1960; Murray & Thal 1960). Using an Ouchterlony precipitation technique, with crude saline extract of normal pooled human pancreas as antigen, they tested the serum of patients with various diseases, and of healthy individuals, for the presence of precipitating antibodies. Positive results were observed in most cases of chronic pancreatitis, cancer of the pancreas and mucoviscidosis, while tests with the serum of 50 blood donors were all negative. Of sera from 228 hospital patients with various diseases, 10 were positive: in some of these, pancreatic disease could not be excluded, and in others the antibody was stated to react also with extracts of other organs. Positive results were reported also in 7 of 80 cases of diabetes mellitus, and review of the features of the 7 positive cases provided no indication that diabetes might have resulted from chronic pancreatitis. The reports containing these findings are lacking in detail. It is stated, for example, that the antibodies reacting with pancreas are organ and species specific, but the evidence for this is not provided, apart from a statement that they do not react with human liver tissue extract. Nor is any evidence provided that the serum factors concerned in the reactions are immunoglobulins. In one report, Thal *et al* (1959a) state that the Ouchterlony precipitation tests are best read after 3–4 weeks at 10°. Besides reducing considerably the possible clinical value of the test, this long period increases the likelihood of various non-immunological precipitates developing in the agar. Thal and his co-workers did not report detailed investigations on the nature of the pancreatic antigens, but they concluded from differential centrifugation experiments that they were associated with the endoplasmic reticulum of pancreatic acinar cells, and that pancreatic juice did not contain them.

In four cases of chronic pancreatitis, serum and pancreatic tissue were available, and autologous tests were performed. In three of the cases, the results were negative, although the sera reacted with the other pancreases: in the fourth case, the autologous reaction was reported as positive.

Positive Ouchterlony precipitation tests for pancreatic antibodies were reported also by Fonkalsrud & Longmire (1961). Like Thal and his co-workers,

they reported positive results in most cases of chronic pancreatitis and cancer of the pancreas, but 5 of 19 'unselected' medical and surgical hospital patients were also found to be positive: 4 of the 5 had marked inflammation in the vicinity of the pancreas (e.g. due to peritonitis, peptic ulceration, or 'cancer of the duodenum'): a weak positive result was also observed in 1 of 7 blood donors tested. Two or more lines of precipitate were usually observed in positive tests, suggesting multiple antigen-antibody systems, but tests with extracts of organs other than pancreas, and attempts to characterize the serum factors as immunoglobulins, were not described. Fonkalsrud and Longmire also prepared a rabbit antiserum to pooled human pancreatic extract, and after absorption with human serum, this was used to detect the presence of pancreatic antigens in the serum of their patients by the Ouchterlony precipitation technique. Positive results were reported with serum from 7 of 16 patients with chronic pancreatitis, and 2 of 4 patients with cancer of the pancreas: most or all of these sera were also positive in tests for pancreatic antibody, but some cases (e.g. a patient with persistent gastro-enteritis and another with gastric cancer) gave a positive test for antigen and a negative test for antibody. In one of the tests for antigen, the formation of two lines of precipitate suggested the presence of two antigens. These findings are consistent with the escape of multiple iso-antigens from pancreatic lesions. However, tests for organ specificity of the antigens were not described, and the interesting possibility of demonstrating precipitation reactions between the sera containing antigens and those containing antibodies was apparently not investigated. These findings are discussed later, together with those in experimental investigations.

Immunological investigation of patients with mucoviscidosis has been reported by Stein, Manlapas, Soike & Patterson (1964) who tested bronchial mucus and serum for antibodies to pancreas and lung tissue. Positive results were reported with the bronchial mucus of 14 out of 16 patients with mucoviscidosis in Ouchterlony precipitation tests with saline extracts of lung tissue from patients with mucoviscidosis: the lines of precipitate were well defined and appeared within 24 hr. Positive tests were also observed with mucoviscidosis pancreatic tissue, but the lines were poorly defined and developed more slowly, and the precipitates with lung and pancreatic tissue extracts were not shown to be identical. Tests of bronchial mucus from mucoviscidosis patients with lung and pancreatic extracts from individuals without mucoviscidosis were reported as negative. The factor in bronchial mucus responsible for the reaction with lung tissue extract resisted heating at 56° for 30 min and was inactivated by trypsin. The precipitation line was found to fuse completely with that produced in the reaction between the bronchial mucus and a goat antiserum to human immunoglobulin, a result which can only be reconciled with the authors' conclusion (that the bronchial mucus contains precipitating antibody to lung tissue) if it is assumed that most of the immunoglobulin in the bronchial mucus

consisted of such antibody. This seems most unlikely, and in the absence of more reliable evidence of the immunoglobulin nature of the mucus factor (e.g. based on DEAE fractionation, salt precipitation or immunoelectrophoresis) the nature of the reaction with lung tissue extract remains in doubt, particularly as tests upon the serum of the patients failed to demonstrate antibodies to mucoviscidosis lung and pancreatic tissues.

Experimentally Produced Pancreatic Antibodies

The investigations of Thal and his co-workers, part of which has been discussed above, included also attempts to stimulate the development of pancreatic antibodies in experimental animals. Pancreatic necrosis was produced in dogs and rabbits by injecting staphylococcal α-toxin directly into the pancreas, and also by intramuscular or subcutaneous injection of homologous pancreatic tissue extract incorporated in Freund's adjuvant (Thal *et al* 1959a). In both groups of animals, antibodies to pancreatic tissue extract were detected by the Ouchterlony precipitation technique; tests of positive sera with an extract of the animal's own pancreas were invariably negative, and the positive reactions were therefore attributed to iso-antibodies. The only pancreatic lesion observed was localized scarring in those animals which had been injected with staphylococcal toxin, although the authors state that they observed pancreatic lesions in chickens and ducks injected with homologous pancreatic extract plus Freund's adjuvant. An investigation by Rose, Metzgar & Witebsky (1960) was reported in much greater detail. They administered to rabbits injections of pooled saline extract of rabbit pancreatic tissue, together with Freund's adjuvant. Antibodies reactive with pooled pancreatic tissue extract were detected in the serum of the experimental animals by Ouchterlony precipitation, complement fixation, and passive cutaneous anaphylaxis tests. At least four distinct iso-antibodies were demonstrated, and these could be removed individually by absorption with pancreatic tissue extracts containing the appropriate iso-antigen. The iso-antibodies resisted heating at 65° for 30 min, and had the electrophoretic mobility of IgG. They were shown to be organ and species specific, and failed to react with autologous pancreatic tissue extract. In another investigation (Witebsky, Rose & Nadel 1960) rabbits were injected with crude saline extracts of hog, dog, beef or human pancreas, together with Freund's adjuvant: antibodies reactive with pancreatic tissue were demonstrated by complement fixation, tanned red cell agglutination and precipitation tests, and were shown to be both species and organ specific.

Further investigation of pancreatic iso-antibodies in animals was reported by Metzgar (1964a) who demonstrated iso-antibodies to pancreatic tissue in rabbits and rhesus monkeys following injections of pooled homologous pancreatic extract plus Freund's adjuvant. Once again, the antibodies were found to be organ and species specific, and to be non-reactive with autologous pancreas. No

pancreatic lesions were observed in the experimental animals, which included monkeys immunized for up to 14 months. Injection of rabbit serum containing pancreatic iso-antibody into normal rabbits (most of which were later shown to have the corresponding pancreatic iso-antigens) did not result in pancreatic lesions. Attempts to induce lesions by transfer of lymphoid cells were also unsuccessful although, as Metzgar points out, no precautions were taken to protect the injected cells against homograft rejection, and the negative result was therefore to be expected. Metzgar describes also immunofluorescence studies with rabbit pancreatic iso-antibodies, using alcohol-fixed sections of pancreatic tissue: fluorescence was confined to the cytoplasm of pancreatic acinar cells, and staining was most intense in the secretory part of the cells. Pancreatic juice collected from rabbits and rhesus monkeys following pilocarpine stimulation was then shown to contain the organ-specific iso-antigens, and Ouchterlony precipitation tests showed reactions of identity between the antigens in the juice and those in pancreatic tissue extracts. The administration of injections of autologous pancreatic extract (obtained by partial pancreatectomy) plus Freund's adjuvant to 10 rabbits did not result in lesions in the residual pancreas, nor were auto- or iso-antibodies to pancreatic tissue detected in the animals' sera. In another report, Metzgar (1964b) refers to difficulties in demonstrating human pancreatic iso-antibodies: these included non-specific reactions in Ouchterlony precipitation tests, and non-reproducibility of complement fixation and tanned red cell agglutination tests. In order to overcome these hazards, he administered homogenates of individual human pancreases, together with Freund's adjuvant, to rhesus monkeys. Complement fixing iso-antibodies developed and were shown by tests with a wide variety of human tissues, including salivary glands, gastro-intestinal mucosa and by absorption tests with red cells, to be organ specific. As in the rhesus monkey and rabbit, the human iso-antigens were shown to be present in pancreatic secretion. Characterization of the human pancreatic iso-antigens was prevented by the strong anti-complementary activity of the immune sera following absorption with individual pancreatic extracts.

Discussion

Considering first the experimental observations, it is apparent that some of the claims to have induced the development of antibodies reactive with pancreas have not been supported by firm evidence of tissue specificity of the observed reactions, nor of the serum factors concerned being immunoglobulins. However, the work of Metzgar is not subject to these criticisms, and provides good evidence that, in the rabbit and rhesus monkey, the pancreatic acinar cells and their secretion contain multiple iso-antigens which are species specific and are apparently absent from a wide range of other tissues. The findings of Thal and his co-workers in experiments on dogs and rabbits are consistent with these

conclusions. All the reported autologous tests have failed to demonstrate the production of pancreatic auto-antibodies in experimental animals. The failure reported by Metzgar to induce pancreatic lesions or antibodies by injecting rabbits with autologous pancreatic tissue in Freund's adjuvant, and the absence of pancreatic lesions in animals which have developed pancreatic iso-antibodies, suggest that the individual animal possesses a high degree of immunological tolerance to the iso-antigens present in its own pancreas. Apart from their location solely in one organ, the pancreatic iso-antigens present features resembling those of the blood group substances secreted in human saliva, gastric juice, etc. However, the reported development of pancreatic iso-antibodies in animals subjected to pancreatic injury (Thal *et al* 1960a) is difficult to explain on this basis, and presents a situation similar to that discussed below in relation to human pancreatic disease.

The investigations of Metzgar (1964b) indicate that in man, as in rabbits, rhesus monkeys, etc., the pancreas contains multiple iso-antigens in the acinar cells and their secretion. The demonstration of circulating iso-antibodies reacting specifically with pancreatic tissue, mainly in patients with pancreatic lesions, requires confirmation and further investigation. On the present evidence, the two most likely explanations of the phenomenon are, firstly, that the observed reactions are non-immunological and that the serum factors concerned are not antibodies, and, secondly, that the reactions are due to antibodies of an auto-allergic nature, developing in relation to pancreatic injury from various causes. If this second possibility is correct, then it is necessary to explain also the failure of the antibodies to react with autologous pancreatic tissue in 3 of the 4 cases tested (Thal *et al* 1959a). One possible explanation, which has already been considered in relation to the AICF reaction (see p. 1099) is that the presence of a pancreatic auto-antibody in the blood may result in blocking of the corresponding auto-antigen in homogenates of that individual's pancreas: on this basis, the presence of multiple iso-antigens in the pancreas, and the development of auto-antibodies against one or more of these in various combinations, would explain the reactivity between homologous serum and pancreatic extract, both of which give negative results in autologous tests. This possible interpretation of the findings in man, which assumes that the observed reactions are attributable to auto-antibodies to pancreatic iso-antigens, would explain also the simultaneous occurrence in the patients' serum of pancreatic antigens and antibodies, as has been described by Fonkalsrud & Longmire (1961).

Conclusions

Tests of human pancreatic antibodies with the patients' own pancreatic extract have been reported in only 4 cases, of which 3 were negative and 1 positive (Thal *et al* 1959a). Attempts to induce pancreatic auto-antibodies in experimental animals have resulted only in iso-antibodies which have not been demonstrated

to react with the animals' own pancreatic iso-antigens: nor have experimentally produced auto-allergic lesions of the pancreas been described. Clearly, the possibility of an auto-allergic pathogenesis for exocrine pancreatic disease is not suggested by the available evidence. The practicability of serum tests for pancreatic antibodies and antigens, and their value in the diagnosis of chronic pancreatitis and other chronic diseases involving the pancreas, cannot be assessed on the present evidence.

Diabetes Mellitus

The evidence that auto-allergy may be concerned in the pathogenesis of diabetes mellitus includes reports suggesting an association between juvenile diabetes and chronic thyroiditis, the reported demonstration in the serum of some diabetics of antibodies to insulin, and immunofluorescence studies which are consistent with a Type III auto-allergic reaction being involved in certain vascular lesions which are common in diabetics.

A 22% incidence of thyroid microsomal antibody in the serum of children with diabetes, as compared with 1.1% in controls, was reported by Pettit, Landing & Guest (1961). A similar result was reported by Landing *et al* (1963) in patients who had developed diabetes before the age of 25 years, but tests upon 'control' individuals were not reported. In both these investigations, the immunofluorescence test for thyroid antibody was apparently performed with normal human thyroid, and not with thyrotoxic thyroid, which is a better source of antigen. In spite of this and also the omission of control tests with other tissues, the results are best interpreted as suggesting an increased incidence of thyroid microsomal antibodies in the diabetics investigated. Similar conclusions were reached by Moore & Neilson (1963) from a well-controlled study in which the microsomal thyroid antibody was detected by a complement fixation test. These authors also provided evidence of an increased incidence of gastric parietal cell antibody in the serum of patients with early-onset diabetes.

The above findings suggest an association between juvenile diabetes and auto-allergic thyroiditis and gastritis. This is supported by the finding of chronic thyroiditis, an uncommon lesion in childhood, in 2 of 11 diabetic children who were examined *post mortem* (Landing *et al* 1963), and by the evidence that manifestations of auto-allergic gastritis, such as reduced gastric secretory activity and pernicious anaemia, are unduly common in diabetics (Arapakis *et al* 1963; Wilkinson 1963).

The development of antibodies to insulin in man and experimental animals has been reviewed by Pope (1966) and also in Chapter 10 of this book. The development of insulin antibodies in diabetic and non-diabetic patients who have received injections of heterologous insulin is of no auto-allergic significance. However, the detection of antibody to insulin in 33% of untreated diabetics, as compared with 4% in healthy blood donors, has been reported by

Páv, Ježková & Škrha (1963) using a technique depending on the consumption of endogenous complement on incubating the patient's serum with added insulin. The immunoglobulin nature of the presumed antibody was not established in this report, and in a similar study Chetty & Watson (1965) reported positive results with 58% of sera from untreated diabetes and 26% of control, non-diabetic sera: they concluded, however, that consumption of complement was attributable to its union with a complex of insulin and a non-immunological carrier protein. Interpretation of the results described in these two investigations, in which heterologous insulins were used as antigens in the antibody tests, is complicated further by the conflicting evidence on the degree of species specificity of antibodies to insulin (see Mancini, Costanzi & Zampa 1964a).

Immunofluorescence studies on diabetic sera were reported by Mancini, Costanzi & Zampa (1964b) and Mancini, Zampa, Vecchi & Costanzi (1965). Using as antigen acetone-fixed sections of fresh human pancreas, they reported positive direct and complement fixation immunofluorescence tests with serum from approximately 50% of treated and 3 of 5 untreated diabetics. The pattern of staining suggested the presence, in positive sera, of antibody reacting with the cytoplasm of the β-cells of the islets of Langerhans. Tests with sections of bovine and pig pancreas were negative, as were tests with sections of human pancreas pretreated with 8% sodium chloride (which removes insulin). Treatment of positive sera with heterologous insulin diminished, but did not abolish, the reaction. From these observations, Mancini and his co-workers concluded that the positive tests were attributable to antibodies to human insulin. Further work, including absorption studies, is necessary to establish the significance of these findings, and in particular there is no mention of antibody tests using unfixed islet tissue, which would be necessary for the detection of organ-specific antibodies to microsomal constituents corresponding to those occurring in patients with auto-allergic thyroiditis and gastritis and idiopathic Addison's disease.

Histological examination of the pancreatic islets in diabetics has provided little evidence to suggest an auto-allergic destructive lesion, but LeCompte (1958) described lymphocytic infiltration, accompanied apparently by loss of β-cells, in the islets of some infants and children dying shortly after the onset of diabetes, and refers to reports of similar observations.

Finally, there is evidence that antibodies to insulin, and also complement, may be present in certain vascular lesions which are commonly present in diabetics. Results of immunofluorescence studies have been summarized by Blumenthal, Berns & Owens (1963) who have reported the binding of fluorescein-conjugated insulin and of fluorescent antibody to complement, by the vascular lesions in the eye, glomerulus, islets of Langerhans, etc., of diabetics. They claim to have demonstrated these reactions in diabetics who have not received insulin injections, and suggest that diabetes may result from the development of auto-allergy

to altered endogenous insulin. They suggest that production and secretion of altered insulin might result from mutations in the β-cells of the islets of Langerhans. While of considerable interest, these findings require confirmation and further investigation. They raise the possibility of vascular injury resulting from a Type III auto-allergic reaction mediated by insulin-antibody complexes which have bound complement.

PROSTATE

Prostatitis in Man

There is no good evidence that prostatic disease results from an auto-allergic reaction, nor that prostatic tissue contains organ-specific antigen. However, subacute or chronic prostatitis is a feature, or at least a frequent complication, of Reiter's syndrome, and may be associated also with ankylosing spondylitis and with uveitis. These associations raise the possibility that prostatitis may result in hypersensitivity manifestations, conceivably of an auto-allergic nature. With this possibility in mind, Grimble (1964) examined the serum of patients with prostatitis, Reiter's syndrome, etc., for the presence of antibodies to prostatic tissue. Prostatic antigen was prepared by extraction of normal prostatic tissue with a hot phenol-water mixture: the watery extract was dialysed, lyophilized, resuspended in water, precipitated by ethanol, resuspended and lyophilized. It was reconstituted in water and used to coat tannic acid treated red cells which then provided the test reagent. Haemagglutination at serum dilutions of 1 in 25 or more was regarded as a positive result: tests were performed for tissue specificity, using the same technique, but with extracts of liver, kidney and colon. Of 21 sera from patients with Reiter's syndrome, 20 reacted with prostate, and of these 15 did not react with any of the other tissue extracts. Specific reactivity for prostatic extract was reported also in uveitis (4 of 8 cases), rheumatoid arthritis (2 of 18 cases) and rheumatic fever (1 of 7 cases). Tests were also performed upon 9 cases of subacute prostatitis, presumably without additional manifestations, and 8 were found to react specifically with prostate.

In a second paper (Grimble & Lessof 1965) the test for prostatic antibody was reported in a larger series of cases: tests with other tissue extracts were not described, but positive sera were retested after absorption with the prostatic antigen, and inhibition was taken to indicate specific reactivity. On this basis, the incidences of specific positive results were: Reiter's syndrome, 54%; ankylosing spondylitis, 39%; prostatitis, 18%. Occasional positive results were observed also among patients with rheumatoid arthritis and with various other diseases, and sera from two females (diagnosis unspecified) were found to be positive. These results must be interpreted with some caution, for the serum factor involved in the reaction was not shown to be associated with the immunoglobulins, and tests for organ specificity were of restricted range in the first paper, and were not reported in the second one. Considering the sensitivity of

tanned red cell agglutination tests in general, the titres (mostly below 1 in 1000) were low. The antigen-antibody nature of the reaction must therefore be regarded as no more than presumptive. Autologous tests, using positive serum and prostatic tissue from the same individual, were not described, and it is unlikely that opportunity arose to perform them. Nor is it stated how many prostates were tested for antigenicity and whether or not they were all reactive. In the absence of such information, the auto-allergic nature of the antibody cannot be regarded as established.

In spite of the above deficiencies, the observations of Grimble and Lessof are of considerable interest. It seems likely that the urethritis of Reiter's syndrome results from a virus infection, extension of which is presumably responsible for the common involvement of the prostate. The evidence, admittedly incomplete, that most patients develop antibody to prostatic tissue, supports the possibility that the polyarthritis, and possibly other complications, may be due to a Type III auto-allergic reaction in which the antigenic stimulus is provided by the inflamed prostate. It is of interest that 8 of 24 cases of Reiter's syndrome were reported by Grimble and Lessof to have a positive latex test for rheumatoid factor. An infective pathogenic agent has long been considered a possibility in rheumatoid arthritis, and has received recent support from the findings of Duthie, Stewart, Alexander & Dayhoff (1967). The joint lesions of rheumatoid arthritis and Reiter's syndrome may have a similar pathogenesis, and the possibilities include the direct action of infective agents, hypersensitivity to infective agents, and auto-allergic reactions initiated by the inflammatory changes of infection.

Experimental Animal Studies

Investigations on the antigenicity of the accessory genital glands of the male rabbit have been reported recently by Shulman and his co-workers (Shulman *et al* 1965, 1966). Injection of saline extract of rabbit prostatic tissue in Freund's adjuvant into rabbits resulted in the development of antibody demonstrable by agar-diffusion precipitation tests and by agglutination of tannic acid treated red cells coated with saline extract of rabbit prostate. The antibody exhibited species specificity and reacted with autologous prostatic tissue. It was shown conclusively that prostatic antigen participating in these reactions was present also in extracts prepared from the coagulating and bulbo-urethral glands and from the seminal vesicles. These accessory glands were apparently stored in the frozen state before being dissected from the prostate and from one another, and it remain possible that the antigen(s) demonstrated by Shulman *et al* is produced by one of the glands and gained access to the others by the common duct system. It is of interest that Weil (1961, 1965) demonstrated, in rabbits and various other species, the secretion by the seminal vesicles of a substance which combined firmly with the spermatozoa: antibodies to this substance were shown to be species specific, and although they reacted with both seminal vesicle and

prostatic tissue, immunofluorescence studies showed the antigen to be present in occasional ducts, but not in the glandular cells, of the prostate, and its presence in the organ was attributable to contamination by seminal fluid.

Pathological changes of the prostate or other male genital accessory glands have not been reported to result from iso-immunization experiments, and the observations on animals thus provide no support for the possibility of organ-specific auto-allergy to prostatic tissue in man.

REFERENCES

Liver

Ahrens E.H. Jr., Payne M.A., Kunkel H.G., Eisenmenger W.J. & Blondheim S.H. (1950) Primary biliary cirrhosis. *Medicine (Baltimore)* **29,** 299.

Anderson J.R., Goudie R.B. & Gray K.G. (1960) A consideration of auto-antigens with special reference to Hashimoto's disease. *Brit. J. exp. Path.* **41,** 364.

Anderson J.R., Gray K.G., Beck J.S. & Kinnear W.F. (1961) Precipitating autoantibodies in Sjögren's disease. *Lancet* **ii,** 456.

Asherson G.L. (1959) Antibodies against nuclear and cytoplasmic cell constituents in systemic lupus erythematosus and other diseases. *Brit. J. exp. Path.* **40,** 209.

Asherson G.L. & Dumonde D.C. (1962) Characterization of auto-antibodies produced in the rabbit by injection of rat liver. *Brit. J. exp. Path.* **43,** 12.

Asherson G.L. & Dumonde D.C. (1963) Autoantibody production in rabbits. II. Organ-specific autoantibodies in rabbits injected with rat tissues. *Immunology* **6,** 19.

Asherson G.L. & Rose M.E. (1963) Autoantibody production in rabbits. III. The effect of infection with *Eimeria stiedae* and its relation to natural antibodies. *Immunology* **6,** 207.

Baggenstoss A.H., Foulk W.T., Butt H.R. & Bahn R.C. (1964) The pathology of primary biliary cirrhosis with emphasis on histogenesis. *Amer. J. clin. Path.* **42,** 259.

Barr M., Buchanan W.W., Doniach D. & Roitt I.M. (1964) The antibody response to tetanus toxoid in patients with auto-immune thyroiditis. *Scot. med. J.* **9,** 295.

Beall G.N. (1963) The nature of the complement-fixing antigen in normal human liver extracts. *J. Lab. clin. Med.* **61,** 67.

Bearn A.G., Kunkel H.G. & Slater R.J. (1956) The problem of chronic liver disease in young women. *Amer. J. Med.* **21,** 3.

Beck J.S. (1963) Auto-antibodies to cell nuclei. *Scot. med. J.* **8,** 373.

Behar A.J. & Tal C. (1959) Experimental liver necrosis produced by the injection of homologous whole liver with adjuvant. *J. Path. Bact.* **77,** 591.

Bennett H.S., Luft J.H. & Hampton J.C. (1959) Morphological classifications of vertebrate blood capillaries. *Amer. J. Physiol.* **196,** 381.

Berkson J. (1946) Limitations of the application of fourfold table analysis to hospital data. *Biometrics* **2,** 47.

Bigley N.J., Dodd M.C. & Geyer V.B. (1963) Immunological specificity of antibodies to liver ribosomes and nuclei. *J. Immunol.* **90,** 416.

Bollag W. (1956) Nachweis von organspezifischer Antikörpern. *Experientia* **12,** 210.

Cherrick G.R., Pothier L., Dufour J.J. & Sherlock S. (1959) Immunologic response to tetanus toxoid inoculation in patients with hepatic cirrhosis. *New Engl. J. Med.* **261,** 340.

D'Amelio V. & Perlmann P. (1960) The distribution of soluble antigens in cellular structures of rat liver. *Exp. Cell Res.* **19,** 383.

DATTA D.V., SHERLOCK S. & SCHEUER P.J. (1963) Post-necrotic cirrhosis with chronic cholestasis. *Gut* **4,** 223.

DEICHER H.R.G., HOLMAN H.R. & KUNKEL H.G. (1960) Anti-cytoplasmic factors in the sera of patients with systemic lupus erythematosus and certain other diseases. *Arth. & Rheum.* **3,** 1.

DIXON F.J., FELDMAN J.D. & VAZQUEZ J.J. (1961) Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis. *J. exp. Med.* **113,** 899.

DODD M.C., BIGLEY N.J., GEYER V.B., MCCOY F.W. & WILSON H.E. (1962) Autoimmune response in rabbits injected with rat and rabbit microsomes. *Science,* **137,** 688.

DONIACH D., ROITT I.M., WALKER J.G. & SHERLOCK S. (1966) Tissue antibodies in primary biliary cirrhosis, active chronic (lupoid) hepatitis, cryptogenic cirrhosis and other liver diseases and their clinical applications. *Clin. exp. Immunol.* **1,** 237.

GAJDUSEK D.C. (1957) An auto-immune reaction against human tissue antigens in certain chronic diseases. *Nature* **179,** 666.

GAJDUSEK D.C. (1958) An 'autoimmune' reaction against human tissue antigens in certain acute and chronic diseases. I. Serological investigations. *Arch. int. Med.* **101,** 9.

GÖKCEN M. (1962) Autoimmunity in liver disease. *J. Lab. clin. Med.* **59,** 533.

GOUDIE R.B., MACSWEEN R.N.M. & GOLDBERG D.M. (1966) Serological and histological diagnosis. *J. clin. Path.* **19,** 527.

HACKETT E., BEECH M. & FORBES I.J. (1960) Autoimmune complement-fixation reaction in 1014 patients. *Brit. med. J.* **2,** 17.

HAVENS W.P. JR., MYERSON R.M. & KLATCHKO J. (1957) Production of tetanus antitoxin by patients with hepatic cirrhosis. *New Engl. J. Med.* **257,** 637.

HAVENS W.P. JR., SHAFFER J.M. & HOPKE C.J. JR. (1951) The production of antibody by patients with chronic hepatic disease. *J. Immunol.* **67,** 347.

HOLBOROW E.J., ASHERSON G.L., JOHNSON G.J., BARNES R.D.S. & CARMICHAEL D.S. (1963) Antinuclear factor and other antibodies in blood and liver diseases. *Brit. med. J.* **1,** 656.

HOTCHIN J.E. (1962) The biology of lymphocytic choriomeningitis infection: virus-induced immune disease. *Cold Spring Harbor Symp. Quant. Biol.* **27,** 479.

HOTCHIN J.E. (1966) Kuru as a persisting tolerated infection. *Lancet* **ii,** 28.

HOTCHIN J.E. & COLLINS D.N. (1964) Glomerulonephritis and late onset disease of mice following neonatal virus infection. *Nature* (*Lond.*) **203,** 1357.

IRONSIDE P.N.J., DE BOER W.G.R.M. & NAIRN R.C. (1966) Smooth-muscle antibody in lupoid hepatitis. *Lancet* **i,** 1210.

JAHIEL R.I. & KOFFLER D. (1961) Hepatic granulomas induced in guinea-pigs by Freund's adjuvant with and without homologous liver. *Brit. J. exp. Path.* **42,** 338.

JOHNSON G.D., HOLBOROW E.J. & GLYNN L.E. (1965) Antibody to smooth muscle in patients with liver disease. *Lancet* **ii,** 878.

JOHNSON G.D., HOLBOROW E.J. & GLYNN L.E. (1966) Antibody to liver in lupoid hepatitis. *Lancet* **ii,** 416.

KIDD J.G. & FRIEDEWALD W.F. (1942) A natural antibody that reacts *in vitro* with a sedimentable constituent of normal tissue cells. I. Demonstration of the phenomenon. II. Specificity of the phenomenon: general discussion. *J. exp. Med.* **76,** 543, 557.

LONGMIRE W.P., JOSEPH W.J., LEVIN P.M. & MELLINKOFF S.M. (1965) Diagnosis and treatment of cholangiolitic hepatitis (primary biliary cirrhosis). *Ann. Surg.* **162,** 356.

MACKAY I.R. & BURNET F.M. (1962) *Autoimmune Diseases,* chap. 11. Springfield, Illinois: Charles C Thomas.

MACKAY I.R. & GAJDUSEK D.C. (1958) An 'autoimmune' reaction against human tissue antigens in certain acute and chronic diseases. II. Clinical correlations. *Arch. int. Med.* **101,** 30.

MACKAY I.R. & LARKIN L. (1959) Non-reactivity of some autoimmune sera with autologous antigens. *Clin. Sci.* **18,** 425.

MACKAY I.R., TAFT L.I. & COWLING D.C. (1959) Lupoid hepatitis and the hepatic lesion of systemic lupus erythematosus. *Lancet* **i,** 65.

MACKAY I.R., WEIDEN S. & HASKER J. (1965) Autoimmune hepatitis. *Ann. N.Y. Acad. Sci.* **124,** 767.

MACKAY I.R., WEIDEN S. & UNGAR B. (1964) Treatment of active chronic hepatitis and lupoid hepatitis with 6-mercaptopurine and azothioprine. *Lancet* **i,** 899.

MACKAY I.R. & WOOD I.J. (1962) Lupoid hepatitis: a comparison of 22 cases with other types of chronic liver disease. *Quart J.. Med.* N.S. **31,** 485.

MACKAY I.R. & WOOD I.J. (1963) The course and treatment of lupoid hepatitis. *Gastroenterology* **45,** 4.

MACLACHLAN M.J., RODNAN G.P., COOPER W.M. & FENNELL R.H. JR. (1965) A clinical, serological and pathological study of 20 patients. *Ann. int. Med.* **62,** 425.

MACMAHON H.E. (1948) Biliary xanthomatosis (xanthomatous biliary cirrhosis). *Amer. J. Path.* **24,** 27.

NORKIN S.N., GOTTLIEB L.S. & ZAMCHECK N. (1962) Effects of liver homogenate and Freund's adjuvant on guinea-pig liver and other organs. *Amer. J. digest. Dis.* **7,** 824.

PAGE A.R., CONDIE R.M. & GOOD R.A. (1964) Suppression of plasma cell hepatitis with 6-mercaptopurine. *Amer. J. Med.* **36,** 200.

PAGE A.R. & GOOD R.A. (1962) Plasma cell hepatitis. *Lab. Invest.* **11,** 351.

PARONETTO F., SCHAFFNER F., MUTTER R.D., KNIFFEN J.C. & POPPER H. (1964) Circulating antibodies to bile ductular cells in various liver diseases. *J. Amer. med. Ass.* **187,** 503.

PARONETTO F., SCHAFFNER F. & POPPER H. (1961) Immunocytochemical reaction of serum of patients with hepatic diseases with hepatic structures. *Proc. Soc. exp. Biol. (N.Y.)* **106,** 216.

PASNICK L.J., BEALL G.N. & VAN ARSDEL P.P. (1962) Complement fixing reactions with liver tissue in human disease. *Amer. J. Med.* **33,** 774.

PINCKARD R.N. & WEIR D.M. (1966) Antibodies against the mitochondrial fraction of liver after toxic liver damage in rats. *Clin. exp. Immunol.* **1,** 33.

POLISH E. & MUSCHEL L.H. (1962) Rheumatoid factor and complement-fixing tissue antibodies in patients with liver disease. *Amer. J. digest. Dis.* **7,** 677.

POPPER H., PARONETTO F. & SCHAFFNER F. (1965) Immune processes in the pathogenesis of liver disease. *Ann. N.Y. Acad. Sci.* **124,** 781.

READ A.E., SHERLOCK S. & HARRISON C.V. (1963) Active 'juvenile' cirrhosis considered as part of a systemic disease and the effect of corticosteroid therapy. *Gut* **4,** 378.

RICHTER M., SARGENT A.U., MYERS J. & ROSE B. (1966) Production of autoantibodies in rats immunized with homologous or heterologous liver. *Immunology* **10,** 211.

ROITT I.M., LING N.R., DONIACH D. & COUCHMAN K.G. (1964) The cytoplasmic autoantigen in the human thyroid. I. Immunological and biochemical characteristics. *Immunology* **7,** 375.

RUBIN E., SCHAFFNER F. & POPPER H. (1965) Primary biliary cirrhosis. Chronic non-suppurative destructive cholangitis. *Amer. J. Path.* **46,** 387.

SHERLOCK S. (1959) Primary biliary cirrhosis (chronic intrahepatic obstructive jaundice). *Gastroenterology* **37,** 574.

SLEEMAN H.K. (1963) Studies on soluble antigenic substances isolated from human liver. *Proc. Soc. exp. Biol. (N.Y.)* **113,** 451.

STEINER J.W. (1961) Investigations of allergic liver injury. I. Light, fluorescent and electron microscopic study of the effects of soluble immune aggregates. *Amer. J. Path.* **38,** 411.

STEINER J.W., CARRUTHERS J.S., BAUMAL R. & KALIFAT S.R. (1961) Experimental immunologic liver injury and the concept of autodestruction. *J. Canad. med. Ass.* **85,** 1369, 1425.

SULITZEANU D., YAGI Y. & PRESSMAN D. (1963) Separation of an antigenic fraction from rat liver responsible for the formation of liver localizing antibodies. *J. Immunol.* **90,** 424.

TAL C. & LAUFER A. (1960) Amyloidosis in mice following injections with Freund's adjuvant, its components separately and homologous liver-adjuvant mixture. *Brit. J. exp. Path.* **41,** 605.

VOGT P.K. (1960) Die Immunologie der Lebermikrosomen. *Ztschr. Naturforsch.* **15B,** 213, 218, 221.

WALKER J.G., DONIACH D., ROITT I.M. & SHERLOCK S. (1965) Serological tests in diagnosis of primary biliary cirrhosis. *Lancet* **1,** 827.

WEIR D.M. (1961) A complement-fixation reaction with serum and tissue extracts after the injection of carbon tetrachloride into rats. *Lancet* **i,** 1147.

WEIR D.M. (1963a) Liver autoantibodies in the rat. *Immunology* **6,** 581.

WEIR D.M. (1963b) Complement fixation after carbon tetrachloride injection in rats. *Lancet* **ii,** 882.

WHITTINGHAM S., MACKAY I.R. & IRWIN J. (1966) Autoimmune hepatitis. *Lancet* **i,** 1333.

WILLIAMS G.E. GREVILLE (1965) Pericholangiolitic biliary cirrhosis. *J. Path. Bact.* **89,** 13.

WILLIAMSON N., HOUSLEY J., HULME A. & WALL P.G. (1966) Autoimmune hepatitis. *Lancet* **ii,** 286.

ADRENAL

ANDERSON J.R., GOUDIE R.B., GRAY K.G. & TIMBURY G.C. (1957) Auto-antibodies in Addison's disease. *Lancet* **i,** 1123.

BARKER N.W. (1929) The pathological changes in twenty-eight cases of Addison's disease. *Arch. Path.* **8,** 432.

BARNETT E.V., DUMONDE D.C. & GLYNN L.E. (1963) Induction of autoimmunity to adrenal gland. *Immunology* **6,** 382.

BLIZZARD R.M., CHEE D. & DAVIS W. (1966) The incidence of parathyroid and other antibodies in the sera of patients with idiopathic hypoparathyroidism. *Clin. exp. Immunol.* **1,** 119.

BLIZZARD R.M. & KYLE M. (1963) Studies of the adrenal antigens and antibodies in Addison's disease. *J. clin. Invest.* **42,** 1653.

BLIZZARD R.M., KYLE M.A., CHANDLER R.W. & HUNG W. (1962) Adrenal antibodies in Addison's disease. *Lancet* **ii,** 901.

BLIZZARD R.M., TOMASI T.B. & CHRISTY N.P. (1963) Autoantibodies against thyroid and adrenal tissue in a patient with multiple primary endocrine deficiencies. *J. clin. Endocr.* **23,** 1179.

BLOODWORTH J.M.B., KIRKENDALL W.M. & CARR T.L. (1954) Addison's disease associated with thyroid insufficiency and atrophy (Schmidt syndrome). *J. clin. Endocr.* **14,** 540.

CENTENO E., SHULMAN S., MILGROM F. & WITEBSKY E. (1965) Immunological studies on adrenal glands. VI. The multiple nature of the rabbit adrenal autoantigens. *Immunology* **8,** 160.

CHRISTY N.P., HOLUB D.A. & TOMASI T.B. (1962) Primary ovarian, thyroidal and adrenocortical deficiencies simulating pituitary insufficiency, associated with diabetes mellitus. *J. clin. Endocr.* **22,** 155.

COLOVER J. & GLYNN L.E. (1958) Experimental iso-immune adrenalitis. *Immunology* **2,** 172.

CSAPÓ G., DÁVID M.A. & KOVÁCS K. (1963) Development of diabetes mellitus in a patient suffering from Addison's disease. *Acta med. scand.* **174,** (Fasc. 3), 321.

DUNLOP D. (1963) Eighty-six cases of Addison's disease. *Brit. med. J.* **2,** 887.

FEYRTER F. & KLIMA R. (1952) Über die Magenveranderungen bei der Addisonschen Krankheit, *Dtsch. med. Wschr.* **77,** 1173.

GASTINEAU C.F., MYERS W.R., ARNOLD J.W. & MCCONAHEY W.M. (1964) Thyroid disorders in Addison's disease. II. Graves's disease. *Proc. Mayo Clin.* **39,** 939.

GOUDIE R.B., ANDERSON J.R., GRAY K.G. & WHYTE W.G. (1966) Autoantibodies in Addison's disease. *Lancet* **i,** 1173.

GRAY S.J., RAMSAY C.G. & THORN G.W. (1956) Adrenal influences on the stomach: peptic ulcer in Addison's disease during active steroid therapy. *Ann. int. Med.* **45,** 73.

HUNG W., MIGEON C.J. & PARROTT R.H. (1963) A possible autoimmune basis for Addison's disease in three siblings, one with idiopathic hypoparathyroidism, pernicious anaemia and superficial moniliasis. *New Engl. J. Med.* **269,** 658.

IRVINE W.J. (1963) A clinical and immunological study of adrenal insufficiency. *J. Endocr.* **26,** xxxii.

KIDD J.G. & FRIEDEWALD W.F. (1942) A natural antibody that reacts *in vitro* with a sedimentable constituent of normal tissue cells. *J. exp. Med.* **76,** 543, 557.

LYMAN DUFF G. & BERNSTEIN C. (1933) Five cases of Addison's disease with so-called atrophy of the adrenal cortex. *Bull. Johns Hopkins Hosp.* **52,** 67.

MANCINI A.M., ZAMPA G.A., VECCHI A. & COSTANZI G. (1965) Histoimmunological techniques for detecting anti-insulin antibodies in human sera. *Lancet* **i,** 1189.

MEAD R.K. (1962) Autoimmune Addison's disease. *New Engl. J. Med.* **266,** 583.

MILCOU S.M., POP A., LUPULESCOU A. & TAGA M. (1959) L'autoimmunisation experimentale de la surrenale chez le lapin. *Ann. Endocr. (Paris),* **20,** 799.

MOORE J.M. & NEILSON J.M. (1963) Antibodies to gastric mucosa and thyroid in diabetes mellitus. *Lancet* **ii,** 645.

MORSE W.I., COCHRANE W.A. & LANDRIGAN P.L. (1961) Familial hypoparathyroidism with pernicious anaemia, steatorrhoea and adrenocortical insufficiency: a variant of mucoviscidosis. *New Engl. J. Med.* **264,** 1021.

SCHMIDT M.B. (1926) Eine Biglandulare Erkrankung (Nebennieren und Schildruse) bei Morbus Addisoni. *Verhandl. Dtsch. Path. Gesellsch.* **21,** 212.

SHULMAN S., CENTENO E., MILGROM F. & WITEBSKY E. (1965) Immunological studies on adrenal glands. V. Chemical studies on the rabbit adrenal-specific autoantigen. *Immunology* **8,** 531.

SIMPSON S.L. (1932) Addison's disease and its treatment by cortical extract. *Quart. J. Med.* N.S. **1,** 99.

SLOPER J.C. (1953) The pathology of the thyroid in Addison's disease. *J. Path. Bact.* **66,** 53.

SMITH A.W.M., DELAMORE I.W. & WYNN WILLIAMS A. (1961) Gastric acid secretion and mucosal appearances in Addison's disease and hypopituitarism. *Gut* **2,** 163.

SOFFER L.J., DORFMAN R.I. & GABRILOVE J.L. (1961) *The Human Adrenal Gland.* London: Kimpton.

STANTON E.R., JONES H.W . & MARBLE A. (1954) Coexisting diabetes mellitus and Addison's disease. *Arch. int. Med.* **93,** 911.

STEINER J.W., LANGER B. & SCHATZ D.L. (1960a) The local and systemic effects of Freund's adjuvant and its fractions. *Arch. Path.* **70,** 424.

STEINER J.W., LANGER B., SCHATZ D.L. & VOLPE R. (1960b) Experimental immunologic adrenal injury. A response to injections of autologous and homologous adrenal antigens in adjuvant. *J. exp. Med.* **112,** 187.

STEWART W.K., GREEN D.M. & LOWE K.G. (1962) Addison's disease and hyperthyroidism. *Acta endocr.* **40,** 613.

TERPLAN K., WITEBSKY E. & MILGROM F. (1963) Histopathology of adrenals in animals immunized with tissue antigens. *Arch. Path.* **76,** 333.

WEGIENKA L.C., WUEPPER K.D., KOMARMY L.E. & FORSHAM P.H. (1966) Cushing's syndrome with adrenal medullary insufficiency and adrenal antibodies. *Lancet* **i,** 741.

WELLS H.G. (1930) Addison's disease with selective destruction of the suprarenal cortex ('suprarenal cortical atrophy'). *Arch. Path.* **10,** 499.

WITEBSKY E. & MILGROM F. (1962) Immunological studies on adrenal glands. II. Immunization with adrenals of the same species. *Immunology* **5,** 67.

PANCREAS

ARAPAKIS G., BOCK O.O.A., WILLIAMS D.L. & WITTS L.J. (1963) Diabetes mellitus and pernicious anaemia. *Brit. med. J.* **1,** 159.

BLUMENTHAL H.T., BERNS A.W. & OWENS C.T. (1963) Insulin antibodies. *Lancet* **ii,** 784.

CHETTY M.P. & WATSON K.C. (1965) Antibody-like activity in diabetic and normal serum. *Lancet* **i,** 67.

FONKALSRUD E.W. & LONGMIRE W.P. JR. (1961) The occurrence of pancreatic antibodies and the experimental production of pancreatitis with pancreatic antiserum. *Surgery* **50,** 134.

LANDING B.H., PETTIT M.D., WIENS R.L., KNOWLES H. & GUEST G.M. (1963) Antithyroid antibody and chronic thyroiditis in diabetes. *J. clin. Endocr.* **23,** 119.

LECOMPTE P.M. (1958) 'Insulitis' in early juvenile diabetes. *Arch. Path.* **66,** 450.

MANCINI A.M., COSTANZI G. & ZAMPA G.A. (1964a) Immunological differences between pancreatic and circulating insulin. *Lancet* **ii,** 44.

MANCINI A.M., COSTANZI G. & ZAMPA G.A. (1964b) Human insulin antibodies detected by an immunofluorescent technique. *Lancet,* **i,** 726.

MANCINI A.M., ZAMPA G.A., VECCHI A. & COSTANZI G. (1965) Histoimmunological techniques for detecting anti-insulin antibodies in human sera. *Lancet* **i,** 1189.

METZGAR R.S. (1964a) Immunologic studies of pancreas-specific isoantigens. *J. Immunol.* **93,** 176.

METZGAR R.S. (1964b) Human pancreas-specific isoantigens. *Nature (Lond.)* **203,** 660.

MOORE J.M. & NEILSON J.M. (1963) Antibodies to gastric mucosa and thyroid in diabetes mellitus. *Lancet* **ii,** 645.

MURRAY M.J. & THAL A.P. (1960) The clinical significance of circulating pancreatic antibodies. *Arch. int. Med.* **53,** 548.

PÁV J., JEŽKOVÁ Z. & SKRHA F. (1963) Insulin antibodies. *Lancet* **ii,** 221.

PETTIT M.D., LANDING B.H. & GUEST G.M. (1961) Antithyroid antibody in juvenile diabetes. *J. clin. Endocr.* **21,** 209.

POPE C.G. (1966) The immunology of insulin. In *Advances in Immunology,* vol. 5, p. 209, ed. DIXON F.J. JR. & HUMPHREY J.H. New York and London: Academic Press.

ROSE N.R., METZGAR R.S. & WITEBSKY E. (1960) Studies on organ specificity. XI. Isoantigens of rabbit pancreas. *J. Immunol.* **85,** 575.

STEIN A.A., MANLAPAS F.C., SOIKE K.F. & PATTERSON P.R. (1964) Specific isoantibodies in cystic fibrosis. *J. Pediat.* **65,** 495.

THAL A.P. (1960) The occurrence of pancreatic antibodies and the nature of the pancreatic antigen. *Surg. Forum* **11,** 367.

THAL A.P., EGNER W. & MURRAY M.J. (1959a) Circulating antibodies in chronic pancreatitis. *Surg. Forum* **10,** 240.

THAL A.P., MURRAY M.J. & EGNER W. (1959b) Isoantibody formation in chronic pancreatic disease. *Lancet* **i,** 1128.

WILKINSON J.F. (1963) Diabetes and pernicious anaemia. *Brit. med. J.* **1,** 676.

WITEBSKY E., ROSE N.R. & NADEL H. (1960) Studies on organ specificity. X. The serological specificity of pancreatic extracts. *J. Immunol.* **85,** 568.

PROSTATE

DUTHIE J.J.R., STEWART S.M., ALEXANDER W.R.M. & DAYHOFF R.E. (1967) Isolation of diphtheroid organisms from rheumatoid synovial membrane and fluid. *Lancet* **i,** 142.

GRIMBLE A. (1964) Auto-immunity to prostate antigen in rheumatic disease. *J. clin. Path.* **17,** 264.

GRIMBLE A. & LESSOF M.H. (1965) Anti-prostate antibodies in arthritis. *Brit. med. J.* **2,** 263.

SHULMAN S., YANTORNO C., BARNES G.W., GONDER M.J., SOANES W.A. & WITEBSKY E. (1965) Studies on autosensitization to prostatic tissue and related antigens. *Ann. N.Y. Acad. Sci.* **124,** 279.

SHULMAN S., YANTORNO C., SOANES W.A., GONDER M.J. & WITEBSKY E. (1966) Studies on organ specificity. XVI. Urogenital tissues and autoantibodies. *Immunology* **10,** 99.

WEIL A.J. (1961) Antigens of adnexal glands of the male genital tract. *Fertil. Steril.* **12,** 538.

WEIL A.J. (1965) The spermatozoa-coating antigen (SCA) of the seminal vesicle. *Ann. N.Y. Acad. Sci.* **124,** 267.

CHAPTER 42

THE SPERMATOZOA AND TESTES IN ALLERGIC DISEASE

PH. RÜMKE

AUTO-ANTIGENICITY OF SPERMATOZOA

The antigenicity of spermatozoa in heterologous species was discovered by Landsteiner and, independently, by Metchnikoff as early as 1899. The formation of immobilizing auto-antibodies to spermatozoa after injection of homologous sperm in guinea-pigs was described by Metalnikoff (1900) and after autologous testis homogenate by Adler (1909). Since then it has been firmly established by many authors that male animals injected with homologous or autologous testis homogenate or sperm, especially when the antigen is emulsified in Freund's adjuvant, may develop, not only immobilizing, but also agglutinating, complement fixing, skin sensitizing and other auto-antibodies which are reactive only with mature testicular and seminal spermatozoa, and thus are organ specific; see reviews by Tyler & Bishop (1963); and Krieg & Eyquem (1964).

AUTO-SPERMAGGLUTININS

In 1921 Wegelin wrote '... it would be possible that antibodies prepare the spermatozoa in the epididymis for phagocytosis, at least if absorption of sperm-substances occurs. This now is not rare in old people and in cases of chronic epididymitis, where sperm can be found in interstitium and lymph vessels of epididymis and rete testis.' Although in 1922 the sera of two sterile *women* were

reported to agglutinate and immobilize spermatozoa of the husbands (Meaker 1922) it was not until 1954 that Wilson and Rümke independently examined serum samples of human males for their capacity to agglutinate and immobilize spermatozoa. During routine clinical investigation of sterile couples, Wilson (1954) encountered two cases of men whose spermatozoa showed spontaneous agglutination and early loss of motility, whereas volume, sperm count, viscosity and initial motility were found to be normal. In a third case, agglutination was present in an oligospermic ejaculate (Wilson 1956). The cause of spontaneous agglutination (but usually of immotile spermatozoa) was till then generally referred to some non-specific condition, such as changes in pH, or salt content (see Mann 1964). Bacterial contamination was also invoked as the cause of rapid immobilization (Rosenthal 1931; Buxton & Wong 1952; Wilson 1956). In Wilson's cases, however, the agglutinating factor was also found in the blood serum when this was incubated with sperm of other donors. Moreover, sperm could absorb the factor from the serum. Therefore it was postulated that agglutination in the ejaculate was the result of the presence of sperm auto-antibodies. The agglutination prevented penetration into the cervical mucus as was shown by post-coital and cervical mucus invasion tests. Since the spouses of these men proved to be fertile after insemination with donor sperm, it could be concluded that the spermagglutination in the ejaculates was the cause of their infertility.

Rümke (1954) described two patients with extreme oligospermia, whose sera possessed spermagglutinins. The serum titres were 4096 and 2048 respectively. The first serum also had immobilizing properties in the presence of complement to a titre of 32. In the following 4 years Rümke examined the sera of 2015 male patients of the Male Infertility Team in Amsterdam along with sera of 416 husbands of pregnant women (Rümke 1959a and b; Rümke & Hellinga 1959). As may be seen in Table 42.1 none of the fertile men possessed agglutinins for human sperm in a titre of 32 or higher, whereas about 3% of the childless males showed agglutinins of at least that titre or higher.

The spermagglutinins were detected with a sensitive agglutination test using 2.5% gelatin as a suspending medium for the sperm at a final density of about 10^7 per ml (Kibrick, Belding & Merrill 1952). The test can be read macroscopically and is simple to perform and therefore is particularly suitable for routine purposes. The donor sperm came from various sources. Small differences in titre (sometimes eight-fold) were found when one serum sample was tested with more than one sperm sample. These differences were attributed to variation in sperm motility and seminal plasma constituents. Also, the end-point reading of a two-fold serial dilution series is often somewhat subjective due to the gradual weakening of the agglutination reaction.

The frequency with which serum spermagglutinins are found with the same technique (and considered 'positive' only if titres are higher than 16) by several

investigators are: 9 out of 75 male patients with inflammatory diseases of the genital tract (Bandhauer 1963), 9 out of 263 (Fjällbrant 1965) and 9 out of 150 (Sobbe, Haferkamp & Doepfmer 1966) males of childless marriages. The difference between 11.9% and 3.1% positive cases when two sperm samples were used for testing 294 serum samples by Tyler and Nakabayashi was also explained on the basis of variation in sperm qualities (Nakabayashi, Tyler & Tyler 1961).

Spermagglutinins can also be detected with a microagglutination technique, which allows both grading of the degree of reaction and determination of the

TABLE 42.1
Sperm agglutinins in the serum of human males

Serum of	Number tested	Negative	Weak positive[1]	Positive[2]	Strong positive[3]	Per cent positive [4]
Patients with a- or oligo-zoospermia (preliminary investigation)	102	96	1	1	4	4.9*
All patients who visited the Male Infertility Team from 1954 to 1958	1913	1836	15	26	36	3.2*
Husbands of pregnant women	416	412	4	0	0	0

Total 'positive'[4] patients: 67

[1] Titre: 4– 16.
[2] Titre: 32–128.
[3] Titre: ⩾256.
[4] Titre: ⩾ 32.
* $P < 0.001$ (compared with husbands of pregnant women).

type of agglutination (Phadke & Padukone 1964; Bandhauer 1966; Tyler, Tyler & Denny 1967; Schwimmer, Ustay & Behrman 1967). The test is, however, less sensitive than the macroagglutination test in gelatin and is possibly more disturbed by 'aspecific' agglutination.

AGGLUTINATION TYPE

Microscopic examination of sperm incubated with spermagglutinating serum reveals various patterns of agglutination. 'Head-to-head' agglutination can be distinguished from 'tail-to-tail' agglutination. Tail-to-tail may start with the end pieces, while in the majority of the sera, the main piece of the tail is predominantly involved. Some sera show both head-to-head and tail-to-tail

agglutination, while sometimes head-to-tail agglutination can be seen (Wilson 1954; Rümke 1959a and b). Tail agglutination was seen more often than head agglutination. In a series of 64 patients tail agglutination predominated 58 times (Rümke 1959b). Phadke & Padukone (1964) found 11 instances of tail, 6 of head and 2 of mixed types. Both Fjällbrant (1965) and Sobbe, Haferkamp & Doepfmer (1966) with their 9 cases found only 1 serum with head agglutinins.

No correlation was found between the presence of immobilizins and the agglutination type (Rümke 1959b).

It is of interest that experimental studies with bull sperm (Henle, Henle & Chambers 1938) and guinea-pig sperm (Pernot, 1956) have demonstrated that heads and tails may contain separate antigens.

Antibody Nature of Spermagglutinins

The spermagglutinating factor has been identified as a true antibody by various means: the factor is resistant to heating to 56°C for 30 min; it can be absorbed by packed sperm; the optimal temperature for absorption seems to be 37°C; it is only present in the γ-globulin fraction as prepared by paper electrophoresis and it can be eluted from a thoroughly washed sediment of a patient's ejaculate (Rümke 1959a and b). Further demonstration of the antibody nature was given with the mixed antiglobulin reaction (Coombs 1962) and with the fluorescent antibody technique (Feltkamp, Kruyff, Ladiges & Rümke 1965). With the latter technique, spermagglutinating sera were shown to contain antibodies of the IgG class, which reacted with various parts of ejaculated spermatozoa or of sperm in testicular sections. The titre of some of the sera studied by Schwimmer, Ustay & Behrman (1967) was reduced by 2-mercaptoethanol, indicating that spermagglutinins may partly belong to the class of IgM antibodies. Sperm-agglutinating sera may also contain immobilizins which are especially active in the presence of complement, indicating that the immobilizing factor is an antibody (Rümke & Hellinga 1959; Fjällbrant 1965).

It is curious that when one observes a patient's ejaculate or the reaction of his serum with normal sperm many spermatozoa often do not join the clumping in spite of ample antibody. This would mean either (1) that the corresponding surface antigens are not present on all spermatozoa, or (2) that non-agglutinating antibodies block the antigenic sites, or (3) that agglutination of two sperm cells will only happen under optimal circumstances of collision. At this moment it is not yet solved which of these possibilities is the most likely explanation. Preliminary fluorescent antibody studies (Rümke unpublished) suggest that certain antigens might be represented unequally on the different spermatozoa of one sample. On the other hand, experimental work with antibody fragments proves that non-agglutinating antibodies, interfering with fertility, may exist (Tyler & Payne 1947; Metz, Schuel & Bischoff 1964).

Relationship between Serum Antibody and Sperm Agglutination in Ejaculate

As might be expected, there is a direct relationship between the detection of the various types of agglutinins and immobilizins in serum and the abnormalities such as agglutination and loss of motility of sperm found in the ejaculates of these patients. Indeed, when the serum spermagglutinin titre is high, agglutination in the ejaculate is often strong and may be complete at first observation shortly after ejaculation, whereas with low serum titres agglutination is either not present or starts so slowly that it is not clearly visible within the first half hour; moreover, the clumps can be disrupted easily by mechanical agitation in these cases.

Judged by post-coital tests and cervical mucus invasion tests *in vitro*, Fjällbrant (1965) found a parallel between the non-ability to invade cervical mucus and serum agglutination and immobilization titres in eleven cases.

However, it has to be stressed that exceptions are not uncommon: some patients have high titred spermagglutinins in the serum without agglutination in the ejaculate (Bandhauer 1966; Schwimmer, Ustay & Behrman 1967). Three patients with serum spermagglutinin titres of 80, 80 and 320 respectively were reported to have impregnated their wives (Phadke & Padukone 1964). Though not stated as such, it is likely that in these cases agglutination of ejaculated sperm was either absent or only partial. This shows why semen analysis is necessary before a prognosis concerning fertility can be made. Only if auto-agglutination is complete and repeatedly found is the chance of fertility nil.

Other patients, on the other hand, with serum titres of 32 or lower, may show complete and early agglutination of the ejaculated sperm.

When seminal plasma and serum titres are compared, generally the former are much lower than the latter (Rümke 1959a and b). This may be partly so because of absorption by the spermatozoa in the ejaculate. Since an azoospermic patient may have a much lower titre in his semen than in the blood serum, it is also likely that the lower titres in seminal fluid are related to the low γ-globulin concentration in seminal fluid—about 1% that of serum (Rümke 1959b). Variation in γ-globulin level of normal and pathological semen samples (Klopstock, Haas & Rimon 1963) may account for differences in the ratio of serum-seminal spermagglutinin titres.

Site of Antibody Production

Since seminal fluid may contain antibodies to bacterial antigens after vaccinations (Katsh & Katsh 1965; Sadri & Rao 1965), it is logical to assume that seminal plasma derives its γ-globulin from the blood plasma. At what site or sites of the genital tract γ-globulin enters is, however, unknown. In a single case the spermagglutinin titre in seminal plasma was found to equal or even to exceed

that in the serum (Rümke 1959a and b). It is likely that in those cases where spermagglutinins are relatively more concentrated than would be expected on account of γ-globulin concentrations, the sperm antibodies are derived from some local source in the genital tract. The degree of passage of antibodies through the barrier between blood and seminal plasma and the local antibody production apparently determine whether, and to what extent, agglutination in the ejaculate will take place.

Other Serological Tests

It would be conceivable that if only spermagglutination and immobilization techniques are used, some antibodies may remain undetected. Other types could well underlie some auto-allergic process to male genital products, although perhaps without having a direct bearing on sperm abnormalities or fertility. Several serological methods are, in principle, available for the detection of such antibodies.

Rao & Sadri (1959) examined two sera of patients with marked agglutination of spermatozoa by the *tanned red cell agglutination technique*, using sheep erythrocytes coated with extracts of freeze-thawed washed spermatozoa. These sera produced passive haemagglutination in high dilutions. In 25 cases of fertile males no antibodies could be detected with this technique. In a survey on 368 serum samples of both males and females of infertile couples Rao (Nakabayashi, Tyler & Tyler 1961) found 36 males and 31 females to be positive, whereas no positive cases were recorded on 10 unmarried females and 72 sera of fertile couples. Southam (1963), however, found 2 of 5 infertile men, whose sera agglutinated spermatozoa-sensitized red cells, subsequently to be fertile.

More detailed studies are necessary to judge the value of this passive haemagglutination test in respect to spermatozoal antigens.

The *fluorescent antibody technique* has proved useful in the detection of autoantibodies to acrosomal antigens of sperm of guinea-pigs or rats with experimentally induced auto-allergic orchitis (p. 1154). Positive fluorescent staining with human spermagglutinating sera was reported by Feltkamp, Kruyff, Ladiges & Rümke (1965). Various parts of the sperm, sometimes in different combinations, were involved in the fluorescent staining. The correlation with the agglutination type, however, was not clear, probably due to the totally different conditions of both tests. For instance, the forces of motility could make it easier for certain parts to agglutinate, while differences in shape of the various parts might influence the clarity by which staining is presented. Some yet unsolved technical difficulties may be responsible for failures with the fluorescent antibody technique (Cruickshank & Stuart-Smith 1959; Sobbe, Haferkamp & Doepfmer 1966).

Precipitin techniques have not given any consistent result. A preliminary finding (Rümke & Hellinga 1959) has not been confirmed (Rümke, unpublished).

Bandhauer (1966) who detected spermagglutinins in 29 out of 448 patients with inflammatory diseases and other conditions of the genital tract, never obtained positive results with these sera when testing them with the double-diffusion agar gel technique against seminal plasma, sperm or prostate extracts. Negative results were also reported by Cruickshank & Stuart-Smith (1959) and Rao (Segal, Tyler, Rao, Rümke & Nakabayashi 1961).

Complement fixing auto-antibodies to spermatozoa are demonstrable in the sera of guinea-pigs injected with testis or sperm in adjuvants (Freund, Lipton & Thompson 1953). With two human spermagglutinating sera the test gave negative results (Cruickshank & Stuart-Smith 1959). Some strong agglutinating sera, however, fixed complement with packed sperm (Rümke, unpublished). No screening has been performed using this test with sera of a larger number of infertile males.

According to the results with a complement fixation test with an alcoholic extract of testis homogenate to which cholesterol was added, Aszódi & Szántó (1935) concluded that sperm resorption in the human male or female quite commonly leads to auto- or iso-antibody production to sperm substances. Such investigations, however, have never been repeated.

Naturally Occurring Antibody-like Factors

Immune-like reactions have been detected between spermatozoa and normal serum from adult males and females of some mammalian species (Edwards 1960; Beck, Edwards & Young 1962; Sell & Edwards, unpublished). These reactions involve complement fixation, immune fluorescence, mixed anti-globulin and immune adherence tests, agglutination and lysis of the acrosome. Complement-dependent cytotoxic activity of adult guinea-pig serum against autologous testicular cells has also been found (Spooner 1964). The factor responsible for these reactions with sperm does not cross the placenta, can be absorbed from serum by sperm or testis but not by other organs and is absent in newly born animals.

Earlier observations of agglutination and immobilization of spermatozoa by fresh serum which can be prevented by inactivation of the serum (Mann 1964, p. 333) also suggest that normal sera may contain antibody-like factors in low concentration. It is mainly because of such agglutinating properties that blood serum does not possess a wider application as a semen diluent.

Such antibody-like factors apparently do not interfere with fertility, presumably because their macromolecular character prevents their passage through the blood-semen barrier. Further work is needed to prove whether these factors might be low-titred IgM antibody specific for spermatozoal antigens and whether they are induced by the normal resorption of sperm products both in male and female animals. In man, the factor is of only minor importance since, in general, serum of the normal adult has no, or only very slight, agglutinating

and no toxic properties for human sperm. Also with fluorescent antibody techniques staining reactions are weak (Edwards, personal communication) or completely negative.

With the macroagglutination technique in gelatin low titred agglutinins were occasionally encountered with sera of fertile men (Table 42.1); therefore only titres of 32 or higher were considered to be 'positive'. Other investigators (Nakabayashi *et al* 1961; Bandhauer 1963, 1966; Fjällbrant 1965; Sobbe *et al* 1966) have followed this arbitrarily chosen limit for what is a true and 'specific' positive. Although this limit may have its practical value for the moment, careful serological studies and follow-up of patients will have to be made to settle whether there is any profit in maintaining this artificial division.

Origin of the Spermatozoal Antigens

Human males immunized with their own testicular tissue incorporated in complete Freund's adjuvant produce antibodies specifically reacting with the man's own germinal cells (Mancini *et al* 1965). This proves that spermatozoa possess organ-specific testicular antigens.

The work of Weil and co-workers, on the other hand, has shown that spermatozoa also derive antigenic substances of the seminal plasma that adhere to the sperm cells (Weil, Kotsevalov & Wilson 1956; Weil & Rodenburg 1960, 1962; Weil 1961, 1965). They showed that spermatozoa cross-react with antisera to seminal plasma and that antisera to carefully washed seminal spermatozoa cross-react with seminal plasma. The spermatozoa-coating antigen (SCA) responsible for the cross-reactions is a product of the seminal vesicles and as such becomes a component of the seminal plasma. It was believed to be organ specific, but recent studies of Hekman & Rümke (1967) have shown that the human seminal vesicle produces a sperm-coating antigen identical with an antigen present in human milk and spleen homogenate. Probably this SCA is lactoferrin which was found by Masson & Heremans (1966) to be an antigen commonly present in seminal plasma and milk. According to Flocks, Bandhauer, Patel & Begley (1962) and Bandhauer (1966) the prostate and the epididymis also contribute to the coating antigens.

In principle it might thus be possible that auto-immunization against testicular as well as male accessory-organ antigens and non-organ-specific substances would lead to the formation of spermagglutinins.

Sperm Resorption

Since the organ-specific antigens of spermatozoa are presumably developing late in life, it might be expected that natural tolerance to these antigens does not exist. It thus is obvious that a possible cause of spermagglutinin formation should be sought in excessive or abnormal resorption of sperm in the genital tract. Resorption is known to occur physiologically. In the bull, for instance,

a constant production of spermatozoa is evident and it is believed that under sexual rest the production rate would be balanced by the sperm resorption rate, which is dependent upon the number of spermatozoa present in the cauda epididymis, where most of the resorption occurs (Amann & Almquist 1962). Rete testis, ductuli efferentes and proximal portion of the ductus epididymis seem to have a combined function in removing from the excretory products of the testis not only excess fluid but also extraneous materials carried with this mass, such that a well-winnowed mass of mature sperm is the product passed on to the more distal portion of the duct system (Mason & Shaver 1951). In the hamster, resorption of extraneous material takes place by phagocytosis and intracellular digestion in the various types of cells lining the sperm pathway (Burgos 1967). In man, spermatozoa are phagocytized by spermiophage cells within the epididymal lumina (Wegelin 1921). It is not known whether under normal circumstances in man any auto-antigenic material, spermatozoa, digestion products or extraneous material whatsoever will have access to cells with immunological capacity. We know, however, that under pathological conditions this may occur. For instance, in cases of obstruction of the efferent ducts, spermatostasis may lead to extravasation of sperm in the epididymis. Sperm extravasation is often accompanied by infiltration of macrophages, lymphocytes and plasma cells. Sperm invasion with inflammatory reactions has been observed in patients who were vasoligated or had testicular trauma in their histories (Friedman & Garske 1949). Impaired resistance of the walls of the tubuli due to infections was believed to be the cause of the sperm extravasation in other patients (Cronquist 1949; King 1955). From experimental work in dogs (Kyrle & Schopper 1914, 1915) and in rats (Mullaney 1962a) it can be concluded that increased intratubular pressure can be the initiating mechanism of extravasation, without infection being a necessary component. Non-specific granulomas are ascribed to sperm extravasation (Sundarasivarao 1955). Zettergren (1957) proposed, therefore, the term *epididymitis spermiostatica granulomatosa*. Several authors have observed that due to obstruction or to acute granulomatous epididymitis sperm was present not only in the interstitium but also in lymph and even in blood vessels (Beneke 1898; Simmonds 1921; Priesel 1924; Oberndorfer 1931; Orsós 1941; Cronquist 1949; Friedman & Garske 1949; King 1955; Glassy & Mostofi 1956; Mullaney 1962b).

Though it is clear that obstruction may lead to sperm extravasation, it certainly is not the rule. Phadke (1964) examined thirty-two epididymal biopsies of patients with azoospermia due to obstruction proximal of the biopsy. In two cases ingestion of spermatozoa by the living epithelial cells and in three instances breached epithelium with extravasation of spermatozoa in the interstitial tissues was found. Perivascular accumulation of plasma cells in the interstitial tissues was observed only twice. In the majority of the cases, however, sperm phagocytosis was witnessed *in* the lumina of the epididymal tubules.

Obstructive Azoospermia and the Presence of Spermagglutinins

The next question is whether patients with obstructive azoospermia do indeed possess spermagglutinins (or other antibodies) in their serum. It is certainly true that the incidence of positive findings is relatively high among patients with proved obstruction. Rümke & Hellinga (1959) came to this conclusion after comparing the incidence and level of serum spermagglutinins in patients with azoospermia due to a- or hypospermatogenesis with those where the cause of the azoospermia was due to an obstruction. Table 42.2 shows that the azoospermic patients *with* spermagglutinins were nearly always patients with obstructive azoospermia, whereas the other cause of azoospermia—impaired

Table 42.2
Correlation between the cause of azoospermia and the presence of spermagglutinins in the serum

Serum of azoospermic patients	Cause of the azoospermia			Total
	A- or hypo-spermatogenesis	Uncertain	Obstruction (with normal spermatogenesis)	
Without spermagglutinins	94	33	45	172
With spermagglutinins	1	4	16	21

spermatogenesis—does not seem to be related to the presence of spermagglutinins. Phadke & Padukone (1964) found spermagglutinins in the serum of 8 out of 25 previously fertile men in whom vasoligation had been carried out as a family limitation measure 2–20 years prior to serum testing. They also found 5 positive cases out of 25 patients with obstructive azoospermia and 6 out of 25 infertile patients in whom a former condition of obstructive azoospermia had been successfully relieved by vaso-epididymostomy.

Patients with congenital absence of the vasa deferentia also had a high incidence (5 out of 11) of spermagglutinins in the serum (Rümke & Kremer 1967).

The question remains as to why not every male with obstruction of the efferent ducts produces spermagglutinins. Several factors might determine this. Most important is the already mentioned observation of Phadke (1964) that in the majority of the cases, phagocytosis takes place in the lumen; this apparently is the normal course of events and presumably does not lead to antibody formation. Of possible significance is the individual's proneness to make antibodies.

This consideration might be sustained by the observation that the 5 'positive' cases out of the 11 forementioned patients with congenital absence of the vasa deferentia all had higher levels of IgA in the serum than the 6 'negatives'. Titres of complement fixing antibodies to measles virus were likewise significantly higher among the 'positives'. There was also a tendency for higher IgG and IgM levels in this group (Rümke 1968). These data, though referring to a small number of patients, possibly indicate that some individuals are more prone to produce antibodies than others, and that they therefore also respond more easily to auto-antigens.

Differences in the way that sperm antigens are broken down and transferred to lymph or blood, or the amount of spermatozoa that are produced (in which persistent, individual differences exist) or the individual's readiness to develop a state of tolerance, all are other factors of possible significance. It is also puzzling why in cases of obstruction or vasectomy some patients have tail-to-tail and others head-to-head or mixed types of agglutinins (Rümke & Hellinga 1959; Phadke & Padukone 1964).

Patients with Spermagglutinins

Not all patients with spermagglutinins have proved obstructions. A survey of sixty-four patients (Rümke & Hellinga 1959; Rümke 1964, 1965) showed that in only about half of the cases did physical examination reveal abnormalities in the epididymis or vas deferens, indicating obstruction of one or both of the efferent ducts. Also in only about half of the cases did the histories of these patients mention a diagnosis that could bear a possible significance. Sometimes, as in the cases of herniorrhaphy in childhood, an operation that often leads to cutting of the tiny vasa deferentia if it is not performed by an experienced surgeon, obstruction may be the only factor involved, whereas in other cases such as gonorrhoea and tuberculosis, inflammatory processes may be responsible both for obstruction and enhanced local immune reactivity and perhaps also for some alteration of the antigens, making them more antigenic. Bandhauer (1966) tested sera of patients with diseases of the urogenital tract. Patients with acute or chronic epididymitis were frequently encountered among the 'positives'. One patient (Rümke & Hellinga 1959) underwent 9 years prior to serum testing a herniorrhaphy at one side. During the operation the funiculus was cut through, which caused an acute bionecrosis and complete atrophy of the testis on that side. In this case resorption of testicular and epididymal sperm antigens present in the necrotic tissue was presumably the cause of the antibody production. Another patient had suffered from mumps orchitis at the age of 11 years. It is, however, questionable whether this had anything to do with the formation of spermagglutinins since no spermagglutinins were found in the serum of thirteen men who had mumps orchitis in adult life 1–12 years prior to serum testing (Rümke 1965).

Many patients had no indication in either their history or physical examination of an abnormality and their sperm count was often normal.

In another series of eleven patients with auto-agglutination of their sperm, no suggestion of the origin of the antibody formation could be found in case histories or clinical findings of ten patients, and only one patient had a significant history of inguinal herniorrhaphy and epididymitis or orchitis (Fjällbrant 1965).

At surgical exploration, performed in three patients with negative histories and clinical findings, sperm appeared to be present in both vasa deferentia and, moreover, these were proved to be patent since the outflow of diluted dyes injected at the site of exploration was normal (Rümke, unpublished).

In such 'idiopathic' cases several possibilities concerning the cause can be suggested: obstruction in a part of the testis leading to local resorption; a minor trauma leading to extravasation of sperm in the epididymis initiating antibody production, with the assumption that normal resorption would subsequently deliver a sufficient antigenic stimulus to continue antibody production; some disturbance of tolerance to the normally resorbed sperm antigens; and lastly the production of antibodies to seminal plasma constituents that coat the spermatozoa on their passage along the genital tract.

Bandhauer (1963, 1966) has found spermagglutinins in the serum of 3 patients with prostate abscesses, 1 with acute prostate vesiculitis and 2 who underwent transurethral electro-resection of the prostate. Whether in such cases a coating antigen is involved in the auto-immune reaction needs further investigation.

When spermagglutinating sera were tested with the indirect fluorescent antibody technique on smears of ejaculated spermatozoa and on testicular sections, they generally stained the spermatozoa of both sources, indicating that in most cases the antibodies in the spermagglutinating sera react with testicular and not with coating antigens (Feltkamp, Kruyff, Ladiges & Rümke 1965; Hamerlynck & Rümke 1967).

Prognosis and Therapy

Patients with strong auto-agglutination in the ejaculate and some others with incomplete agglutination have been infertile as long as their cases have been followed, which for some of them was for more than 10 years. Other males with weak agglutination, especially those with the lower serum spermagglutinin titres have impregnated their wives after some time (Rümke 1964, 1965). Other investigators have observed that spermagglutinins may disappear spontaneously (Bandhauer 1966; Schwimmer, Ustay & Behrman 1967). Particularly when inflammations of genital organs subside spermagglutinins may disappear (Bandhauer 1966).

It has to be stressed again that since occasionally high spermagglutinin titres have been found in the serum of patients with seemingly normal ejaculates, a prognosis can be made only on account of repeated semen analysis.

Treatment with ACTH and corticosteroids in moderate doses for a period of 2 months has not resulted in any observable decrease in serum antibody titre or auto-agglutination in the ejaculate (Rümke & Hellinga 1959).

Surgical treatment in order to remove sites of sperm resorption seems to be promising. Bandhauer (1966) reported of two such cases: one with an epididymal cyst and another patient with an old tuberculous inflammation of one epididymis whose spermagglutinins disappeared after removal of cyst or epididymis.

EXPERIMENTAL AUTO-ALLERGIC ORCHITIS

Auto-allergic orchitis, with reversible or irreversible aspermatogenesis, can be induced experimentally in guinea-pigs, rats and other animals, by injections of auto- or homologous testis homogenate or sperm emulsified in complete Freund's adjuvant. The original studies of Freund, Lipton & Thompson (1953) have been repeated and extended by many others. Their work has been reviewed by several authors (Freund *et al* 1955; Katsh & Bishop 1958; Waksman 1959; Bishop, Narbaitz & Lessof 1961; Tyler & Bishop 1963; Bishop & Carlson 1965). The testis lesions are confined to the germinal cells of the seminiferous tubules, and may lead to complete aspermatogenesis. The early stages of testicular cytopathology may involve interstitial lymphocytic infiltration. The damage can be reversible or result in total atrophy of the testis. It is believed that the cellular type of allergic response is mainly responsible for the development of the orchitis. Leucocytes from sensitized rats are able to induce similar changes in non-immunized male rats (Laurence, Carpuk & Perlbachs 1965).

Humoral antibodies do occur in the immunized animals, but in general no correlation exists between the severity of the lesions and the titre and type of antibody. Moreover, when immunization takes place with the spermatic antigen emulsified in Freund's adjuvant without added acid-fast mycobacteria, humoral antibodies are found, but there are no signs of testicular lesions. Multiple transfer of serum from actively sensitized guinea-pigs to recipients also failed to induce testicular defects (Bishop 1963). Humoral antibodies may even protect the animal from testicular damage when challenged with the antigen in complete adjuvant (Brown, Glynn & Holborow 1963; Chutná & Rychlíková 1964a). However, it has recently been found that the antibodies which are cytotoxic in testicular tissue cultures (Chutná & Rychlíková 1964b) and those giving the Arthus type of hypersensitivity (Toullet 1965) if combined with the cellular type of hypersensitivity become highly effective in inducing the lesions. The effect—protection or enhancement of orchitis—may well depend on the presence of either the γ_1 or the γ_2 type of IgG antibodies.

The aspermatogenic antigenic factor in guinea-pigs has been localized in the acrosome of mature germ cells. A polysaccharide-polypeptide complex possesses potent aspermatogenic capacity (Freund, Thompson & Lipton 1955). At least three auto-antigens can evoke auto-antibody formation, but only one—a

glycoprotein—seems to be responsible for the formation of antibodies giving the Arthus-type reaction, the cellular hypersensitivity and the induction of aspermatogenic orchitis (Toullet 1965). Purification of the aspermatogenic antigenic factor of guinea-pig testes has resulted in a glycoprotein capable of inducing circulating antibody, delayed skin reactivity and a testis lesion on injection of as little as 5 μg into guinea-pigs (Brown, Holborow & Glynn 1965). Guinea-pig testicular and spermatozoal antigens reveal a close similarity in amino-acid content, and human testicular antigen bears a striking similarity to those from the guinea-pig (Kirkpatrick & Katsh 1964).

Although induction of an experimental auto-allergic aspermatogenesis in human volunteers suffering from prostatic carcinoma has been described (Mancini *et al* 1965), so far no naturally occurring disease of the testes has been proved to be due to an auto-allergic process. As 15 out of 17 patients with spermagglutinins showed normal testes on biopsy, and as over 100 patients with impaired spermatogenesis had no spermagglutinins there is nothing to suggest a condition comparable to experimental auto-allergic orchitis (Rümke & Hellinga 1959). In the future other techniques will have to be used to examine sera of patients whose testis biopsy shows a- or hypospermatogenesis with focal infiltration of mononuclears to prove that such conditions are indeed of immunological origin.

REFERENCES

Adler H. (1909) Über Autospermotoxine. *Z. Immunitätsforsch.* **3,** 447.

Amann R. & Almquist J. (1962) Reproductive capacity of dairy bulls. *J. Reprod. Fertil.* **3,** 260.

Aszódi Z. & Scántó G. (1935) Über gegen Spermien gebildeten Antikörper. *Z. Ges. Exp. Med.* **97,** 485.

Bandhauer K. (1963) Untersuchungen über immunbiologische Ursachen der männlichen Sterilität. *Klin. Med. (Wien)* **4,** 204.

Bandhauer K. (1966) Immunreaktionen bei Fertilitätsstörungen des Mannes. *Urol. Int.* **21,** 247.

Beck J.S., Edwards R.G. & Young M.R. (1962) Immune fluorescence technique and the isoantigenicity of mammalian spermatozoa. *J. Reprod. Fertil.* **4,** 103.

Beneke (1898) In the discussion of a paper of Simmonds. 70 Versamml. Deutsch. Naturforsch. u. Ärzte, Düsseldorf. *Med. Abt.* **2,** 2.

Bishop D.W. (1963) Antitestes antibody concentration in sensitized guinea pigs. *Abstr. Biophys. Soc. (N.Y.)* **1,** 1.

Bishop D.W. & Carlson G.L. (1965) Immunologically induced aspermatogenesis in guinea pigs. *Ann. N.Y. Acad. Sci.* **124,** 247.

Bishop D.W., Narbaitz R. & Lessof M. (1961) Induced aspermatogenesis in adult guinea pigs injected with testicular antigen and adjuvant in neonatal stages. *Develop. Biol.* **3,** 444.

Brown P.C., Glynn L.E. & Holborow E.J. (1963) The pathogenesis of experimental allergic orchitis in guinea pigs. *J. Path. Bact.* **86,** 505.

Brown P.C., Holborrow E.J. & Glynn L.E. (1965) The aspermatogenic antigen in experimental allergic orchitis in guinea-pigs. *Immunology* **9,** 255.

BURGOS M.H. (1967) Places of selective absorption of colloidal particles along the sperm pathway. 8th Conf. Int. Planned Parenthood Fed. Santiago, Chile. p. 342.

BUXTON C.L. & WONG A.S.H. (1952) Spermicidal bacteria in cervix as a cause of sterility. *Amer. J. Obstet. Gynec.* **64,** 628.

CHUTNÁ J. & RYCHLÍKOVÁ M. (1964a) Prevention and suppression of experimental autoimmune aspermatogenesis in adult guinea-pigs. *Folia Biol.* **10,** 177.

CHUTNÁ J. & RYCHLÍKOVÁ M. (1964b) A study of the biological effectiveness of antibodies in the development and prevention of experimental autoimmune aspermatogenesis. *Folia Biol.* **10,** 188.

COOMBS R.R.A. (1962) Mixed agglutination reactions in relation to spermatozoa. *Proc. Conf. Immuno. Reprod. La Jolla, Calif.* p. 127. New York: Population Council.

CRONQUIST S. (1949) Spermatic invasion of the epididymis. *Acta Path. Microbiol. Scand.* **26,** 786.

CRUICKSHANK B. & STUART-SMITH D.A. (1959) Orchitis associated with spermagglutinating antibodies. *Lancet* **1,** 708.

EDWARDS R.G. (1960) Complement-fixing activity of normal rabbit serum with spermatozoa and seminal plasma. *J. Reprod. Fertil.* **1,** 268.

FELTKAMP F.E.W. KRUYFF K., LADIGES N.C.J.J. & RÜMKE PH. (1965) Auto-spermagglutinins. II. Immunofluorescent studies. *Ann. N.Y. Acad. Sci.* **124,** 702.

FJÄLLBRANT B. (1965) Immunoagglutination of sperm in cases of sterility. *Acta Obstet. Gynec. Scand.* **44,** 474.

FLOCKS R.H., BANDHAUER K., PATEL C. & BEGLEY G.J. (1962) Studies on spermagglutinating antibodies in anti-human-prostate-sera. *J. Urol.* **87,** 475.

FREUND J., LIPTON M.M. & THOMPSON G.E. (1953) Aspermatogenesis in the guinea-pig induced by testicular tissue and adjuvants. *J. Exp. Med.* **97,** 711.

FREUND J., THOMPSON G.E. & LIPTON M.M. (1955) Aspermatogenesis, anaphylaxis and cutaneous sensitization induced in the guinea-pig by homologous testicular extract. *J. Exp. Med.* **101,** 591.

FRIEDMAN N.B. & GARSKE G.L. (1949) Inflammatory reactions involving sperm and the seminiferous tubules: extravasation, spermatic granulomas and granulomatous orchitis. *J. Urol.* **62,** 363.

GLASSY F.J. & MOSTOFI F.K. (1956) Spermatic granulomas of the epididymis. *Amer. J. Clin. Path.* **26,** 1303.

HAMERLYNCK J.V.T.H. & RÜMKE PH. (1967) Unpublished.

HEKMAN A. & RÜMKE PH. (1967). Proc. Int. Symp. Immunol. Sperm. Fertil., Varna, Bulgaria.

HENLE W., HENLE G. & CHAMBERS L.A. (1938) Studies on the antigenic structure of some mammalian spermatozoa. *J. Exp. Med.* **68,** 335.

KATSH S. & BISHOP D. (1958) The effect of homologous testicular and brain and heterologous testicular homogenates combined with adjuvant upon the testis of guinea-pigs. *J. Embryol. Exp. Morph.* **6,** 94.

KATSH S. & KATSH G.F. (1965) Perspectives in immunological control of reproduction. *Pacif. Med. Surg.* **73,** 28.

KIBRICK S., BELDING D.L. & MERRILL B. (1952) Methods for the detection of antibodies against mammalian spermatozoa. *Fertil. Steril.* **3,** 430.

KING E.S.J. (1955) Spermatozoal invasion of the epididymis. *J. Path. Bact.* **70,** 459.

KIRKPATRICK C.H. & KATSH S. (1964) Amino-acid content of antispermatogenic antigen. *Nature* **201,** 197.

KLOPSTOCK A., HAAS R. & RIMON A. (1963) Immunoelectrophoretic analysis of seminal plasma. *Fertil. Steril.* **14,** 530.

KRIEG H. & EYQUEM A. (1964) Immunologie und Sterilität. *Gynäk. Rdsch.* **1,** 243.

KYRLE J. & SCHOPPER K.J. (1914) Ueber auffällige Befunde bei experimentellen Studien an Nebenhoden. *Klin. Wschr. (Wien)* **27,** 969.

KYRLE J. & SCHOPPER K.J. (1915) Ueber Regenerationsvörgange im tierischen Nebenhoden. *Virchow Arch. Path. Anat.* **220,** 1.

LANDSTEINER K. (1899) Zur Kenntnis der spezifisch auf Blutkörperchen wirkende Sera. *Zbl. Bakt.* **25,** 546.

LAURENCE K.A., CARPUK O. & PERLBACHS M. (1965) Transfer of testicular lesions by leucocytes from testes-immunized rats. *Int. J. Fertil.* **10,** 13.

MANCINI R.E., ANDRADA J.A., SARACENI D., BACHMANN A.E., LAVIERI J.C. & NEMIROVSKY M. (1965) Immunological and testicular response in man sensitized with human testicular homogenate. *J. Clin. Endocr.* **25,** 859.

MANN TH. (1964) *Biochemistry of Semen and of the Male Reproductive Tract.* London: Methuen & Co. Ltd.

MASON K.E. & SHAVER S.L. (1952) Some functions of the Caput epididymis. *Ann. N.Y. Acad. Sci.* **55,** 585.

MASSON P.L. & HEREMANS J.F. (1966) In *Molecular Biology of Human Proteins,* vol. I, p. 836, ed. SCHULTZE H.E. & HEREMANS J.F. Elsevier Publ. Comp.

MEAKER S.R. (1922) Some aspects of the problem of sterility. *Boston Med. Surg. J.* **187,** 535.

METALNIKOFF S. (1900) Etudes sur la spermatoxine. *Ann. Inst. Pasteur* **14,** 577.

METCHNIKOFF E. (1899) Etudes sur la résorption des cellules. *Ann. Inst. Pasteur* **13,** 737.

METZ C.B., SCHUEL H. & BISCHOFF E.R. (1964) Inhibition of the fertilizing capacity of the sea-urchin sperm by papain-digested, nonagglutinating antibody. *J. Exp. Zool.* **155,** 261.

MULLANEY J. (1962a) Experimental production of spermatic granuloma in rats. *Nature* **194,** 487.

MULLANEY J. (1962b) Granulomatous lesions associated with spermatozoal invasion of the interstitial tissues of the epididymis. *Brit. J. Urol.* **34,** 351.

NAKABAYASHI N.T., TYLER E.T. & TYLER A. (1961) Immunologic aspects of human infertility. *Fertil. Steril.* **12,** 544.

OBERNDORFER S. (1931) Spermaretention, Spermaresorption, Spermiophagie, Spermagglutination, Riesenzellbildungen in Hodenkanälchen und abführenden Samenwegen. *Henke-Lubarsch's Handbuch der speziellen Path. Anat. und Histologie* **6,** 708.

ORSÓS F. (1941) Die Sperminvasion. *Virchow Arch. Path. Anat.* **307,** 352.

PERNOT E. (1956) Récherches sur les constituants antigéniques des spermatozoïdes de cobayes. *Bull. Soc. Chim. Biol.* **38,** 1041.

PHADKE A.M. (1964) Fate of spermatozoa in cases of obstructive azoospermia and after ligation of vas deferens in man. *J. Reprod. Fertil.* **7,** 1.

PHADKE A.M. & PADUKONE K. (1964) Presence and significance of auto-antibodies against spermatozoa in the blood of men with obstructed vas deferens. *J. Reprod. Fertil.* **7,** 163.

PRIESEL A. (1924) Ueber das Verhalten von Hoden und Nebenhoden bei angeborenen Fehlen des Ductus deferens, zugleich ein Beitrag zur Frage des Vorkommens von Zwischenzellen im menschlichen Nebenhoden. *Virchow Arch. Path. Anat.* **249,** 246.

RAO S.S. & SADRI K.K. (1959) Immunological studies with human semen and cervical mucus. *Proc. 6th Conf. Planned Parenthood, New Delhi,* p. 313.

ROSENTHAL L. (1931) Spermagglutination by bacteria. *Proc. Soc. exp. Biol. Med.* **28,** 827.

RÜMKE PH. (1954) The presence of sperm antibodies in the serum of two patients with oligozoospermia. *Vox Sang.* **4,** 135.

RÜMKE PH. (1959a) Autoantibodies against spermatozoa in sterile men. *1st Int. Symp. Immunopathol. Basel, 1958,* p. 145.

RÜMKE PH. (1959b) Auto-antilichamen tegen spermatozoën als oorzaak van onvruchtbaarheid bij de man. Thesis, Amsterdam.

RÜMKE PH. (1964) Spermagglutinating auto-antibodies. Their relation to male infertility. *Proc. 2nd Int. Congr. Endocrinol. London*, p. 906.

RÜMKE PH. (1965) Auto-spermagglutinins. A cause of infertility in men. *Ann. N.Y. Acad. Sci.* **124**, 696.

RÜMKE PH. (1968) *Proc. Roy. Soc. Med.* (In press).

RÜMKE PH. & HELLINGA G. (1959) Auto-antibodies against spermatozoa in sterile men. *Amer. J. Clin. Path.* **32**, 357.

RÜMKE PH. & KREMER J. (1967) Unpublished.

SADRI K.K. & RAO S.S. (1965) Personal communication.

SCHWIMMER W.B., USTAY K.A. & BEHRMAN S.J. (1967) An evaluation of immunologic factors of infertility. *Fertil. Steril.* **18**, 167.

SEGAL S., TYLER E.T., RAO S., RÜMKE PH. & NAKABAYASHI N. (1961) Immunologic factors in infertility. In *Sterility*, p. 386, ed. TYLER E.T. New York: McGraw-Hill.

SIMMONDS M. (1921) Ueber das Verhalten des Menschlichen Hodens bei narbigem Verschlusz des Samenleiters. *Verh. Deutsch. Path. Ges.* **18**, 201.

SOBBE A., HAFERKAMP O. & DOEPFMER R. (1966) Serologische und immunhistologische Untersuchungen an Sperma und Seren von Männern steriler Ehen. *Deutsch. Med. Wschr.* **91**, 1234.

SOUTHAM A.L. (1963) Clinical significance of antibodies to spermatozoa and seminal plasma. *J. Reprod. Fertil.* **5**, 458.

SPOONER R.L. (1964) Cytolytic activity of the serum of normal guinea-pigs against their own testicular cells. *Nature* **202**, 915.

SUNDARASIVARAO D. (1955) Spermatozoal granuloma of the epididymis. *J. Path. Bact.* **69**, 324.

TOULLET F. (1965) Recherches sur la nature et sur les propriétés immunologiques des auto-antigènes de spermatozoïdes de cobaye. Thèse de Sciences, Paris.

TYLER A. & BISHOP D.W. (1963) Immunological phenomena. In *Mechanisms Concerned with Conception*, p. 397. ed. HARTMAN C.G. Pergamon Press.

TYLER A., TYLER E.T. & DENNY P. (1967) Concepts and experiments in immuno-reproduction. *Fertil. Steril.* **18**, 153.

TYLER E.T. & PAYNE S. (1947) Spermatogenesis and the therapy of infertility. *J.A.M.A.* **134**, 770.

WAKSMAN B.G. (1959) A histologic study of the autoallergic testis lesion in the guinea-pig. *J. exp. Med.* **109**, 311.

WEGELIN C. (1921) Ueber Spermiophagie im menschlichen Nebenhoden. *Beitr. Path. Anat.* **69**, 281.

WEIL A.J. (1961) Antigens of the adnexal glands of the male genital tract. *Fertil. Steril.* **12**, 538.

WEIL A.J. (1965) The spermatozoa-coating antigen (SCA). *Ann. N.Y. Acad. Sci.* **124**, 267.

WEIL A.J., KOTSEVALOVO & WILSON L. (1956) Antigens of human seminal plasma. *Proc. Soc. Exp. Biol. Med.* **92**, 606.

WEIL A.J. & RODENBURG J.M. (1960) Immunological differentiation of human testicular (spermatocele) and seminal spermatozoa. *Proc. Soc. Exp. Biol. Med.* **105**, 43.

WEIL A. J. & RODENBURG J.M. (1962) The seminal vesicle as the source of the spermatozoa-coating antigen of seminal Plasma. *Proc. Soc. exp. Biol. Med.* **109**, 567.

WILSON L. (1954) Spermagglutinins in human semen and blood. *Proc. Soc. Exp. Biol. Med.* **85**, 652.

WILSON L. (1956) Sperm agglutination due to auto-antibodies. A new cause of sterility. *Fertil. Steril.* **7**, 262.

ZETTERGREN L. (1957) Epididymitis spermiostatica granulomatosa. *Acta Chir. Scand.* **114**, 150.

CHAPTER 43

ALLERGIC REACTIONS OF THE EYE*

ARTHUR M. SILVERSTEIN

INTRODUCTION

It is not often appreciated that the tissues of the eye and its adnexae provide an extremely fertile ground for the entire spectrum of immunologic and allergic reactions of which the body is capable. Both within and around the eye may be encountered anaphylactic, Arthus, and delayed-type hypersensitivity reactions. Transplantation immunity is manifested occasionally following corneal grafting, while auto-allergic mechanisms operate against lens and uveal antigens. Curiously, the tissues of the eye may even be involved to a considerable extent in the local formation of antibody. The eye thus represents, in some respects, an immunologic microcosm in which all mechanisms may operate. But these ocular immunologic reactions are of more than academic interest to the immunologist or ophthalmologist; each of them may function to produce clinically significant disease.

By virtue of several anatomic and physiologic factors peculiar to the eye,

* This work was supported by a grant-in-aid from the Council for Research in Glaucoma and Allied Diseases, Alfred P. Sloan Foundation, Inc., by Grant No. NB 05366 from the National Institute of Neurological Diseases and Blindness, N.I.H., and by an Independent Order of Odd Fellows Research Professorship.

certain of its tissues respond differently in a given immunologic situation. For example, the lack of vascularization and of lymphatics in the normal cornea affect the course of *antibody-mediated* inflammatory reactions, and make the cornea a favoured tissue for the receipt of homografts. The relative isolation of the eye from the rest of the body, mediated by the so-called blood-aqueous barrier, inhibits the free exchange of substances between the ocular humours and the blood, giving rise to the almost unique ability of the eye to develop a state of local organ hypersensitivity. Again, the anatomic isolation of the lens permits it to harbour, without untoward consequences in the normal way, an assortment of 'foreign' protein antigens.

The eye possesses two other attributes which make it interesting to the immunologist. The clear cornea permits a ready observation of the intraocular tissues, so that certain immunopathologic reactions may be observed with the ophthalmoscope and slit lamp in a manner impossible in other tissues. To watch the development of an Arthus or delayed hypersensitivity reaction in the iris is in some respects like looking at the analogous response in the skin, but through a window. There is this additional point about the eye: it is almost exquisitely sensitive to functional alterations which affect the sight of the individual. Thus a mild inflammation elsewhere in the body which might go unrecognized as a subclinical entity could well represent in the anterior uvea a painful or disturbing disease requiring medical attention. This point will be returned to below, since there are certain processes such as antibody formation which are generally regarded as physiologic and benign when they result only in a slight peripheral lymphadenopathy, but which in the eye may *be* disease.

ANAPHYLACTIC REACTIONS

As might be expected, ocular tissues share, with related tissues elsewhere in the body, in systemic anaphylactic and atopic allergies. The challenge of an allergic individual with the appropriate allergen will result in an inflammatory reaction at the site of ocular insult. The conjunctiva is an especially sensitive tissue in this respect, since it is exposed to so many potential allergens borne either in the air or self-imposed as in drugs or cosmetics. Indeed, the instillation of an allergen into the conjunctival sac has been employed as an extremely sensitive test for allergy, since the reaction when present is both rapid and quite marked. The response takes the form of a greater or lesser degree of chemosis, i.e. of vasodilatation and oedema. Theodore & Schlossman (1958) discuss at length the characteristics of these types of conjunctivitis. An intraocular analogue of the cutaneous anaphylactic wheal and flare exists also, probably as a component of many cases of uveitis. Here, the response may be observed experimentally in the sensitized animal following anterior chamber challenge. The vasodilatation presents as a hyperaemia of the iris and ciliary body, while the oedematous

component takes the special form of the leakage of proteinaceous fluid into the normally protein-free aqueous, producing a positive Tyndall flare in the anterior chamber.

The participation of the conjunctiva in hay fever and related syndromes is too ubiquitous to warrant further comment. It may be mentioned in this connection that the conjunctiva may also participate in systemic responses to food allergens (Rowe 1935), and occasionally may constitute the only clinical sign of involvement.

The eyelids are another site of potential involvement in atopic reactions in the sensitive individual, a role shared with other cutaneous surfaces. Allergic oedema of the eyelids, however, is generally more impressive an occurrence than that elsewhere, because of the laxity of the skin at this site and the fact that the swelling can only proceed forward. This involvement is seen occasionally as a component of generalized urticaria or angioneurotic oedema. In addition to the lids, uveal or neuroretinal involvement is occasionally encountered in these situations.

Vernal Conjunctivitis

Vernal conjunctivitis (Biegelman 1950) is a recurrent bilateral interstitial inflammation of the conjunctiva which, as its name implies, occurs during the warmer months of the year. There is a marked cellular infiltration of the substantia propria characterized by eosinophils, with plasma cells appearing in numbers during periods of exacerbation of the disease. In advanced cases, there is a striking increase in connective tissue, with hyaline degeneration and an impressive proliferation of epithelium.

Threading through all discussions of this disease is the possibility that it may be based upon an allergic pathogenesis. In addition to its seasonal occurrence and the eosinophilia, there is a high incidence of familial and individual allergy. Many of these patients suffer from concurrent pollen atopies and possess circulating anti-pollen antibodies (Allansmith & Frick 1963), while sensitivity to food allergens has been implicated (Alimuddin 1955). Oguchi (1954) found that instillation of pollens into the conjunctival sacs of patients with the disease might induce a recurrence. While the pathogenesis of this disease remains unclear, the possibility must be retained that allergy may play a role, perhaps as one of several mechanisms capable of triggering the process.

ARTHUS REACTIONS

The experimental observations of Waksman & Bullington (1956) and of Silverstein & Zimmerman (1959) have shown that ocular tissues, and especially the uvea, are able to support the typical Arthus phenomenon. These inflammatory responses may be elicited both in actively and in passively sensitized

animals. Following fairly rapidly on the heels of an intraocular challenge injection, a more or less severe uveitis develops, depending upon the degree of sensitization of the animal. There is generally a marked chemosis and iridocyclitis, often with proteinaceous exudate and cells in the anterior chamber. Petechial haemorrhages are often seen in the iris, while the cellular infiltrate is almost exclusively polymorphonuclear, especially in the passively sensitized animals. Identifiable thromboses are generally absent in these lesions. As occurs in other sites, the inflammation resolves to a considerable plasmacytosis, more pronounced and more rapidly in the actively sensitized animals. Of course, a suitable antigenic insult in the neighbouring extraocular tissues of a suitably sensitized recipient would similarly result in an Arthus-type inflammatory response.

The cornea has played an important role in fundamental studies aimed at elucidating the mechanism of the Arthus phenomenon. Rich & Follis (1940) studied the Arthus reaction in the cornea and suggested that blood vessels are required for the development of the inflammation. They were unable to elicit a keratitis upon introduction of protein antigens into the normal cornea of the sensitized animal, but typical reactions appeared if the cornea had previously been vascularized. This observation appeared to them to be in line with the classic suggestion that antigen-antibody union does not involve primary destruction of parenchymal cells, but results only from insult upon vascular elements. Of interest in this connection is the report of Germuth *et al* (1958) on the effect of antigen-antibody interaction upon the avascular cornea. These workers demonstrated that intracorneal injection of protein antigen into the passively sensitized animal would result in inflammation, but of a curious type. Concentrically located around the site of injection in the cornea was a ring of what proved to be antigen-antibody precipitate, resulting presumably from the outwardly diffusing antigen meeting and reacting with antibody diffusing inward from the limbus. Along this line of precipitation there occurred an alteration of the keratocytes and ground substance, and an intense local infiltration by inflammatory cells. A similar phenomenon was demonstrated with elegance by the injection of antigen into one side of the cornea of a normal animal and of antibody into the other side of the same cornea, analogous to the Ouchterlony double-diffusion test for antigen-antibody interactions. Where the reactants met, a straight line of precipitation developed, which was bordered by an inflammatory infiltrate.

DELAYED HYPERSENSITIVITY REACTIONS

The delayed, tuberculin-type allergic responses are not uncommon in and around the eye. With only minor variations, these are similar to the analogous reactions observed elsewhere, being characterized by perivascular round-cell

infiltrates. Only in the delayed corneal reaction (see below) is the polymorphonuclear response predominant. The conjunctiva has proved to be an extremely sensitive terrain for this type of inflammatory response. This is illustrated by the well-known Calmette test, involving the instillation of tuberculin into the conjunctival sac of the sensitized individual, leading to a readily discernible conjunctivitis. Woods (1937, 1949) has indicated that sensitivities to staphylococcal antigens are responsible for many cases of recurrent conjunctivitis, while sensitivities to fungi and helminths are not unknown.

The intraocular tissues, and especially the uveal tract, also furnish a fertile ground for the development of delayed inflammatory lesions. These reactions may be elicited in the eyes of animals sensitized either to bacterial antigens such as tuberculin (Rich & Follis 1940), or to simple bland protein antigens such as ovalbumin and bovine serum albumin (Silverstein & Zimmerman 1959). While the customary response to intracameral challenge with antigen is a non-granulomatous round-cell infiltration of the iris and ciliary body, often with an accompanying focal choroiditis, there may also be present a perivasculitis of the optic nerve and retina (Silverstein & Zimmerman 1959). In the experimental situation the reaction may spread to include the sclera and episclera. It has been suggested (Silverstein, Welter & Zimmerman 1961) that the earliest component of the spontaneous uveitis which follows the experimental injection of antigen into the vitreous (see below) may be a delayed hypersensitivity response to antigen leaking slowly out of the depot formed at the injection site.

In his classification of endogenous uveitis, Woods (1961) suggested that the non-granulomatous form may in large measure be due to delayed hypersensitivity to bacterial products, mainly streptococcal. Woods marshals a very convincing array of experimental and clinical evidence to justify the conviction that hypersensitivity mechanisms play an important if not predominant role in the pathogenesis of this disease. In contrast to non-granulomatous uveitis, usually arising from a sterile insult, Woods has defined granulomatous uveitis as the chronic disease resulting from active infection by non-pyogenic organisms. The aetiologic agents generally conceded to be important in this disease are toxoplasma, histoplasma, tubercle bacilli, brucella, treponema, and an occasional nematode or fungus (Woods *et al* 1954). In each instance the invader stimulates a retinitis or chorioretinitis at the focus of infection. Since each of these pathogens is known to be highly antigenic, infection often being accompanied by positive delayed skin reactivity, it must be supposed that hypersensitivity mechanisms contribute significantly to the development of the granulomatous lesions involved. In addition, the non-granulomatous anterior segment inflammation which generally accompanies the posterior lesion may well represent a hypersensitivity response to antigen which has diffused forward from the focus of infection in the hypersensitive individual.

We may finally mention contact dermatitis-type reactions in our catalogue

of delayed ocular inflammatory phenomena (Theodore & Schlossman 1958). These occur predominantly on the skin of the eyelids and are not infrequent, due to the sensitivity of this cutaneous surface and its exposure to cosmetics, drugs and contaminated fingers. The response on the lids differs in no major respect from that met with elsewhere on the body, and need concern us no further. Contact reactions of the conjunctiva are often encountered, usually secondary to lid involvement, while corneal contact responses have been diagnosed. In the experimental situation, however, it proved impossible to elicit a *primary* contact conjunctivitis or keratitis, although inflammation apparently secondary to a lid reaction was occasionally observed in these tissues (Silverstein, Welter & Zimmerman 1960).

Corneal Reactions

By virtue of its avascularity and accessibility, the cornea provides a unique tissue for the study of hypersensitivity mechanisms. It has already been mentioned that diffuse corneal inflammation cannot be incited in Arthus-sensitized animals by intracorneal challenge with the appropriate antigen. In contrast, even minute amounts of antigen may induce an acute interstitial keratitis in the delayed-sensitive animal. These lesions may be elicited by a variety of different antigenic substances. In view of the difference in corneal response in the delayed-hypersensitive as opposed to the Arthus-sensitive animal, intracorneal challenge has often been employed in the differentiation of the two types of hypersensitivity.

In the case wherein the intracorneally injected allergen is soluble, the keratitis is found to subside within a matter of days or at most of a few weeks, leaving little permanent damage. It might be anticipated, however, that persisting non-soluble stimuli such as bacteria would result in a continuously developing, chronic lesion which would not abate so long as the insulting agent remained. Eventually, an epithelioid formation resembling a type of tubercle may develop.

Phlyctenulosis is believed by many to be due in large measure to a pathogenetic mechanism such as that mentioned above. It has always been considered significant that a high incidence of positive tuberculin hypersensitivity is encountered in cases of phlyctenular disease (Weekers 1909; Sorsby 1942). As early as 1909, Weekers demonstrated experimentally that phlyctenules might be produced in tuberculin-sensitive rabbits following the instillation of this substance into the conjunctiva. Other pathogens have been implicated aetiologically in the development of this disease as a result of its experimental production in the eyes of animals made hypersensitive to staphylococci (Funaishi 1923), and, curiously, even to horse serum (Riehm 1928).

LOCAL OCULAR HYPERSENSITIVITY AND UVEITIS

The parenteral administration of an antigen results in its fairly rapid exit from

the site of injection and its dissemination throughout the body. By the time the immunologic response has started to function fully, there is usually no antigen remaining at the original site. In general, no lesion results from this first exposure. The eye, on the other hand, responds differently. A week or so following the injection of soluble protein antigen into the vitreous body a spontaneous uveitis develops in the injected eye. This inflammation is characterized histologically (Woods, Friedenwald & Wood 1955; Zimmerman & Silverstein 1959) by lymphocytes and monocytes in the early stage, with a marked plasmacytosis appearing a few days later. It has been shown that the vitreous body forms a natural depot from which antigenic proteins can only slowly escape (Fernando 1960; Rodenhäuser 1959). Thus, antigen persists in the eye while hypersensitivity is developing in the body, a perfect setting for the appearance of a 'spontaneous' inflammation. The situation is somewhat analogous to a repeatedly administered immunization, the earliest response to which seems to be a delayed-hypersensitivity state, followed some days later by the appearance of antibody (Gell & Hinde 1954). It has been demonstrated (Silverstein 1963, 1964) that the inflammatory cells which participate in these uveal responses arise outside the eye, rather than from the stimulation of autochthonous elements.

Perhaps more significant is the condition of the injected eye after recovery from the initial inflammatory response to antigen. At this time the eye is often clinically and even histologically normal. The imprint of the previous exposure to the antigen remains, however, so that a recurrence of the inflammatory reaction may be provoked by parenteral administration of additional antigen. This may be accomplished by intravenous or intracutaneous injection, or even by the ingestion of the offending allergen (Seegal, Seegal & Khorazo 1933). The eye in this situation appears to have a heightened degree of hypersensitivity, so that it alone of all organs will respond to the new antigenic stimulus. (It may be mentioned that a parallel situation exists in the response of the cornea to interlamellar injection of proteins, including both the primary spontaneous inflammation and also the response to intravenous challenge (v. Szily 1914; Leibowitz & Elliott 1965). Much of the following discussion will bear also on this phenomenon in the cornea.)

The ability to incite a recurrent uveitis in these experimental animals may persist for many months, and the response may be invoked repeatedly upon reintroduction of the proper antigen. The specificity of the immunologic mechanism may be impressively demonstrated by the injection of a different antigen into each eye of the test animal. Later intravenous injection of the suitable antigen will then light up either eye at will. The importance of this experimental model and of the concept of local organ hypersensitivity in the pathogenesis of clinical uveitis has been discussed at length by Woods (1961). While some cases of recurrent non-granulomatous uveitis have been traced to the ingestion of or exposure to known allergens, it has generally been extremely

difficult to make an aetiologic diagnosis in this field. An exception to this, and one which provides a striking analogy to the experimental situation, is encountered in the so-called focal reaction observed occasionally in clinical practice. It sometimes happens during attempts at desensitization with tuberculin or with streptococcal vaccines that injection of the antigen causes a flare-up of ocular disease (Weizenblatt 1949; Miller & Smerz 1956). This may be an exacerbation of either non-granulomatous or of granulomatous disease. Kaufman (1960) has reported that as long as 1 year following experimental ocular toxoplasma infection, the injection of killed organisms would rekindle an old lesion.

In the normal course of events, the blood-aqueous barrier does not permit the entry of substances into the ocular tissues, so that local sensitization of the eye does not accompany intravenous injection of antigens. We may reasonably inquire how, then, the antigen may, in the natural setting, accomplish its entry into the eye and its sensitization of ocular tissue. The most obvious possibility involves antigens which are endogenous to the eye, such as lens proteins (see below). Another possibility is that during the course of even a mild bacteraemia accompanying an infection elsewhere, a few organisms may lodge in the uvea and reside there for a time. Even if the resulting infection were subclinical, local sensitization might ensue. Finally, it has been well established that as a result of trauma or of other ocular inflammation, there is a breakdown of the blood-aqueous barrier. Any antigenic substances borne in the blood at this time might thus be able to gain entry into and sensitize the eye (Taylor & Suie 1956).

ANTIBODY FORMATION IN THE EYE

In studying the nature of the antibody response mechanism, most investigators have been preoccupied with the response of the organism as a whole to antigenic stimulus, with the emphasis placed upon the most obvious tissues, the spleen and lymph nodes. It should be noted, however, that the production of antibodies is not limited to these organs, but may occur at any site of the body capable of supporting an inflammatory response. This point has special significance in ocular immunopathology.

It has been mentioned that following the intraocular injection of antigen there will develop a marked plasmacytosis of the iris, the ciliary body and at the optic disc. This phenomenon is strongly suggestive of a local production of antibody within the eye in response to an initial antigenic stimulus; later exposure to the same antigen induces a new phase of plasmacytosis. Witmer (1955) has identified antibody-producing cells in the uvea, employing the fluorescent-antibody technique. Wolkowicz and co-workers (1960) have been able to show the continuing production of antibody by ocular tissues transferred to tissue culture after antigenic stimulus. Even the cornea appears able to support the active production of antibody (Oakley, Batty & Warrack 1955; Schwab 1959;

Burkl & Schwab 1959; Leibowitz & Elliott 1965). The significance of these demonstrations of local antibody formation in the eye, accompanied by plasmacytosis, is perhaps heightened by recalling that the typical histologic picture of a subsiding non-granulomatous uveitis is one of an often remarkable degree of plasmacytosis in the anterior uvea.

It would thus seem that the presence of antibody produced locally in the eye and unable to escape readily would constitute an important factor in what we have called above 'local ocular hypersensitivity'. This might account for the ability in some cases to produce uveitis with antigenic challenge at a distant site. This ability persists, however, for long periods of time, so that often the 'sensitive' eye shows no clinical or histologic signs of this sensitivity, i.e. local antibody production seems to have ceased for all practical purposes. But the eye may still be inflamed by intravenous challenge. Since this eye has produced antibody previously, it should be capable of reacting to newly introduced antigen with an anamnestic response, involving the proliferation and differentiation of immunologically competent cells. In a lymph node, this act would pass unnoticed; in the iris and ciliary body it might well appear as inflammation and clinical disease. We would thus suggest that the simple *act* of antibody production in the eye may represent a contributing factor in the pathogenesis of uveitis (Silverstein 1964). The significance of ocular antibody formation for the aetiologic diagnosis in uveitis cases has also been stressed repeatedly (Witmer 1964).

An interesting footnote to the problem of local ocular hypersensitivity and antibody production involves the question of clinical desensitization in uveitis. In many instances, as has been hinted, the allergen probably reaches the sensitive ocular tissue via the circulation. The aim, then, of desensitization in the face of local ocular hypersensitivity might not be to 'neutralize' antibody within the eye, but rather to prevent antigen from ever reaching the sensitive intraocular tissues. This goal can be achieved in practice by immunization such that there exists a high enough level of circulating antibody to neutralize intravascular antigen before it reaches ocular tissue. The familiar course of desensitization in this instance may thus serve to create an antibody barrier around the eye.

OCULAR COMPLICATIONS OF SYSTEMIC ALLERGIC DISEASE

As might be anticipated, a systemic disorder which affects a particular type of anatomic structure or a specific physiologic function may well affect the same substrates when and where they exist in the eye. The tissues of the eye are not so clearly divorced from those of the rest of the body that they will be excepted from participation in certain generalized disease processes.

An example of such participation is found in the ocular changes which sometimes accompany serum sickness. This generally takes the form of a transient anterior uveitis, more often bilateral than unilateral (Theodore & Lewson 1939; Hoover 1956; Sedan & Guillot 1955). In addition papilloedema and retinal changes have been noted in a moderate proportion of serum sickness cases (Brown 1925). Periarteritis nodosa is also accompanied by ocular changes. The most consistent of these alterations represent not local ocular periarteritis but rather a hypertensive retinopathy or neuroretinitis secondary to kidney and circulatory damage (Boeck 1956; Goldsmith 1946). Less frequently, there are present choroidal foci of periarteritis nodosa, and only rarely are retinal nodules noted (Goldsmith 1946). Scleral nodules are not infrequent, and keratitis and corneal ulcers have also been reported (Wise 1952).

The most frequent ocular complication of rheumatoid arthritis is a moderately severe, recurrent non-granulomatous anterior uveitis. This is more often bilateral than unilateral. What may be an experimental analogue of this condition and indicate a possible pathogenetic mechanism is suggested by a study by Waksman & Bullington (1960) of experimental arthritis in rats. These investigators showed that together with symptoms of arthritis, a number of animals responded to footpad injections of bacteria in adjuvants with the development of ocular lesions. These included an essentially non-granulomatous uveitis, conjunctivitis, keratitis and episcleritis. The authors discuss the lesions in relation to the incidence of uveitis in Reiter's syndrome and in ankylosing spondylitis. The ocular complications of collagen diseases in general have been reviewed elsewhere by Lebas (1959) and by Manschot (1960).

There is yet another experimental model which is not without interest in the field of ocular immunopathology. It has been shown (Waksman 1959) that the injection of animals with homologous brain tissue would lead to the development of what has been termed allergic encephalomyelitis. The injection of optic nerve or other central nervous tissue may also give rise to ocular lesions. In addition to the expected allergic encephalomyelitis, the animals were found to have developed an optic neuritis and iridocyclitis (Bullington & Waksman 1958). There seems to be little doubt as to the immunologic nature of this disease, the ocular complications appearing presumably as a result of antigens which are common to the eye and to nervous tissue elsewhere.

AUTO-ALLERGIC DISEASES OF THE EYE

There appear to be two possible bases for the development of auto-allergic phenomena; on the one hand, the immune response may be stimulated by autochthonous proteins which are truly antigenic for the host, and on the other hand the response may be to altered substances which in their native state are

Burkl & Schwab 1959; Leibowitz & Elliott 1965). The significance of these demonstrations of local antibody formation in the eye, accompanied by plasmacytosis, is perhaps heightened by recalling that the typical histologic picture of a subsiding non-granulomatous uveitis is one of an often remarkable degree of plasmacytosis in the anterior uvea.

It would thus seem that the presence of antibody produced locally in the eye and unable to escape readily would constitute an important factor in what we have called above 'local ocular hypersensitivity'. This might account for the ability in some cases to produce uveitis with antigenic challenge at a distant site. This ability persists, however, for long periods of time, so that often the 'sensitive' eye shows no clinical or histologic signs of this sensitivity, i.e. local antibody production seems to have ceased for all practical purposes. But the eye may still be inflamed by intravenous challenge. Since this eye has produced antibody previously, it should be capable of reacting to newly introduced antigen with an anamnestic response, involving the proliferation and differentiation of immunologically competent cells. In a lymph node, this act would pass unnoticed; in the iris and ciliary body it might well appear as inflammation and clinical disease. We would thus suggest that the simple *act* of antibody production in the eye may represent a contributing factor in the pathogenesis of uveitis (Silverstein 1964). The significance of ocular antibody formation for the aetiologic diagnosis in uveitis cases has also been stressed repeatedly (Witmer 1964).

An interesting footnote to the problem of local ocular hypersensitivity and antibody production involves the question of clinical desensitization in uveitis. In many instances, as has been hinted, the allergen probably reaches the sensitive ocular tissue via the circulation. The aim, then, of desensitization in the face of local ocular hypersensitivity might not be to 'neutralize' antibody within the eye, but rather to prevent antigen from ever reaching the sensitive intraocular tissues. This goal can be achieved in practice by immunization such that there exists a high enough level of circulating antibody to neutralize intravascular antigen before it reaches ocular tissue. The familiar course of desensitization in this instance may thus serve to create an antibody barrier around the eye.

OCULAR COMPLICATIONS OF SYSTEMIC ALLERGIC DISEASE

As might be anticipated, a systemic disorder which affects a particular type of anatomic structure or a specific physiologic function may well affect the same substrates when and where they exist in the eye. The tissues of the eye are not so clearly divorced from those of the rest of the body that they will be excepted from participation in certain generalized disease processes.

An example of such participation is found in the ocular changes which sometimes accompany serum sickness. This generally takes the form of a transient anterior uveitis, more often bilateral than unilateral (Theodore & Lewson 1939; Hoover 1956; Sedan & Guillot 1955). In addition papilloedema and retinal changes have been noted in a moderate proportion of serum sickness cases (Brown 1925). Periarteritis nodosa is also accompanied by ocular changes. The most consistent of these alterations represent not local ocular periarteritis but rather a hypertensive retinopathy or neuroretinitis secondary to kidney and circulatory damage (Boeck 1956; Goldsmith 1946). Less frequently, there are present choroidal foci of periarteritis nodosa, and only rarely are retinal nodules noted (Goldsmith 1946). Scleral nodules are not infrequent, and keratitis and corneal ulcers have also been reported (Wise 1952).

The most frequent ocular complication of rheumatoid arthritis is a moderately severe, recurrent non-granulomatous anterior uveitis. This is more often bilateral than unilateral. What may be an experimental analogue of this condition and indicate a possible pathogenetic mechanism is suggested by a study by Waksman & Bullington (1960) of experimental arthritis in rats. These investigators showed that together with symptoms of arthritis, a number of animals responded to footpad injections of bacteria in adjuvants with the development of ocular lesions. These included an essentially non-granulomatous uveitis, conjunctivitis, keratitis and episcleritis. The authors discuss the lesions in relation to the incidence of uveitis in Reiter's syndrome and in ankylosing spondylitis. The ocular complications of collagen diseases in general have been reviewed elsewhere by Lebas (1959) and by Manschot (1960).

There is yet another experimental model which is not without interest in the field of ocular immunopathology. It has been shown (Waksman 1959) that the injection of animals with homologous brain tissue would lead to the development of what has been termed allergic encephalomyelitis. The injection of optic nerve or other central nervous tissue may also give rise to ocular lesions. In addition to the expected allergic encephalomyelitis, the animals were found to have developed an optic neuritis and iridocyclitis (Bullington & Waksman 1958). There seems to be little doubt as to the immunologic nature of this disease, the ocular complications appearing presumably as a result of antigens which are common to the eye and to nervous tissue elsewhere.

AUTO-ALLERGIC DISEASES OF THE EYE

There appear to be two possible bases for the development of auto-allergic phenomena; on the one hand, the immune response may be stimulated by autochthonous proteins which are truly antigenic for the host, and on the other hand the response may be to altered substances which in their native state are

non-antigenic. Both of these mechanisms may be operative in the eye. The former has been well established in the case of sensitization to lens antigens, while the latter may be concerned with auto-allergy to uveal antigens.

Phaco-anaphylaxis

The clinical condition known as endophthalmitis phaco-anaphylactica was first described by Verhoeff & Lemoine (1922) to be the result of autosensitization to one's own lens antigens. Classically, the event follows the initial release of lens material during an uneventful extracapsular cataract extraction, or as a result of traumatic rupture of the capsule. If lens material remains in this eye, or if lens antigen is released subsequently in the fellow eye, then uveitis may ensue if sensitization has occurred. In some instances (Courtney 1942; deVeer 1953) the disease may appear bilaterally, and may be confused with sympathetic ophthalmia.

The early demonstrations (Uhlenhuth 1903; Hektoen & Schulhof 1924) that lens antigens are antigenic in their host, and are organ rather than species specific, established the lens in a somewhat unique position in immunology. The lens, and therefore its protein antigen constituents, becomes anatomically isolated from the rest of the body at a quite early stage of embryonic development. They are thus out of contact with the tissues which are involved in the development of immunologic competence, and in this sense are 'foreign' substances, to which the immune mechanism may subsequently respond. What may represent an interesting by-product of this anatomic isolation involves the antigenic interrelationship among the lens proteins of many species (Halbert & Manski 1963; Halbert, Manski & Auerbach 1961). It may be that such isolation has protected these antigens from many of the evolutionary alterations to which most other proteins have been subjected.

An experimental analogue of phaco-anaphylaxis has been produced in laboratory animals (Burkey 1934; Müller 1952) which is similar to the natural disease both clinically and histologically. In both, the lesion occurs predominantly in the anterior segment, where the reaction develops around the disrupted lens. Polymorphonuclear leucocytes form an abscess which in turn is walled off by epithelioid cells in a fibrovascular zone. Finally, the reaction site is surrounded by lymphocytes and plasma cells, which also occur abundantly as the principal component of the iridocyclitis.

Sympathetic Ophthalmia

Sympathetic ophthalmia typically follows a perforating injury of the globe in which the uveal tract is involved. Its incidence, however, is very much lower following surgical procedures. After a time lag of some weeks, the injured eye may develop what in most respects is a typical granulomatous endophthalmitis. There are mutton-fat keratic precipitates present, and the lesion is histologically

one of diffuse and often massive lymphocytic infiltration of uveal tissues, with areas of epithelioid cell infiltrations and often giant cells. At any time from about the third week to several years following the trauma, a spontaneous lesion may appear in the sympathizing eye, similar in all respects to that in the first eye.

An allergic theory of this disease was advanced as early as 1910 by Elschnig, who demonstrated that uveal emulsions were auto-antigenic and who suggested that uveal pigment was the offending antigen. The postulate gained important support from the investigations of Woods (1921, 1956), who was able to identify anti-uveal antibodies in patients with perforating injuries of the globe. Woods also showed that extracts of uveal tissue would elicit a positive intradermal hypersensitivity test in these patients. Friedenwald (1934) showed that the histology of the skin test response was strikingly similar to that of the ocular lesion. Collins (1949, 1953) injected emulsions of uveal tissue in Freund's adjuvant into guinea-pigs and observed the development of diffuse inflammatory reactions in the choroid of many of these animals. Using the same approach, Aronson, Hogan & Zweigert (1963) were able to induce the development of spontaneous uveitis in experimental animals, using both pigmented and albino uveal tissue. It is still unfortunately unclear whether these observations on the production of experimental auto-immune uveitis bear any meaningful relationship to the clinical entity of sympathetic ophthalmia, since the histopathologic pictures differ so markedly in the two cases.

There is another observation that may be pertinent to the present discussion. The recent popularity of auto-immune processes to explain the pathogenesis of a wide variety of diseases has prompted an examination of the sera of patients with uveitis. In two such studies (Hallett *et al* 1962; Aronson *et al* 1964) a significantly greater incidence of anti-uveal antibodies has been reported in such patients. The relationship of these findings to the pathogenesis of the ocular disorders remains to be established.

In sum, the temporal aspects of sympathetic ophthalmia, the delayed skin test response, and much of the experimental evidence, all combine to suggest that allergic mechanisms and autosensitization play an important role in the pathogenesis of sympathetic ophthalmia, although the evidence here is not quite as compelling as that which exists in the earlier discussed phaco-anaphylaxis.

THE HOMOGRAFT REACTION AND KERATOPLASTY

The homograft rejection reaction is of interest in ophthalmology due to its potential participation in the rejection of corneal transplants. The factors which affect the survival of corneal grafts have been reviewed in some detail by Maumenee (1960). He discussed the reasons for the relative infrequency of

immune reactions to corneal homografts, and points out that the cornea constitutes a privileged transplant in certain respects. Corneal tissue appears to be only weakly antigenic, especially after being denuded of its epithelium, a standard practice. In addition, the wide separation of corneal stromal cells by the ground substance apparently lessens the ability of the keratocyte to initiate an immune response. It has been shown (Algire, Weaver & Prehn 1957; Woodruff 1957; Merwin & Hill 1954) that sensitization to a homograft usually requires vascularization of the donor tissue. If the corneal button is placed upon a relatively avascular recipient bed and if subsequent vascularization does not occur, then initial sensitization may well not follow.

There are several additional reasons to explain why the lamellar graft (and the experimental interlamellar graft) are even less frequently rejected than is the penetrating graft. The lamellae at the base of these grafts may be sufficient to separate the stromal cells of the donor from blood vessels in the recipient bed, thus preventing sensitization. In addition, the endothelium of the penetrating graft seems to be more sensitive than the stromal cells to damage leading to corneal clouding. In the absence of transplanted endothelium, the lamellar graft would be less prone to disturbance of its physiology. In contrast to the opinion popular only a few years ago, it is currently believed that there is no rapid replacement of donor stromal cells by those of the host. Basu, Miller & Ormsby (1960), using sex chromatin as a biological marker, were able to show survival of donor keratocytes for at least several months while Hanna & Irwin (1962) and Polack *et al* (1964), employing tritiated thymidine-labelling techniques, demonstrated that donor stromal and endothelial cells survive for longer than a year in the recipient bed.

So great are the advantages of the cornea as a transplantation site, and of the lamellar graft as the approach of choice, that it has proved possible to obtain successful heterografts of corneal tissue (Chavan & King 1960; Payrau, Pouliquen & Faure 1961) even in the human. Payrau *et al* have reviewed this subject, and have indicated that only certain donor-recipient species relationships permit of successful heterografting.

REFERENCES

Algire G.H., Weaver J.M. & Prehn R.T. (1957) Studies on tissue homotransplantation in mice using diffusion chamber methods. *Ann. N.Y. Acad. Sci.* **64,** 1009.

Alimuddin M. (1955) Treatment of vernal conjunctivitis. *Brit. J. Ophth.* **39,** 540.

Allansmith M. & Frick O.L. (1963) Antibodies to grass in vernal conjunctivitis. *J. Allergy* **34,** 535.

Aronson S.B., Hogan M.J. & Zweigart P. (1963) Homoimmune uveitis in the guinea pig. I. General concepts of auto- and homoimmunity, methods, and manifestations. *Arch. Ophth.* **69,** 105.

Aronson S.B., Yamamoto E., Goodner E.K. & O'Connor G.R. (1964) The occurrence of antiuveal antibody in human uveitis. *Arch. Ophth.* **72,** 621.

BASU P.K., MILLER I. & ORMSBY H.L. (1960) Sex chromatin as a biological marker in the study of corneal transplants. *Amer. J. Ophth.* **49,** 514.

BIEGELMAN M.N. (1950) *Vernal Conjunctivitis.* Los Angeles: University of Southern California Press.

BOECK J. (1956) Ocular changes in periarteritis nodosa. *Amer. J. Ophth.* **42,** 567.

BROWN A.L. (1925) Ocular manifestations of serum sickness. *Amer. J. Ophth.* **8,** 614.

BULLINGTON S.J. & WAKSMAN B.H. (1958) Uveitis and experimental encephalomyelitis: results produced by injection of nervous tissue in adjuvants. *Arch. Ophth.* **59,** 435.

BURKY E.L. (1934) Experimental endophthalmitis phacoanaphylactica in rabbits. *Arch. Ophth.* **12,** 536.

BURKL W. & SCHWAB F. (1959) Histologischer und immunohistologischer Nachweis der lokalen Antikörperbilding in der Hornhaut. *v. Graefe's Arch. Ophth.* **161,** 168.

CHAVAN S.B. & KING J.H. (1960) Experimental lamellar heterografts. *Amer. J. Ophth.* **49,** 1387.

COLLINS R.C. (1949, 1953) Experimental studies on sympathetic ophthalmia. *Amer. J. Ophth.* **32,** 1687; **36,** 150.

COURTNEY R.H. (1942) Endophthalmitis with secondary glaucoma accompanying absorption of the crystalline lens. *Trans. Amer. Ophth. Soc.* **40,** 355.

DEVEER J.A. (1953) Bilateral endophthalmitis phacoanaphylactica: pathologic study of the lesion in the eye first involved and, in one instance, the secondarily implicated or 'sympathizing' eye. *Arch. Ophth.* **49,** 607.

ELSCHNIG A. (1910) Studien zur sympathischen Ophthalmie. II. Die antigenische Wirkung des Augenpigmentes. *v. Graefe's Arch. Ophth.* **76,** 509.

FERNANDO A.N. (1960) Immunological studies with I^{131} labeled antigen in experimental uveitis. *Arch. Ophth.* **63,** 515.

FRIEDENWALD J.S. (1934) Notes on the allergic theory of sympathetic ophthalmia. *Amer. J. Ophth.* **17,** 1008.

FUNAISHI S. (1923) Experimentelle untersuchungen über die Aetiologie der phlyktänulären Augenentzündungen. *Klin. Monatsbl. Augenh.* **71,** 141.

GELL P.G.H. & HINDE I.T. (1954) Observations on the histology of the Arthus reaction and its relation to other known types of skin hypersensitivity. *Int. Arch. Allergy* **5,** 23.

GERMUTH F.G., MAUMENEE A.E., PRATT-JOHNSON J., SENTERFIT L.B., VAN ARNAM C.E. & POLLACK A.D. (1958) Observations on the site and mechanism of antigen-antibody interaction in anaphylactic hypersensitivity. *Amer. J. Ophth.* **46,** 282.

GOLDSMITH J. (1946) Periarteritis nodosa with involvement of choroidal and retinal arteries. *Amer. J. Ophth.* **29,** 435.

HALBERT S.P. & MANSKI W. (1963) Organ specificity with special reference to the lens. *Progr. Allergy* **7,** 107.

HALBERT S.P., MANSKI W. & AUERBACH T. (1961) Lens antigens and evolution. In *The Structure of the Eye,* p. 249, ed. SMELSER G.K. New York: Academic Press.

HALLETT J.W., WOLKOWICZ M.I., LEOPOLD I.H., CONAMUCIO C. & WIJEWSKI E. (1962) Autoimmune complement fixation test in endogenous uveitis. *Arch. Ophth.* **68,** 168.

HANNA C. & IRWIN E.S. (1962) Fate of cells in the corneal graft. *Arch. Ophth.* **68,** 810.

HEKTOEN L. & SCHULHOF K. (1924) Further observations on lens precipitins. *J. Infect. Dis.* **34,** 433.

HOOVER R.E. (1956) Nongranulomatous uveitis, a complication of serum sickness. *Amer. J. Ophth.* **41,** 534.

KAUFMAN H.E. (1960) The effect of corticosteroids on experimental ocular toxoplasmosis. *Amer. J. Ophth.* **50,** 919.

LEBAS P. (1960) Les Syndromes Oculo-cutanes. *Bull. Soc. belge d'Ophtal.* No. 124, Fasc. 1.

LEIBOWITZ H.M. & ELLIOTT J.H. (1965) Antibody production in corneal hypersensitivity. *Arch. Ophth.* **73,** 687.

MANSCHOT W.A. (1960) The eye in relation to collagen diseases. *Trans. Ophth. Soc. U.K.* **80,** 137.

MAUMENEE A.E. (1960) The immune reaction to corneal homografts. In *The Transparency of the Cornea,* ed. DUKE-ELDER S. Oxford: Blackwell Scientific Publications.

MERWIN R.M. & HILL E.L. (1954) Fate of vascularized and nonvascularized subcutaneous homografts in mice. *J. Nat. Cancer Inst.* **14,** 819.

MILLER R.K. & SMERZ A. (1956) Bilateral posterior uveitis complicating a positive tuberculin cutaneous reaction. *Arch. Ophth.* **56,** 896.

MÜLLER H. (1952) Tierexperimentelle Untersuchungen zur Ophthalmia phakogenetika. *v. Graefe's Arch. Ophth.* **153,** 1.

OAKLEY C.L., BATTY I. & WARRACK G.H. (1955) Antibody production in the rabbit cornea. *J. Path. Bact.* **70,** 349.

OGUCHI M. (1954) Allergic reactions of skin and conjunctiva with pollens in vernal catarrh. *Acta Soc. Ophth. Japan* **58,** 735 (cited in THEODORE & SCHLOSSMAN (1958)).

PAYRAU P., POULIQUEN Y. & FAURE J.P. (1961) Hétérogreffes de la cornée: étude expérimentale et premiers résultats cliniques. *Ann. Oculist* **194,** 1.

POLACK F.M., SMELSER G.K. & ROSE J. (1964) Long-term survival of isotopically labeled stromal and endothelial cells in corneal homografts. *Amer. J. Ophth.* **57,** 67.

RICH A.R. & FOLLIS R.H. (1940) Studies on the site of sensitivity in the Arthus phenomenon. *Bull. Johns Hopk. Hosp.* **66,** 106.

RIEHM W. (1928) Über experimentelle anaphylaktische Keratoconjunctivitis am Kaninchenauge. *Arch. Augenheilk.* **99,** 438.

RODENHÄUSER J.H. (1959) Über die Resorption und Verteilung Isotopen markierte Allergene im Auge normergische Kaninchen. *v. Graefe's Arch. Ophth.* **161,** 45.

ROWE A.H. (1935) Food allergy: a common problem in practice. *Sth. Med. J.* **28,** 261.

SCHWAB F. (1959) Untersuchungen über den Antikörpergehalt (Präcipitine) der Hornhaut. *v. Graefe's Arch. Ophth.* **160,** 592.

SÉDAN J. & GUILLOT P. (1955) Uvéites médicamenteuses. *Bull. Soc. franç. Ophtal.* **68,** 317.

SEEGAL B.C., SEEGAL D. & KHORAZO D. (1933) Local organ hypersensitivity. V. The fate of antigens and the appearance of antibodies during the development of hypersensitivity in the rabbit eye. *J. Immunol.* **25,** 207 (and earlier papers).

SILVERSTEIN A.M. (1963) Effect of X-irradiation on the development of immunogenic uveitis. *Invest. Ophthal.* **2,** 58.

SILVERSTEIN A.M. (1964) Ectopic antibody formation within the eye: pathologic implications. In *Immunopathology of Uveitis,* p. 83, eds. MAUMENEE A.E. & SILVERSTEIN A.M. Baltimore: Williams & Wilkins.

SILVERSTEIN A.M., WELTER S. & ZIMMERMAN L.E. (1960) Studies on experimental ocular hypersensitivity to simple chemicals. *Amer. J. Ophth.* **50,** 937.

SILVERSTEIN A.M., WELTER S. & ZIMMERMAN L.E. (1961) A progressive immunization reaction in the actively sensitized rabbit eye. *J. Immunol.* **86,** 312.

SILVERSTEIN A.M. & ZIMMERMAN L.E. (1959) Immunogenic endophthalmitis produced in the guinea-pig by different pathogenetic mechanisms. *Amer. J. Ophth.* **48,** 435.

SORSBY A.P. (1942) The aetiology of phlyctenular ophthalmia. *Brit. J. Ophth.* **26,** 159, 189.

TAYLOR F.W. & SUIE T. (1956) Effect of trauma on the production of experimental uveitis. *Amer. J. Ophth.* **41,** 299.

THEODORE F.H. & LEWSON A.C. (1939) Bilateral iritis complicating serum sickness. *Arch. Ophth.* **21,** 828.

THEODORE F.H. & SCHLOSSMAN A. (1958) *Ocular Allergy.* Baltimore: Williams & Wilkins.

UHLENHUTH P.T. (1903) Zur Lehre von der Unterscheidung verscheidener Eiweissarten mit Hilfe spezifischer Sera. *Festschr. zum 60 Geburtstag von Robert Koch*, pp. 49–74. Jena: Fischer.

VERHOEFF F.H. & LEMOINE A.N. (1922) Endophthalmitis phacoanaphylactica. *Int. Congr. Ophth. Washington*, p. 234.

VON SZILY A. (1914) *Die Anaphylaxie in der Augenheilkunde*. Stuttgart: Ferdinand Enke.

WAKSMAN B.H. (1959) *Experimental Allergic Encephalomyelitis and the 'Auto-allergic Diseases'*. Basel: Karger.

WAKSMAN B.H. & BULLINGTON S.J. (1956) A quantitative study of the passive Arthus reaction in the rabbit eye. *J. Immunol.* **76,** 441.

WAKSMAN B.H. & BULLINGTON S.J. (1960) Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. III. Lesions of the eye. *Arch. Ophth.* **64,** 751.

WEEKERS L. (1909) L'exanthème de la conjonctivite phlycténulaire envisagé comme une toxi-tuberculide. *Arch. Ophthal.* **29,** 294.

WEIZENBLATT S. (1949) Allergic ocular reactions to the tuberculin test: bilateral cyclitis and neuroretinitis. *Arch. Ophth.* **41,** 436.

WISE G.N. (1952) Ocular periarteritis nodosa. *Arch. Ophth.* **48,** 1.

WITMER R. (1955) Antibody formation in the rabbit eye studied with fluorescein-labeled antibody. *Arch. Ophth.* **53,** 811.

WITMER R. (1964) Origin of ocular antibodies. In *Immunopathology of Uveitis*, p. 111, eds. MAUMENEE A.E. & SILVERSTEIN A.M. Baltimore: Williams & Wilkins.

WOLKOWICZ M.I., HALLET J.W. & LEOPOLD I.H. (1960) Studies on antibody production in the rabbit eye. I. Antibody formation *in vitro* after intraocular injection of typhoid bacilli. *Amer. J. Ophth.* **50,** 126.

WOODRUFF M.F.A. (1957) Cellular and humoral factors in the immunity to skin homografts. *Ann. N.Y. Acad. Sci.* **64,** 1014.

WOODS A.C. (1921) Immune reactions following injuries to the uveal tract. *J. Amer. Med. Assoc.* **77,** 1317.

WOODS A.C. (1937) Clinical problems of allergy in relation to conjunctivitis and iritis. *Arch. Ophth.* **17,** 1.

WOODS A.C. (1949) The diagnosis and treatment of ocular allergy. *Amer. J. Ophth.* **32,** 1457.

WOODS A.C. (1961) *Endogenous Inflammations of the Uveal Tract*. Baltimore: Williams & Wilkins.

WOODS A.C., FRIEDENWALD J.S. & WOOD R.M. (1955) The histopathology of acute and chronic ocular hypersensitivity reactions in the experimental rabbit. *Amer. J. Ophth.* **40,** 631.

WOODS A.C., JACOBS L., WOOD R.M. & COOK M.K. (1954) A study of the role of toxoplasmosis in adult chorioretinitis. *Amer. J. Ophth.* **37,** 163.

ZIMMERMAN L.E. & SILVERSTEIN A.M. (1959) Experimental ocular hypersensitivity. Histopathologic observations. *Amer. J. Ophth.* **48,** 447.

SECTION V

IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY

CHAPTER 44

PROPHYLAXIS OF MICROBIAL DISEASES

C.L. OAKLEY

NATURALLY ACQUIRED IMMUNITY

Recovery from infectious disease caused by a particular bacterium or virus very commonly carries with it a decreased susceptibility to subsequent attack by the same organism. This relative insusceptibility, usually called immunity, may be partial, in which case exposure to the organism is followed by disease less, or far less, severe than that suffered by inexperienced persons similarly exposed, or complete, in which apparently adequate exposure provokes no symptoms whatever. In many, perhaps most, infectious diseases this immunity depends on the production by the infected host of antibodies—protein carrier substances

directed fairly specifically against the antigens of the infecting organisms and capable of combining with these antigens, and particularly with the soluble antigens with which the infecting organism damages its host (see Chapters 15 and 16).

If antibody production occurred only during infection, it would be of little practical importance; the antibody response to first experience of an antigen is small, and more important, slow (Glenny & Sudmersen 1921; Burnet & Fenner 1949). Since antibodies are globulins, they are destroyed at the rate normal for these proteins, and would therefore, once the disease was over, fall fairly soon to ineffective concentrations were it nor for two things—that antibody synthesis, once started, apparently continues in the absence of fresh antigen (Barr & Glenny 1949a), and that experience of an antigen almost always conditions the antibody-producing mechanism to produce antibody faster and in higher concentration when it is again exposed to the same antigen, even when the interval between the first and subsequent exposures is considerable (Glenny & Sudmersen 1921; Burnet & Fenner 1949; Barr & Sachs 1955; Boyd 1960; Evans 1965). Taken together these provide a reasonable, though probably partial, explanation of acquired immunity to infectious disease.

This improvement with experience in capacity to deal with infecting organisms has obvious advantages, but it does not always occur—it may even be prevented by the measures taken to treat the infection—and even when it does, the immunity dependent on it may be obtained at the expense of a prolonged and debilitating illness, and the benefit to some individuals exposed to the disease may be offset, from the community's point of view, by the deaths of others less fortunate.

Immunity Due to Subclinical Infection

Far luckier are those who develop immunity as a result of subclinical infection, in which, possibly because the infecting dose of organisms is small, or for other imperfectly understood reasons, attack by an organism produces immunity but no symptoms. Subclinical infections are also probably the means by which immunity to some diseases is maintained in the population; thus in measles, an exceedingly common disease in this country, the virus is probably almost always available to settle in the respiratory tract of persons rendered immune by previous experience of the disease, and so to reinforce their immunity by symptomless infection.

Quantitative Character of Immunity

Acquired immunity, because it depends in part at least on antibody, is for that reason quantitative rather than absolute; in theory we could always find a dose of bacteria or virus that would still produce the disease, however high the anti-

body concentration in the host, or in the cases where immunity is mainly antitoxic, we could always find a dose of toxin sufficient to kill the animal, however high its antitoxin concentration. In practice, however, we shall be satisfied if the 'immune' person is satisfactorily protected against the infective risks of an ordinary existence.

Defects of Naturally Acquired Immunity

Even this degree of protection, however, may not be obtainable from natural infection. Diseases vary enormously in the degree of immunity they evoke in patients who have recovered from them. In measles, for instance, immunity in the recovered patient is solid and lifelong; only in the aged, possibly because of breakdown of the whole antibody-producing mechanism, are second attacks of measles likely to be experienced, and even in them the chance is extremely small. By comparison, tetanus (Barr & Sachs 1955) and the common cold confer a most disappointing degree of immunity. In tetanus the determining factor is probably the very small amount of antigen to which the patient is exposed, and the correspondingly trifling antigenic stimulation; the amounts of tetanus toxin necessary for good antibody production would almost certainly be fatal to the inexperienced patient. In other cases the antigens may be only poorly antigenic, so that even though large amounts of them are produced in the patient, little antibody is synthesized in consequence; but far more often failure to develop immunity is more apparent than real. Antibodies usually show some spread of specificity in the antigens with which they will combine, but this range is ordinarily very narrow, so that any considerable change in the antigenic structure of an organism may render antibodies against the unmodified form ineffective against the modified one.

Influenza virus is an organism of remarkable antigenic plasticity, showing considerable variation of some of its antigens over very short periods of time. In many instances the antigenic change is insufficient to make much difference to the effectiveness of the antibody previously acquired or synthesized during the attack; but sometimes, as in the recent epidemic of 'Asian' influenza, the change was so great that no evidence of immunity due to previous attacks of influenza was demonstrable (Editorial 1957).

The dependence of immunizing power on antigenic correspondence between the strains used to produce vaccines and those currently producing infection is very well shown in whooping-cough outbreaks. Vaccines made from strains of the type whose antigens were 1,2 were so satisfactory that infection by these strains almost disappeared; but these vaccines gave only poor protection against strains whose antigens were 1,3 or 1,2,3 (Manchester Public Health Laboratory 1965; Preston 1966).

Moreover, antigenic change in an organism may affect only few antigens,

leaving the others unaffected. As we shall see later when we consider combined prophylactics, the presence in an antigenic mixture of antigens to which the patient has already reacted may greatly depress his response to those in the mixture of which he has had no previous experience; so that a limited but important antigenic change in an infecting organism may in this indirect way reduce the antibody response to an essential antigen.

Naturally acquired immunity, excellent as it is in many respects, has therefore considerable defects; it may involve illness, perhaps death in the less fortunate patient; it may, from one cause or another, never develop at all; and it may be too sharply specific to be of great future value when it does. Obviously our aim should be to acquire immunity without suffering from serious disease, preferably indeed without discomfort, and to so arrange the specificity of the immune response that it will be practically effective under the kind of conditions that may reasonably be expected.

PROPHYLAXIS

Exactly how we attempt to acquire or provide immunity will depend on the nature of the diseases with which we are concerned, the risk of suffering from them, the state of the person requiring immunization, and the time and money at our disposal. We could, as we do in many virus diseases, attempt to produce a mild attack of the disease, from which the patient would infallibly recover, endowed thereby with a solid immunity; but in bacterial diseases this is rarely practical politics, and what we try to do is to provide the patient with experience of the antigens of the infecting organism without subjecting him to the symptoms of infection, so that he can, in a subclinical infection, produce antibodies against them without risk. We then have to decide what antibodies are necessary.

Nature of the Disease

It is convenient to divide infectious diseases into two groups: (1) those in which the local infective lesion is of little more than mechanical importance compared with the damage produced at a distance by soluble poisons—toxins—secreted by the infecting organism, and (2) those in which the presence of the organism is essential, in the untreated disease at least, for the production of lesions. The first group may be called toxin-dependent diseases, and include diphtheria, tetanus, botulism and scarlet fever in man, lamb dysentery, infectious enterotoxaemia of sheep and goats and infectious icterohaemoglobinuria of cattle; the second, the organism-dependent group, includes enteric infections, tuberculosis, plague, cholera, whooping cough and diseases due to viruses. The distinction is far from absolute, and some diseases might fit into either group; obviously, apart from the peculiar case of virus diseases, any damage done to tissues by

organisms must be due to toxic substances secreted by them or produced by their autolysis, so that in a sense all infectious diseases are toxin-dependent, but in most organism-dependent diseases these toxic substances are either absorbed locally or secreted in too low concentrations to produce any effect at a distance from the infecting organisms.

Toxin-dependent Diseases

Botulism

It is perhaps a matter of semantics to determine whether botulism is an infective disease or not; true infections with *Clostridium botulinum* rarely occur (Davis, Mattman & Wiley 1951; Thomas, Keleher & McKee 1951), and the great majority of cases of botulism are due to ingestion of the preformed toxin, produced by *Cl. botulinum* during the cooling stage of preservation of infected foods that have been heated to a temperature inadequate to kill the heat-resistant spores of the organism. The presence of the organism is not, therefore, essential to the development of the flaccid paralysis of botulism, and all that is necessary for immunization is to protect against the toxin.

Tetanus

This disease can be produced by injection into animals of bacteria-free filtrates of *Cl. tetani*, but under natural conditions infection with the organism is essential. Spores are introduced into wounds with the soil in which *Cl. tetani* normally survives, and if sufficient damage is done for the eH of the tissues to fall to 0.01 volts at pH7 these spores will germinate and grow (Fildes 1929). Growth of *Cl. tetani* is entirely local, except that the organisms may occasionally be carried to other tissues, particularly the lungs, in detached clots, but the main manifestations of the disease are the tonic convulsions due to the effect of the toxin formed in the wound on the nerve cells in the anterior horn of the spinal cord (Wright, Morgan & Wright 1950; Baylis, Mackintosh, Morgan & Wright 1952). Some antibody may be necessary for killing, or assisting to kill, organisms in the wound area, but only antitoxin will neutralize locally formed toxin and prevent it from reaching the nervous system.

Diphtheria

Here both local infection and distant symptoms are due to toxin elaborated by the organism. When *Corynebacterium diphtheriae* settles in the pharynx or nose or larynx or in a wound in the skin, it must grow on the surface and secrete toxin before any macroscopic lesion is produced (Amies 1954). When sufficient toxin has been secreted, it damages tissue to such an extent as to cause the production of the characteristic 'membrane', and the organism grows in the damaged tissue and produces more toxin, which as it spreads through the body causes myocardial degeneration, paralysis and adrenal changes. The local lesion may be

mechanically important, but the distant toxic lesions are those that lead to death. To give protection antitoxin is essential, though we cannot be certain that it is the only antibody we need.

Scarlet fever

Here the local lesion is the streptococcal sore throat, with the peripheral rash due to the erythrogenic toxin of the scarlet fever streptococcus. Scarlet fever is no longer the serious disease it used to be, and now responds very well to chemotherapy, but there is some support for the view that the possession of antitoxin by the patient is of value (Wilson & Miles 1955a).

Lamb dysentery

This disease, together with the previous one, may perhaps be regarded as a bridge to the organism-dependent diseases. It is an affection of newborn lambs, occurring mostly in the first few days of life, hardly ever after the first week. *Clostridium welchii* type B is picked up by the lamb from the infected fleece of the ewes, passes through the stomach of the lamb, which possesses little effective pepsin or hydrochloric acid, and produces a haemorrhagic enteritis, with local punched-out ulcers in the intestinal mucosa. These ulcers are due to *Cl. welchii* β-toxin, but antitoxin against this antigen does not confer complete protection against the disease; antitoxin against the ε-toxin of *Cl. welchii* is also necessary, but as even mixtures of these two antitoxins derived from *Cl. welchii* types C and D do not afford complete protection, it is clear that some other antibody, possibly antibacterial, is essential as well as the antitoxins.

ORGANISM-DEPENDENT DISEASES

Much less is known about the antigens responsible for the lesions provoked by organisms such as *Salmonella typhi*, *Bordetella pertussis*, *Pasteurella pestis*, *Vibrio cholerae* and *Mycobacterium tuberculosis*, except that in all cases the effective antigens are probably associated with the organism, and require fairly vigorous procedures or autolysis for their extraction. If we knew what the effective antigen was, we might hope to use it to immunize animals or men, and some progress has been made, e.g. with *Pasteurella pestis* (Crumpton & Davies 1956), but in the majority of cases our ignorance is such that we have to obtain antibodies against the whole organism in order to include the antibodies we really need for protection.

ON ACTIVE IMMUNIZATION AGAINST TOXIN-DEPENDENT DISEASES

If the effective organism is isolated in pure culture from the infected area or any other convenient source, it can then usually be grown in artificial media so as to

produce adequate yields of toxin. In theory persons who needed to be immunized against the toxin could receive injections of minute amounts of toxin, but this process is so dangerous that the method has been completely abandoned and modified toxins are now universally employed. By great good fortune, most bacterial toxins, when they are treated with appropriate concentrations of formaldehyde, lose their toxicity but retain their antigenicity; more important, the antibodies to the toxin so modified (called toxoid in Great Britain, but anatoxine in France) combine not only with toxoid but also with toxin, neutralizing in the process all its poisonous activities (Glenny & Sudmersen 1921; Glenny & Hopkins 1923). Injections of toxoid into man or animals will then provoke the production of antitoxins, which in pure toxin-dependent diseases will provide adequate immunity. Moreover, the use of toxoids makes it possible to inject far greater amounts of antigen than would be possible if toxins were used, so that, in tetanus, for example, injection of tetanus toxoid may provoke higher antitoxin concentrations than are ever produced in the course of the natural disease.

Further Modifications of Bacterial Toxoids

One difficulty in the effective use of some bacterial toxoids is their relatively low molecular weight; as a consequence they are readily excreted in the urine, and may remain in the body for a time inadequate to provoke effective antibody synthesis. The period over which they are effective as antigens may be greatly increased by adsorbing them on insoluble carriers, such as aluminium hydroxide or phosphate or on mixtures of the two (Glenny 1930), or on killed bacteria, as in some combined prophylactics. This adsorption on insoluble carriers greatly increases the antibody response to a constant dose of toxoid, and at the same time tends to iron out the differences between different batches of the same toxoid. It is also possible that adsorbed toxoids provoke a more vigorous histiocyte reaction than unadsorbed toxoids do.

Another perhaps less obvious form of carrier for toxoid is antitoxin; mixtures of toxoid and antitoxin in appropriate concentrations yield precipitates of toxoid-antitoxin 'floccules' (TAF) which if 'underneutralized' (i.e. if they contain less antitoxin than would neutralize the toxin from which their toxoid was produced) are excellent antigens, particularly if we require a partially purified antigen (Glenny & Pope 1927).

Purification of antigens has been much to the fore lately, partly because of improved techniques that make the necessary processes easier, and partly because of the feeling that the 'reactions' that occasionally follow injections of toxoid are due to non-toxoid impurities. This attitude is to a large extent justified, and modern methods of purification of diphtheria toxoid for example have certainly reduced the incidence of reactions, but two points must be made. First, some

persons become sensitive even to the purest toxoids available (Agusstion & Toomey 1948), so there may be no great gain in extreme degrees of purification of toxoid; second, we can never be sure that the only effective antigen in a formolized filtrate is the toxoid—other substances may be important. Indeed, in their report to the Medical Research Council, Evans *et al* (1962) have shown that highly purified diphtheria formol toxoid is a very poor antigen for primary immunization in children.

Factors Affecting the Antitoxin Response to Toxoids

The antitoxin response to toxoids is affected by (1) the capacity of the patient to respond, (2) the immune state of the patient, (3) the efficiency of the prophylactic itself, (4) the size of the first dose, (5) the intervals between doses and (6) the presence of other antigens in the prophylactic.

The Capacity of the Patient to Respond

When large numbers of persons or animals of the same age and general characteristics are given the same doses of the same preparation of toxoid, the antitoxin responses are not uniform; they usually lie over a log normal curve, i.e. a curve in which the frequencies of particular antitoxin concentrations are more or less 'normal' or Gaussian when they are plotted against the logarithm of those concentrations. However high the geometric mean antitoxin concentration in such a population was, we could, if we made the population large enough, find a few who had no detectable antitoxin at all, and these would require further injections to immunize them. Most of them would respond.

Failure of synthesis of antibody

There are, however, a few unfortunate persons who are apparently unable to synthesize more than traces of γ-globulin, and are therefore unable to produce useful amounts of antibody (see Chapter 12). Such persons, usually said to be agammaglobulinaemic or hypogammaglobulinaemic, are particularly liable to suffer from repeated severe bacterial infections to which they seldom develop any active immunity (Bruton 1952). For them only passive immunization or treatment of the infections is likely to be of much value.

The Immune State of the Patient

Obviously a patient with some basic immunity developed during an illness or as a result of previous immunization will, if his serum antitoxin concentration is sufficiently low, respond very much better to injection of the toxoid than an inexperienced patient. It is, however, possible for high serum antitoxin concentrations to interfere with the response to injected toxoid, presumably by overneutralizing it. This may occur in two situations.

Pre-existing active or passive immunity

In the first, the patient has a high circulating antitoxin concentration produced by himself; injections of toxoid into such a patient may lead to a very small rise in circulating antibody, or to none at all. This, however, is of very little practical moment compared with the interference with active immunity produced by passive immunization. We shall deal with passive prophylaxis later; all that it is necessary to say here is that it is produced by injecting into a patient antibody produced in another person or in another species.

A patient possessing antitoxin so obtained may show a poor active antitoxin response either to infection with the organism producing the corresponding toxin or to injection of toxoid; effective active immunization cannot be produced until the concentration of passively acquired antitoxin has fallen to a sufficiently low level. The exact time required for this is disputed. With homologous serum it is probably about 6 weeks, though this will depend on the highest serum antitoxin concentration reached in the recipient; with heterologous antitoxin, i.e. that derived from another species, this period is shorter, and is likely to be determined rather by administrative compromise than by scientific reasoning. It should be emphasized, however, that any patient immunized passively against a particular infection should be actively immunized afterwards; and this is particularly true when heterologous antitoxin has been employed.

It will be clear from this that combined active and passive immunization against the same antigen is unlikely to be successful, at least when plain toxoid is used. Recently, however, claims have been made by Smith *et al* (1963) that almost all patients given tetanus antitoxin in one site and adsorbed tetanus toxoid in another, followed by a second injection of adsorbed toxoid, show an excellent active antitoxin response.

Arrangements in foetuses and newborn children

The second situation is that shown by the foetus of an immune mother. In many animals (horse, pig, sheep, cattle) the placenta is impervious to serum globulin, and the young are consequently born without circulating antibodies (Mason, Dalling & Gordon 1930). These they subsequently acquire with the colostrum, in which antibodies are present in high concentration; as the young have little or no effective digestive enzymes for a short period after birth, they are able to absorb antibodies from the alimentary canal, which is for this period permeable to protein (Mason *et al* 1930).

In man and many other animals the position is different: the placenta is freely permeable to protein, and the foetus therefore receives antibody derived passively from its mother. Moreover, the foetus is apparently able to concentrate antibody so obtained, so that as pregnancy advances the foetal serum concen-

tration of, for example, diphtheria antitoxin may be much higher than that in the mother (Barr, Glenny & Randall 1950).

If the mother possesses high concentrations of antitoxin, their transfer to the foetus may significantly reduce the response of the newborn or very young child to the corresponding antigens (Barr *et al* 1950); but as this passively acquired antitoxin is lost at the normal rate for human γ-globulin and is not replaced, we need only wait for a reasonable time for the concentration of this antitoxin to fall below that inhibiting the active response. This time is about 6 weeks; for children with the higher initial antitoxin concentrations it will be a little more, for those with low ones, rather less (Barr *et al* 1950).

This is the reason why official immunization schemes (Parish *et al* 1960) provide immunization against diphtheria and tetanus starting with a first injection 2–6 months after birth. No doubt some children would respond if the antigens were injected a little earlier, but it is obviously administratively more convenient to commence immunization at the same time for all children, even if a few are left exposed to a rather small risk in consequence.

Tolerance

Little is known about the phenomenon of tolerance of antigens in man, but the development of blood group chimaeras (Dunsford, Bowley, Hutchison & Thompson 1953; Woodruff & Lennox 1959) is sufficient evidence that it can occur. Briefly, an animal may become tolerant to an antigen, i.e. to a substance to which the great majority of animals of the same species would produce antibody, when it has experience of it at a time at which it does not 'recognize' it as foreign. This is particularly liable to occur when the animal is still setting up systems for synthesizing normal body constituents, and particularly, therefore, the enzymes necessary for their construction and breakdown. The main period for this is foetal and early neonatal life, and structures developed after this, e.g. myelin, spermatozoa (King 1955), are normally, to avoid production of antibody against them, segregated from antibody-producing cells. Experience of a normally antigenic substance during this period may therefore cause it to be regarded as a normal body constituent (possibly because an enzyme system is developed for its destruction), and not only will no antibody be produced against it, but in some way subsequent injection of the same antigen, at any rate within short periods, evokes no antibody response (Billingham, Brent & Medawar 1953).

Thus pure-bred mice of one strain, which normally reject grafts of skin from pure-bred mice of another strain, will accept such grafts if they have during late foetal or early post-natal life received transfusion of blood from mice of the other strain: they have become tolerant to the antigens of the grafts (Billingham *et al* 1953; see also Chapter 17).

In some animals (Owen 1945) and rarely in man (Dunsford *et al* 1953;

Woodruff & Lennox 1959), twin non-identical foetuses may have some vascular connections which may allow transfusion and even implantation of bone marrow from one foetus to another. Such foetuses may possess red cells of two different blood groups, one set their own, the other those derived from the implant of bone marrow from their twin. Normally red cells of one group (except group O) are antigenic in a person of another group; in these blood group chimaeras the recipient of the transfusion has become tolerant.

Most examples of tolerance are derived from cases in which the antigenic material is cellular and therefore self-reproducing; there are, however, a few cases in which tolerance to non-cellular antigens has been established. Only one example of partial tolerance to a toxoid is known, and in this the response of adult chickens to a primary stimulus with diphtheria alum-precipitated toxoid was shown to be delayed if diphtheria APT had been injected into the yolk sac of the developing egg. The primary response to tetanus APT, of which the chickens had had no previous experience, was unaffected, and the secondary response to both antigens was normal in control and in experimental birds (Gowland & Oakley 1960).

It seems likely, therefore, that any tolerance to toxoids developed in man as a result of injection soon after birth would be very slight; but it might add an additional justification to those previously given for delaying the first injection of an immunizing series for 2 months or so after birth.

The Efficiency of the Prophylactic

In the early days of diphtheria prophylaxis, many of the prophylactics used were of poor and irregular quality, and though they passed the legal test for antigenicity then in force, provided poor and uncertain immunization (Fulton, *et al* 1942; Barr & Glenny 1949b). Since that time the legal tests have been greatly strengthened, and few prophylactics of poor quality are produced. It is evident that up to a point increase in the amount of antigen present will improve the response to it, and the regulations for most toxoids require a specified final concentration of toxoid in the product.

Other things being equal, a 'carrier' prophylactic, i.e. adsorbed on material relatively insoluble in tissue fluid, is more effective than an unadsorbed prophylactic. What determines whether a prophylactic is efficient or not may not depend entirely on its capacity to provoke high-level antibody production: carrier prophylactics may be regarded as less efficient from the community's point of view because they provoke 'reactions', because they may on rare occasions lead to 'provocation poliomyelitis' or because they do not lead to a satisfactory response after a single injection. We may deal with the last point here.

From an administrator's point of view it is extremely inconvenient that most children, and many adults for that matter, dislike hypodermic injections, even

when they are done for their good. Defaulting is therefore a great nuisance in immunization schemes and though, for very good reasons, 'one-shot' immunization is regarded as unsatisfactory, some people running such schemes tend to regard a prophylactic that will give reasonable immunity after one injection as preferable to one that gives very little. If anyone defaults after the first injection, such a prophylactic will give him better protection than one that requires two injections for the provision of solid immunity. This was really the main argument in favour of Holt's purified toxoid aluminium phosphate precipitated (PTAP) as compared with the usual diphtheria alum-precipitated toxoid (APT): it provoked a better response to a single injection (Holt 1950).

The Size of the First Dose of Toxoid

The size of the dose is not necessarily the volume, but rather the amount of active antigen it contains. Experimental work on animals and some on babies suggests that there is a minimum size for the dose of prophylactic used for the primary stimulus before all (or almost all) the persons given a secondary stimulus give their maximum response (Barr & Glenny 1947; Barr & Llewellyn-Jones 1951). Earlier it had been supposed, though there was little evidence to support it, that very small amounts of antigen would give adequate primary stimulation if the interval between primary and secondary stimulation was sufficient. The main argument against the use of very large doses of antigen as a primary stimulus is the tendency they have to provoke the production of non-avid antitoxin (Barr & Glenny 1931).

The Interval between Doses

Repetition of injections of a prophylactic has two objects: to lay down basic immunity and to maintain a reasonable concentration of circulating antibody. These may usefully be dealt with separately. Injection of an effective dose of diphtheria toxoid leads to a characteristic response: no diphtheria antitoxin is detectable in the serum of the animal injected for a rather variable period, usually for about 3 weeks; the serum antitoxin concentration then rises slowly to a maximum over about 3 weeks, and then slowly falls. This is the *primary response* (Glenny & Sudmersen 1921).

If another injection of diphtheria toxoid is given a month or more after the first, a wholly different response is obtained. At first there is, if the animal when injected possessed some detectable circulating antitoxin, a slight fall in its concentration—the negative phase: by about 3 days this concentration shows a rise, and it increases rapidly to a maximum about 10 days after injection, after which it declines relatively rapidly, though seldom to a level below that at which the secondary stimulus was given (Glenny & Sudmersen 1921).

Though this response is subject to a good deal of variation from animal to

animal, it always differs sufficiently from that to a primary stimulus to justify the use of the term *secondary response*. Not, be it noted, *second* response; the response to all antigenic stimuli after an efficient first stimulus is, with one exception, secondary in character, though the time-scales for different antigens may be different. The one exception is the response to rickettsial antigens, which do not seem to differ whether the stimulus is a primary or subsequent one (Burnet & Fenner 1949).

Thus the response to the primary stimulus consists not only of the production of antibody, but the alteration of the antibody-producing mechanism so that this will, on subsequent experience of the same antigen, produce antibody faster and in higher concentration than it did after its first experience. The actual concentration of antibody produced after primary stimulation is, however, small and subsequent injections are essential to raise it to a level that will, allowing for natural loss and replacement of antibody, give reasonable immediate protection over a long period. The word 'subsequent' necessarily involves consideration of the time between the stimuli. It seems clear that the shorter the period, the poorer the response to the secondary stimulus, and the more likely is non-avid antitoxin to be produced, i.e. antitoxin that is readily dissociated from toxin by dilution.

Recommendations for practice

For diphtheria toxoid with or without carrier, 1 month between the first and second injections is usually recommended: if no carrier is added three injections should be given at intervals of a month. For tetanus toxoid three injections are usually given, with 6 weeks between the first and second, and 6 months between the second and third (Barr & Sachs 1955; Boyd 1960).

In both cases these injections will provide a sound basic immunity, and all we have to do is to keep the antitoxin concentration at a reasonable level by subsequent injections, i.e. by 'booster' doses of antigen. For protection against diphtheria, a booster dose of toxoid is usually given at school entry and at 8 or 9 years, mainly to keep up the serum antitoxin level, and similar boosters are given for protection against tetanus (Boyd 1960).

Provision for booster doses of diphtheria prophylactic in immunization schemes is probably based on the unexpressed belief that now diphtheria is uncommon the chance of receiving a boosting dose of toxin from organisms picked up from carriers or cases of diphtheria is very small, and that artificial means must be substituted; it is difficult to come to any conclusion on the available evidence. The chance of such inapparent infection in tetanus is very small, and practice has usually depended on the degree of risk. It has been suggested, for instance, that soldiers, gardeners, farmers and others particularly exposed to the risk of tetanus might be given tetanus toxoid every 5 years (Barr & Sachs 1955), whilst others might be satisfied with the basic course of three injections,

and might be given toxoid only when they suffered a wound likely to give rise to tetanus.

In general the response to tetanus toxoid many years after the basic immunization is excellent, and the recommendation for persons not normally exposed to risk is probably correct. The only difficulty is that few persons are not at risk nowadays, and in consequence some have suggested that the proposed practice for soldiers, gardeners and farmers might be extended to all civilians. In so uncommon a disease as tetanus it is not easy to come to any justifiable conclusion: my own feeling, for what it is worth, is that the only groups for which repeated boosters are really necessary are the armed forces and perhaps footballers. (For a rather different view, see Parish & Cannon 1962, and the extensive associated correspondence.)

Effect of Other Antigens in the Prophylactic

Direct interference

It might reasonably be expected—especially if Burnet's clonal selection theory (Burnet 1959) was sound—that mixtures of two or more antigens injected simultaneously into an inexperienced animal would provoke the same response as if each antigen was injected into a separate inexperienced animal. In fact it is found that the response to mixtures of antigens depends on their composition (Barr & Llewellyn-Jones 1953a). Thus if we take a constant amount of diphtheria APT and add to it a range of amounts of tetanus APT, adjust each mixture to the same volume and inject it into a guinea-pig as primary and later secondary stimulus, we find that beyond a certain amount of added tetanus APT the secondary response to tetanus APT steadily rises. This is highly satisfactory; what is not so satisafctory is that the response to diphtheria APT falls. Evidently the composition of a combined prophylactic must be very carefully adjusted to give the optimum result to all antigens, and to minimize interference between them.

Indirect interference

So far we have dealt with the response to combined prophylactics in the inexperienced animal. A further complication is introduced if the animal possesses antibody to one of the antigenic components of the mixture. It would be expected that if an animal possessed actively acquired antibody to tetanus toxoid, but none to diphtheria toxoid, injection of a mixture of diphtheria and tetanus toxoids would provoke a primary response to diphtheria and a secondary response to tetanus toxoid; what is much less to be expected is that though the secondary response to tetanus toxoid is elicited, the primary response to diphtheria toxoid is greatly reduced or even totally abolished (Barr & Llewellyn-Jones 1953b). Moreover, a further injection of the combined prophylactic after a month's interval, though it yields a good response to tetanus toxoid, provokes

a very much reduced secondary response to diphtheria toxoid. Only after a third injection of the combined prophylactic are the expected maximal secondary responses to both antigens obtained. Nor is it necessary for both components to be toxoids; animals immunized with killed bacterial vaccines—e.g. typhoid vaccine—and subsequently given a mixture of typhoid vaccine and tetanus toxoid—events that might very well happen in practice to man—show a much reduced response to tetanus toxoid (Barr & Llewellyn-Jones 1953b).

Clearly if combined prophylactics are to be used—and the administrative and practical arguments are all in their favour—it is essential that they should be used so that each component is given an equal chance. A child who has previously been given diphtheria toxoid alone should not be given diphtheria and tetanus toxoids with pertussis vaccine subsequently; it would be much better to complete his basic diphtheria immunization, and then give him tetanus toxoid and pertussis vaccine together.

In all immunizing processes on any scale, recommendations like these are ideal; they may not be easily practicable, and we may have to put up with suboptimal immunizing schedules so that everyone may benefit; but it is a good thing to know what the optimum arrangement is.

IMMUNIZATION AGAINST ORGANISM-DEPENDENT DISEASES

This will depend, as in most instances we have no idea what the antigens determining pathogenicity are, on exposing the animal or person to be immunized to as much of the antigenic material of the infecting organism as is possible without producing a severe infection or a severe reaction. For this the immunizing material may be live organism or dead vaccine.

Immunization with Living Organisms

What we have to do is to so modify the antigenic structure of the organism that antigens responsible for the production of extensive infections are so changed that the organism can give rise to a self-limiting infection only, but still immunize against the fully virulent organism. In the outstanding example of this, in protection against tuberculosis with BCG (Bacille Calmette-Guérin), Calmette and Guérin exposed a bovine strain of the tubercle bacillus to repeated culture in an unfavourable but not a fatal environment—a medium containing glycerol, potato extract and bile salts—in the expectation that the organism would be so hard put to it to survive in these conditions that it would abandon the complex machinery necessary for vigorous pathogenicity. Their expectation was realized, for the organism in time lost its capacity to produce a generalized infection in guinea-pigs, and produced only a limited infection; in man the infection that normally follows intradermal injection of a BCG suspension consists of a local

lesion, with swelling, sometimes ulceration, and final healing of a tuberculoma of skin, often with involvement of the draining lymphatic glands. About 6 weeks after injection of the organisms originally tuberculin-negative persons become tuberculin-positive, so that some degree of cross-reaction with the products of tubercle bacilli is evident. The original immunizing experiments in France were carried out in a period when proper recording and statistical examination of results were little understood. In consequence, though millions of children were immunized, little information is available about the efficacy of the process. Indeed, it was not until quite recently that proper trials were carried out in Europe; these make it clear that inoculation with BCG does provide some protection against tuberculous infection (M.R.C. 1956).

Obviously there is no point in immunizing tuberculin-positive persons with BCG; any immunization that is possible in them has been developed by their own experience of the tubercle bacillus.

Dead Bacterial Vaccines

In the early days of immunology, when vaccines were first introduced, there was far more tendency to believe in their efficacy than could be justified by evidence. No doubt in many instances they acted merely as placebos or non-specific factors increasing resistance. With the development of more efficient methods of treating disease, and with a clearer appreciation of statistical methods for assessing the value of prophylactics, the number of vaccines in general use as immunizing agents has dwindled: those that survive are generally regarded as of proved efficacy. These are typhoid and paratyphoid vaccines, pertussis vaccines and perhaps cholera vaccines. They illustrate extremely well the problems that arise in preparing vaccines and assessing their value.

Tests for Effectiveness of Vaccines

If we are to kill bacteria to make vaccines, we must do so in such a way as to produce the minimum change in their antigenic make-up; and to find out what methods we should use is an extremely complicated matter. Ideally we should carry out clinical trials on all modified vaccines, to see whether they are effective in the field, and base our procedure on the results. In practice clinical trials are expensive, they take a long time, and they are often none too easy to interpret. It is natural, therefore, to attempt to devise some laboratory procedure that may be used instead, which, it is hoped, will give results parallel to those obtained in clinical trials. These laboratory procedures may be demonstrations that exposure to the vaccine produces, in laboratory animals, resistance to infection with virulent organisms—that is that inoculated animals withstand doses of these organisms that would kill uninoculated animals, and that the differences are statistically significant. Or, less directly, they may show that laboratory animals so inoculated possess some particular antibody—an agglutinin, a bacteriolysin,

an anti-haemagglutinin or a precipitin. If the disease is not very common, or if it occurs in rare explosive outbreaks, it may never be possible to compare the results of laboratory experiment with those of clinical trial.

Capacity to induce resistance to infection may seem a reasonable characteristic to expect from a vaccine, yet proof of it bristles with difficulties, not the least of which is the problem of producing in laboratory animals bacterial diseases resembling those of man. Thus typhoid baccilli injected intraperitoneally will produce an infection in mice, but this infection is a septicaemia throughout, with no localization to the intestine, and bears little resemblance to typhoid fever in man. It is not unlikely, therefore, that vaccines capable of protecting mice against experimental typhoid infection, and indeed designed to be effective in such tests, may be far less effective in man.

For instance, the capacity of typhoid bacilli to produce a fatal infection in mice appears to depend on the presence of a particular antigen called Vi (Felix 1941). This antigen is normally partly destroyed when suspensions of typhoid bacilli are killed with phenol—the usual method for preparing typhoid vaccines —but is preserved when they are killed with alcohol. Alcohol-killed typhoid vaccines are much better than phenol-killed vaccines in protecting mice against artificial typhoid infections, but there is no real evidence that they are more effective in man. Some believe that the main effect of typhoid vaccines is to reduce the incidence of the disease, and that they have no effect on the mortality if an inoculated person contracts it (Wilson & Miles 1955b). If this is so, it seems likely that the main effect of typhoid vaccine in man is to prevent typhoid bacilli from penetrating the intestine and producing a septicaemia; in the mouse this stage is by-passed by the method of producing the infection. The diseases we have to prevent are therefore quite different, so that conclusions drawn from mice may have little bearing on the disease in man. Moreover, alcohol-killed vaccines, even if they were as effective as phenol-killed vaccines against typhoid in the field, are more difficult to make and to maintain in an active state (Felix & Anderson 1951)—obvious arguments in favour of phenolized vaccines.

Recent trials of typhoid vaccines in Jugoslavia (Report 1957, 1962) and British Guiana (Report 1964) have, however, shown that though both phenolized and alcoholized vaccines are effective in the field, phenolized vaccines are superior; acetone-killed vaccines are apparently better than either (Report 1964).

Typhoid vaccines raise a further problem—that of antigenic competence. By this is meant that the antigenic composition of the organisms used for vaccines should be sufficiently close to that of those currently causing infections to offer reasonable protection against them. As we usually do not know which antigens are of consequence in deciding this point, many hold that 'wild' strains, i.e. strains recently isolated from cases, should be included in vaccines along with old and well-tried strains, so that we can cover any gross antigenic variation in the organisms at present responsible for infection. Not only in typhoid vaccines

but in others, practice in choice of strains is, in this country at any rate, rather conservative. Immunity is short lived, whatever the vaccine used.

Pertussis Vaccines

These illustrate very well the problems that may arise in producing vaccines against a very common and debilitating infectious disease of man. We may treat them under the headings growth, killing and testing.

Growth

Originally *Bordetella pertussis*, when required in bulk for vaccine production, was grown on solid media; even though the organism is not really fastidious, growth is slow, even on media to which blood has been added, so special precautions are necessary to avoid contamination. Moreover, the blood in the solid media is often partially washed out when the organisms are washed off the surface, and, since it is antigenic, may attract to itself a considerable part of the antibody response. Nor, since much of the pertussis antigen is set free by autolysis in the washing fluid, can the organism be freed from blood products by washing in saline and centrifugation.

These problems have largely been solved by growing the organism in its phase-I form in blood-free media such as that of Cohen & Wheeler (1946).

Killing

Phenol and formaldehyde have both proved in trials to produce too great an antigenic change in pertussis vaccines; Thiomersalate is now used for this purpose (Pittman 1952).

Testing

In man whooping-cough is characterized by a sticky mucoid exudate in the smaller bronchi, in which the infecting organisms occur in large numbers; secondary infection often complicates the picture. Comparable changes can be produced in mouse lungs by intranasal injection of suspensions of *Bordetella pertussis* under ether anaesthesia, and this might very well have been expected to provide an admirable challenge test for vaccines (Burnet & Timmins 1937). Unfortunately the test gives very inconstant results, partly no doubt because a variable amount of the intranasal challenge dose is swallowed, and the test now usually employed is an intracerebral challenge test (Kendrick, Eldering, Dixon & Misner 1947; Kendrick, Updyke & Eldering 1949). As this is very different from anything that occurs in natural whooping-cough in man, it might be expected to give results having little bearing on protection against whooping-cough in practice, but surprisingly enough clinical trials have shown that good vaccines, as judged by the intracerebral challenge tests, are usually good for preventing whooping-cough.

More recently suggestions have been made that intratracheal challenge might be more satisfactory (Te Punga & Preston 1958), since a smaller amount of the challenge dose might be lost by swallowing, and it has been shown that the results obtained do run parallel with those obtained in clinical trials. Suggestions that agglutinin production in response to injection of vaccine might be used as a test for efficacy (Evans & Perkins 1953) have received less support, as one effective whooping-cough prophylactic (Pillemer's SPA) does not provoke any production of agglutinin (Evans & Perkins 1955). The discrepancies have now been resolved, for later work based on the demonstration by Andersen (1963) and Eldering *et al* (1957, 1962) of antigenic types in *Bordetella pertussis* has made it clear that the protective power of vaccines does run parallel to their capacity to provoke a good agglutinin response to the antigens of the challenge organism, and that absence of a particular agglutinogen may sharply reduce the effectiveness of a vaccine in the field (Preston 1963, 1965, 1966; Manchester Public Health Laboratory 1965; Preston & Garrity 1967). If Pillemer's SPA is tested as a vaccine against organisms of the same antigenic composition as those from which it was made, it proves to be both immunogenic and capable of provoking an agglutinin response. Histamine-sensitizing factor has little or no immunogenic power (Preston & Garrity 1967).

Unquestionably effective pertussis prophylactics have now been produced, since clinical trials have shown that under conditions of equal exposure to infection the incidence of the disease in the vaccinated is only about 40% of that in the unvaccinated, and that the disease in the vaccinated is much less severe than that in the controls. Indeed, the effectiveness of some vaccines is so evident to the public that it is now difficult to obtain a control group for a new pertussis prophylactic, and new trials will evidently have to be done against a known effective vaccine as control.

THE UNSATISFACTORY SIDE OF ACTIVE IMMUNIZATION

Speed

Active immunization is a slow process. In those instances in which we can measure production of the antibody believed to be responsible for protection, as in diphtheria and tetanus prophylaxis, it is seldom possible to demonstrate antibody production in less than a fortnight after the first injection of prophylactic. This may be longer than the incubation period in either disease, and in general, in unimmunized persons exposed by contact or injury to the risk of an infection with a short incubation period, passive immunization is necessary.

Reactions

Persons given an injection of any prophylactic, but particularly bacterial vaccines, may develop reactions. These vary from local swelling, sometimes with sterile

'abscess' formation, to a general reaction with fever, a good deal of local pain and sometimes, as in some children given whooping-cough vaccines, cerebral symptoms (Byers & Moll 1948; Toomey 1949; Anderson & Morris 1950). They are seldom of serious importance: the sterile abscesses can usually be avoided by giving smaller doses of prophylactic or by deep intramuscular injection; reactions to alcohol- or acetone-killed typhoid vaccines are less severe than those to phenol-killed vaccines.

Some, at least, of the reactions are due to the development of sensitivity to bacterial proteins. Thus, for instance, many adults have become sensitive to diphtheria bacterial protein without becoming immune to diphtheria; injection of alum-precipitated diphtheria toxoid into such persons is likely to give rise to 'reactions'—usually local swelling, occasionally general symptoms with fever. For adults, therefore, a purer prophylactic with less bacterial protein content such as toxoid-antitoxin floccules, or purified toxoid, is to be preferred.

When tetanus toxoid was produced by growing tetanus bacilli on media containing Witte peptone and toxoiding the toxin from the culture, a few severe anaphylactic reactions were produced in persons into whom it was injected. These were due, not to any substance produced by the tetanus bacillus, but to residual Witte peptone present in the toxoid (Parish & Oakley 1940). Since the use of Witte peptone for this purpose has ceased, reactions to tetanus toxoid no longer seem to occur, though some people seem to be sensitive to the purest tetanus toxoid so far produced.

Provocation poliomyelitis

A rare complication of active immunization, particularly when the prophylactic contains killed *Bordetella pertussis*, is the conversion of non-paralytic into paralytic poliomyelitis (McCloskey 1950, 1951; Knowelden 1960). It is now clear that in most outbreaks of poliomyelitis far more people are infected with virus than show symptoms, and far more show slight symptoms than are paralysed. If, during a symptomless infection with poliomyelitis virus, persons receive injections of relatively insoluble material, e.g. carrier prophylactics, in a very minute proportion of them (1 in 20,000) the injection of the material into the muscle produces an increased susceptibility to poliomyelitis of the cord segments supplying the injected. muscles, and these and their neighbourng muscles may become paralysed. The observation was first made because it was noticed that persons who had received injections of prophylactic within 7 days or so of the onset of poliomyelitis showed paralysis of the injected limb more frequently than would have been expected from the distribution of paralysis in uninjected cases. How this increased susceptibility is produced is far from clear; perhaps it is due to increased vascularity of the cord segments supplying the injected area.

Several suggestions have been made to deal with this problem. The idea that

subcutaneous injection of carrier prophylactics might be free from this danger has proved ill-founded (Knowelden 1960), so that we have a choice of using non-carrier prophylactics (e.g. purified formol toxoid) with the necessarily increased number of injections they require or of injecting carrier prophylactics during the period, i.e. winter and spring, in which poliomyelitis is not prevalent. Success has been claimed for both, but the condition is so uncommon that it is not easy to reach satisfactory conclusions. Nowadays, however, the problem has been solved by the production of effective poliomyelitis vaccines; if children are immunized with these before other prophylactics are injected, the risks of provocation poliomyelitis can be reduced to negligible levels.

The need for repetition

The bacterial toxoids usually provide a very solid basal immunity, lasting for a very long time, and providing an excellent response to subsequent experience of an antigen; booster doses are given in many cases to provide a high level of circulating antibody as well. Bacterial vaccines are usually much less satisfactory; more doses have to be given, and though it is difficult to be certain of this, it is believed that immunity wanes much faster, and needs more frequent boosting in persons particularly at risk. Two injections at an interval of a week or fortnight are considered necessary for typhoid immunization; a longer interval might perhaps give a better effect, though the local reaction would probably be worse. But whatever is done, repeat vaccination every 3 years is recommended for those particularly exposed. For pertussis vaccination three injections are given at an interval of a month, and another at 15–18 months, after which the chance of infection is less. The variation in the number of doses necessary for effective immunization has a considerable effect on immunization schedules for children.

COMBINED PROPHYLAXIS

The increasing number of prophylactics that it is now considered necessary to inoculate into children, and the increased number of injections this would entail if the prophylactics were injected separately, has led to a considerable demand for combined prophylactics, i.e. mixtures of two or more immunizing agents that can be given together by a single injection (Bair 1960). Some consideration has already been given to the optimum composition of such mixtures, and all that is necessary here is to refer to two important points. First, the mixture must be compatible—it must not, early or late, undergo physical changes that make it useless, or chemical changes that inactivate any of its components. Secondly, it must be administered under such conditions that all its components have a reasonable chance of producing their effect. In other words its composition should be optimum and the recipient should have equal experience (which may

include no experience) of all the antigens it contains. For if he has had previous experience of *some* of the antigens, he will respond much better to these, and poorly, if at all, to the others, and may need further injections of prophylactic to make up for this.

IMMUNIZATION SCHEDULES

Arrangement of Active Immunization Schedules

Schemes for the active immunization of children are necessarily compromises between immunizing efficiency and administrative efficiency; they attempt to obtain optimum, safe immunization with the minimum number of injections in the minimum number of visits by the doctor. Difficulties arise from the diverse nature of the antigens used; some, like the bacterial vaccines, need at least three injections; most bacterial toxoids need only two, at most three; if formolized poliomyelitis vaccine continues to be used, it will need four. But if, to allow for this, we give one dose of pertussis prophylactic, then one of pertussis and tetanus prophylactic and then one of diphtheria, tetanus and pertussis prophylactic, we shall get considerable interference with the responses, and may very well have to give more injections to obtain a satisfactory degree of immunity. Far better to give all three prophylactics together from the start—that is, give three injections of diphtheria, tetanus and pertussis combined triple prophylactic at intervals of a month. An alternative possibility, if a child has been immunized with one bacterial toxoid, is to complete this immunization before proceeding with the others.

At the present time, mainly owing to the success of oral poliomyelitis vaccine, immunization practice is in a state of flux. Roughly speaking, three immunization schedules are in use (Ministry of Health 1963, 1965, 1966). In schedule P, with its amendments, three doses of combined diphtheria, tetanus and pertussis prophylactic are given first, over the first 6 months of life, followed by three doses of oral poliomyelitis vaccine over the 7th to 11th months. In schedule Q, with its amendments, three doses of oral poliomyelitis vaccine are given over the first 6–10 months, followed by three doses of diphtheria, tetanus and pertussis prophylactic over the 11th to 13th months. On logical grounds I prefer schedule Q, as the preliminary immunization with poliomyelitis vaccine might reasonably be expected to protect against provocation poliomyelitis during the subsequent immunization against diphtheria, tetanus and whooping-cough. It has, however, the disadvantage that immunization against diphtheria, tetanus and whooping-cough is somewhat delayed (though this seems to be of less importance than it was in the past); the third schedule in use therefore provides for immunization against diphtheria, tetanus, whooping-cough, *and* polio-

myelitis (with oral vaccine) over the 3rd to 5th months of life. There is a general feeling that the immune response of infants to poliomyelitis prophylactics is better at 6 months than at 3 months, and to allow for this a fourth dose of oral poliomyelitis vaccine is given between 18 and 24 months, and a fifth at school entry (5 years). The administrative convenience of this schedule is obvious, and it appears to be gaining ground.

All three schedules require a further dose of diphtheria, tetanus and pertussis prophylactic to be given between 18 and 21 months of age; boosting doses for diphtheria and tetanus protection are given at school entry, when there is a particular risk of these diseases, and though there is some dispute about the necessity for it, at some time between 8 and 12 years.

Vaccination against smallpox is best done separately, at any time within the first 5 years (preferably within the first two), to avoid having to do primary vaccination in adolescents or adults. If, as is now likely, the child is still tuberculin-negative at 15 years, BCG vaccination may be given.

Passive Immunization

In active immunization the person or animal immunized produces his own antibody; this soon enters his circulation, and injections of his serum or plasma will then transfer the antibody to animals of his own or other species. By this means —passive immunization—antibody can be provided for inexperienced animals or man at a speed much greater than is practicable by active immunization.

A number of antibodies may be produced in man by subclinical or clinical infection (e.g. measles antibody) or by deliberate active immunization (e.g. tetanus antitoxin) and used for passive immunization of other persons. They have the great advantage that they are homologous proteins; they are therefore destroyed at the usual rate for these proteins, and do not excite the antibody response necessarily provoked by the corresponding foreign proteins. Their main defect is their low concentration in the plasma. By appropriate concentration processes measles antibody can be effectively concentrated in serum, but there seems little hope of obtaining other antibodies in sufficiently high unitage.

This difficulty has been overcome in the past by immunizing horses against the appropriate antigen—particularly against bacterial toxins, and using their serum for passive prophylaxis. A straightforward case occurs in prophylaxis against tetanus. When a non-immunized person suffers a wound likely to give rise to tetanus, the only useful way he can be protected against this risk is to immunize him passively with tetanus antitoxin prepared in the horse, in which antitoxin levels of 300 units per ml or more, which can be raised to 1500 units per ml or more by refining and concentration, can readily be obtained. By this means, if the serum is injected intramuscularly or subcutaneously, high concentrations of circulating antitoxin can be provided within 24 hr or less, and there is good reason to suppose that they offer considerable protection.

In certain circumstances, when new-born children are exposed to serious risk of infection, it may be of value to immunize them passively by actively immunizing their mothers. Thus in some parts of northern Africa it is customary to dress the umbilical cord with dung paste, and neonatal tetanus is therefore common. Attempts to change long-established custom are unlikely to be effective, and immunization of the mothers against tetanus with tetanus toxoid, with consequent placental transfer of tetanus antitoxin to the foetus, is far more likely to protect the newborn child. In order to provide high levels of passively acquired antitoxin at birth and for about 10 days afterwards, the second or third dose of toxoid should be injected 10–14 days before the expected date of birth. Subsequent active immunization will be necessary for the child (see below).

A similar method is sometimes used for preventing lamb dysentery; pregnant ewes are immunized with *Clostridium welchii* type-B toxoid, so that the ewe reaches the maximum of her secondary response at the time of the birth of the lamb; high concentrations of antibody are secreted in the colostrum, from which the lamb is able to absorb it. As lamb dysentery is a disease of the first few days of life only, no subsequent active immunization of the lamb is necessary. This method has obvious advantages compared with the other at one time in common use—immunization of newborn lambs with *Clostridium welchii* type-B horse antiserum—and as *Cl. welchii* type-B toxoids have increased in potency, has largely superseded it.

Defects of Passive Immunization

Length

Passive immunization is short lived; it lasts as long as an effective concentration of antibody is present in the circulation, and unlike active immunization, it confers on the patient no increased capacity to respond to subsequent experience of the antigen. Furthermore, the patient may respond to the injection of horse protein by producing antibody against it. This may have two effects; subsequent injection of horse antitoxin may cause such rapid production of antibody against it that the antitoxin may be removed from the circulation and rendered ineffective long before it has had any chance to protect the patient (Littlewood, Mant & Wright 1954), and, secondly, combination of horse antitoxin with precipitating antibody in the tissues may lead to serum reactions or even, though rarely, to anaphylactic shock or death.

Reactions to horse serum

When crude horse serum was used for prophylaxis or treatment, some 60% of persons treated with it developed some kind of serum reaction, usually, if they were being given serum for the first time, 7–10 days after the injection. Ammonium-sulphate concentration of the serum, by removing considerable

amounts of unwanted antigenic protein, greatly reduced the incidence of serum reactions, and the introduction of the 'refining' process, by which albumin was destroyed and the antitoxin molecule split into two, with heat-denaturation of the non-antitoxic moiety, has brought it down to the present level of $2\frac{1}{2}$ to 5%.* Most of these reactions, whatever the kind of serum used, consist of urticarial rashes, swelling of joints and swelling of lymph glands. It is well worth remembering that a patient given serum to protect him against tetanus may develop a temporomandibular 'arthritis', which may easily be mistaken for trismus if his history is not known.

Some patients who have, as far as is known, never had injections of serum previously, develop severe reactions on their first injection (for a particularly complete account of such a reaction see Czekalowski 1965); reactions to subsequent injections are likely to be more severe and to occur earlier. Their prevention and treatment is the same whether serum is used prophylactically or therapeutically.

Horse antitoxin should never be injected intravenously, only intramuscularly or subcutaneously, and no patient given antitoxin should ever be left alone for the first 20 min after injection. A syringe containing 1/1000 adrenaline armed with a sharp needle should always be at hand, and at the slightest sign of pallor or general reaction 0.2 ml of the adrenaline should be injected intravenously. Other action will depend on symptoms.

Most patients who develop serum sickness 8–10 days after injection of horse tetanus antitoxin will recover completely without treatment; cases with more severe symptoms are best given an intramuscular injection of 80 units of hydrocortisone gel.

Some evidence that tetanus antitoxin is not very effective as a prophylactic in patients with wounds likely to give rise to tetanus, and much stronger evidence that its use is dangerous (see Czekalowski 1965), have led some workers (Cox, Knowelden & Sharrard 1963; Sharrard 1965a and b) to suggest that it should be used only where the wound is more than 24 hr old and is very likely to be contaminated with tetanus bacilli; other cases should be treated by surgical treatment of the wound and injection of adsorbed tetanus toxoid, with or without antibiotics, according to severity. It should be noted, however, that in experimental tetanus in mice penicillin has little effect 8 hr after infection, whereas antitoxin is effective up to 24 hr (Smith 1965); how far this applies to man is unknown. Ellis (1965a and b) uses antitoxin much more freely, draws attention to the difficulties attending wound excision, and makes the interesting suggestion that when antitoxin and procaine penicillin are injected together, severe immediate reactions to antitoxin are very rare. He also draws attention to

* Cox, Knowelden & Sharrard (1963) suggest that a thorough follow-up would show an incidence of delayed general reactions of about 8%.

misunderstandings of the views of Cox *et al* (1963), which have led some people to replace injection of antitoxin with a single injection of toxoid, without provision for further injections. Ellis is far from satisfied that prophylactic antitoxin is useless, and cites extensive clinical experience in Nigeria to the contrary. Batten (1965), with similar experience, offers considerably less convincing evidence against prophylactic antitoxin, which he frequently found ineffective, and is in favour of antibiotics.

I hold that every case of wounding should be treated on its merits, though my practice would probably be nearer to that of Ellis; a good account of the two sides of the controversy is given by Ellis (1965b) and Sharrard (1965b). It is clear enough, however, from Ellis's (1965a, p. 225) figures that no method, single or combined, that does not involve effective previous immunization with tetanus toxoid will give guaranteeable protection against tetanus; and my own view is that the question 'Shall I give antitoxin or not?' has no satisfactory answer, and should be by-passed by adequate immunization of the entire population against tetanus. Prophylactic tetanus antitoxin should never be given to patients adequately immunized against tetanus with toxoid; an injection of tetanus toxoid is the only *specific* measure needed if they suffer wounds likely to give rise to tetanus.

Necessity for Subsequent Active Immunization

It is evident from what has been said above that persons immunized passively not only cease to be immune when their passively acquired antibody is exhausted, but may be unable to profit by subsequent passive immunization. It is often suggested that if this is necessary, bovine antitoxin might be used; but it is not easily available, and it is by no means certain that it is of any value. Only human serum can be used safely on more than one occasion. It follows, therefore, that anyone subjected to passive immunization should be actively immunized thereafter against any diseases for which horse serum might be used prophylactically or therapeutically, against which he has not been previously actively immunized; in practice the only likely ones are diphtheria and tetanus.

The question naturally arises: how long after passive immunization shall we wait before we immunize actively? Ideally, no doubt, we should wait till all traces of passive antitoxin have disappeared, i.e. for about 6 weeks. In practice, if such long intervals are allowed, patients often disappear and do not bother doctors in their new area for active immunization; I am therefore in favour of a shorter period—about 10 days, though a month would be better—which though less satisfactory, theoretically is far more likely to be effective. Further injections will be necessary 6 weeks and 8 months after the first, but it will be easier to give them if the first has already been given. The use of adsorbed tetanus toxoid,

given at the same time as tetanus antitoxin, but in a different site, with a second injection a month or so later, is said to give excellent active immunization against tetanus (Smith *et al* 1963).

REFERENCES

AGUSSTION H. & TOOMEY J.A. (1948) Purogenated toxoid reactions. *Archs Pediat.* **65,** 405.

AMIES C.R. (1954) The pathogenesis of diphtheria. *J. Path. Bact.* **67,** 25.

ANDERSON, ELSE K. (1953) Serological studies on *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*. *Acta path. microbiol. scand.* **33,** 202.

ANDERSEN I.M. & MORRIS D. (1950) Encephalopathy after combined diphtheria-pertussis inoculation. *Lancet* **i,** 537.

BARR, MOLLIE (1960) in *A Symposium on Immunization in Childhood* p. 92. Ed. Cannon D.A. & others. Edinburgh, E. & S. *Livingstone.*

BARR, MOLLIE & GLENNY A.T. (1931) Further observations on qualitative differences in antitoxin fractions prepared from the same sample of plasma. *Brit. J. exp. Path.* **12,** 337.

BARR, MOLLIE & GLENNY A.T. (1947) The delayed immunity response. *Lancet* **ii,** 647.

BARR, MOLLIE & GLENNY A.T. (1949a) Factors affecting the maintenance of antitoxin immunity. *Lancet* **i,** 646.

BARR, MOLLIE & GLENNY A.T. (1949b) Qualitative differences among toxins and toxoids. *J. Hyg. (Lond.)* **47,** 107.

BARR, MOLLIE, GLENNY A.T. & RANDALL K.J. (1950) Diphtheria immunization in young babies. *Lancet* **i,** 6.

BARR, MOLLIE & LLEWELLYN-JONES, MONA (1951) Factors affecting the development of potential immunity and the characters of the secondary response. *Brit. J. exp. Path.* **32,** 231.

BARR, MOLLIE & LLEWELLYN-JONES, MONA (1953a) Some factors affecting the response of animals to immunization with combined prophylactics. *Brit. J. exp. Path.* **34,** 12.

BARR, MOLLIE & LLEWELLYN-JONES, MONA (1953b) Interference with antitoxic responses in immunization with combined prophylactics. *Brit. J. exp. Path.* **34,** 233.

BARR, MOLLIE & SACHS A. (1955) *Report on the Investigation into the Prevention of Tetanus in the British Army*. London: The War Office.

BATTEN R.L. (1965) Tetanus prophylaxis. *Proc. Roy. Soc. Med.* **58,** 229.

BAYLIS J.G., MACKINTOSH, JANET, MORGAN R.S. & WRIGHT G.P. (1952) The effect of sclerosis of the nerve trunk on the ascent of tetanus toxin in the sciatic nerve of rabbits and on the development of local tetanus. *J. Path. Bact.* **64,** 33.

BILLINGHAM R.E., BRENT L. & MEDAWAR P.B. (1953) 'Actively acquired tolerance' of foreign cells. *Nature (Lond.)* **172,** 603.

BOYD J.S.K. (1960) In *A Symposium on Immunization in Childhood*, p. 83, ed. CANNON D.A. and others. Edinburgh: E. & S. Livingstone.

BRUTON O.C. (1952) Agammaglobulinaemia. *Pediatrics* **9,** 722.

BURNET F.M. (1959) *The Clonal Selection Theory of Acquired Immunity*. Cambridge: Cambridge University Press.

BURNET F.M. & FENNER F. (1949) *The Production of Antibodies*. Melbourne: Macmillan.

BURNET F.M. & TIMMINS, CECILY (1937) Experimental infection with *Haemophilus pertussis* in the mouse by intranasal inoculation. *Brit. J. exp. Path.* **18,** 83.

BYERS R.K. & MOLL F.C. (1948) Encephalopathies following prophylactic pertussis vaccine. *Pediatrics* **1,** 437.

COHEN, SOPHIA M. & WHEELER, MARY W. (1946) Pertussis vaccines prepared with phase-I cultures grown in fluid medium. *Amer. J. publ. Hlth* **36,** 371.

COX, COLLEEN A., KNOWELDEN J. & SHARRARD W.J.W. (1963) Tetanus prophylaxis. *Brit. med. J.* **ii,** 1360.

CRUMPTON M.J. & DAVIES D.A.L. (1956) An antigenic analysis of *Pasteurella pestis* by diffusion of antigens and antibodies in agar. *Proc. roy. Soc. B* **145,** 109.

CZEKALOWSKI J.W. (1965) A case of serum sickness in man. *J. Path. Bact.* **90,** 607.

DAVIS J.B., MATTMAN L.H. & WILEY M. (1951) *Clostridium botulinum* in a fatal wound infection. *J. Amer. med. Ass.* **146,** 66.

DUNSFORD I., BOWLEY C.C., HUTSHISON A.M. & THOMPSON J.S. (1953) A human blood-group chimaera. *Brit. med. J.* **ii,** 81.

EDITORIAL (1957) Influenza virus variants. *Brit. med. J.* **i,** 1517.

ELDERING, GRACE, EVELAND W.E., & KENDRICK, PEARL L. (1962) Fluorescent antibody staining and agglutination reactions in *Bordetella pertussis* cultures. *J. Bact.* **83,** 745.

ELDERING, GRACE, HORNBECK C. & BAKER, JULIA (1957) Serological study of *Bordetella pertussis* and related species. *J. Bact.* **74,** 133.

ELLIS M. (1965a) Tetanus prophylaxis. *Proc. Roy. Soc. Med.* **58,** 224.

ELLIS M. (1965b) Prophylaxis of tetanus. *Prescrib. J.* **4,** 17.

EVANS D.G. (1965) Tetanus prophylaxis. *Proc. Roy. Soc. Med.* **58,** 222.

EVANS D.G. & PERKINS F.T. (1953) An agglutinin-production test in the study of pertussis vaccines. *J. Path. Bact.* **66,** 479.

EVANS D.G. & PERKINS F.T. (1955) Tests for agglutinin production by pertussis protective antigen, SPA. *J. Path. Bact.* **69,** 329.

EVANS D.G. *et al* (1962) Inefficiency of purified diphtheria formol toxoid in primary immunization against diphtheria. *Brit. med. J.* **ii,** 149.

FELIX A. (1941) A new type of typhoid and paratyphoid vaccine. *Brit. med. J.* **i,** 391.

FELIX A. & ANDERSON E.S. (1951) The immunizing potency of alcohol-preserved typhoid vaccine after storage for ten years. *J. Hyg. (Lond.)* **49,** 288.

FILDES P. (1929) Tetanus—IX. The oxidation-reduction potential of the subcutaneous tissue fluid of the guinea-pig; its effect on infection. *Brit. J. exp. Path.* **10,** 197.

FULTON F., TAYLOR J., MOORE B., WELLS A.Q. & WILSON G.S. (1942) Immunization against diphtheria with alum-precipitated toxoid. *Brit. med. J.* **i,** 315.

GLENNY A.T. (1930) Insoluble precipitates in diphtheria and tetanus immunization. *Brit. med. J.* **ii,** 244.

GLENNY A.T. & HOPKINS, BARBARA E. (1923) Diphtheria toxoid as an immunizing agent. *Brit. J. exp. Path.* **4,** 283.

GLENNY A.T. & POPE C.G. (1927) Diphtheria toxoid-antitoxin floccules. *J. Path. Bact.* **30,** 587.

GLENNY A.T. & SUDMERSEN H.J. (1921) Notes on the production of immunity to diphtheria toxin. *J. Hyg. (Lond.)* **21,** 176.

GOWLAND G. & OAKLEY C.L. (1960) Acquired immunological tolerance to diphtheria alum-precipitated toxoid in the domestic fowl. *J. Path. Bact.* **80,** 373.

HOLT L.B. (1950) *Development in Diphtheria Prophylaxis,* p. 83. London: Heinemann Ltd.

KENDRICK, PEARL, ELDERING, GRACE, DIXON M.J. & MISNER J. (1947) Mouse protection tests in the study of pertussis vaccine. *Amer. J. publ. Hlth* **37,** 803.

KENDRICK, PEARL, UPDYKE, ELAINE L. & ELDERING, GRACE (1949) Comparison of pertussis cultures by mouse protection and virulence tests. *Amer. J. publ. Hlth* **39,** 179.

KING E.S.J. (1955) Spermatozoal invasion of the epididymis. *J. Path. Bact.* **70,** 459.

KNOWELDEN J. (1960) The risks of immunization. In *A Symposium on Immunization in Childhood,* p. 11, ed. CANNON D.A. and others. Edinburgh: E. & S. Livingstone.

LITTLEWOOD A.M., MANT A.K. & WRIGHT G.P. (1954) Fatal tetanus in a boy after prophylactic tetanus antitoxin. *Brit. med. J.* **ii,** 444.

McCloskey B.P. (1950) The relation of prophylactic injections to the onset of poliomyelitis. *Lancet* **i,** 659.

McCloskey B.P. (1951) The relation of prophylactic inoculation to the onset of poliomyelitis; a study of 620 cases in the Victorian epidemic of poliomyelitis. *Med. J. Aust.* **i,** 613.

Manchester Public Health Laboratory (1965) *Mon. Bull. Minist. Hlth* **24,** 104.

Mason J.H., Dalling T. & Gordon W.S. (1930) Transmission of maternal immunity. *J. Path. Bact.* **33,** 783.

Ministry of Health (1963) Active Immunization Against Infectious Disease. London.

Ministry of Health (1965) Letter to practitioners C.M.O. 14A/65.

Ministry of Health (1966) Letter to practitioners C.M.O. 4A/66.

M.R.C. Tuberculosis Vaccines Clinical Trials Committee (1956) B.C.G. and vole bacillus vaccines in the prevention of tuberculosis in adolescents. *Brit. med. J.* **i,** 413.

Owen R.D. (1945) Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* **102,** 400.

Parish H.J. & Cannon D.A. (1962) Prevention of tetanus. *Brit. med. J.* **i,** 868.

Parish H.J. & Oakley C.L. (1940) Anaphylaxis after injection of tetanus toxoid. *Brit. med. J.* **i,** 294.

Parish H.J. and others (1960) In *A Symposium on Immunization in Childhood* p. 116 Ed. Cannon D.A. and others, Edinburgh E. & S. Livingstone.

Pittman, Margaret (1952) Influence of preservatives, of heat and of irradiation on mouse protective activity and detoxification of pertussis vaccine. *J. Immunol.* **69,** 201.

Preston N.W. (1963) Type-specific immunization against whooping cough. *Brit. med. J.* **ii,** 724.

Preston N.W. (1965) Effectiveness of pertussis vaccines. *Brit. med. J.* **ii,** 11.

Preston N.W. (1966) Potency tests for pertussis vaccines: doubtful value of intracerebral challenge in mice. *J. Path. Bact.* **91,** 173.

Preston N.W. & Garrity, Pamela (1967) Histamine-sensitizing factor of *Bordetella pertussis* differentiated from immunogens by neutralization and passive protection tests. *J. Path. Bact.* **93,** 483

Report (1957) *Bull. Wld Hlth Org.* **16,** 897.

Report (1962) *Bull. Wld Hlth Org.* **26,** 357.

Report (1964) *Bull. Wld Hlth Org.* **30,** 623, 631, 635.

Sharrard W.J.W. (1965a) Tetanus prophylaxis. *Proc. Roy. Soc. Med.* **58,** 221.

Sharrard W.J.W. (1965b) Prophylaxis of tetanus. *Prescrib. J.* **4,** 120.

Smith J.W.G. (1965) Experimental studies of tetanus prophylaxis. *Proc. Roy. Soc. Med.* **58,** 226.

Smith J.W.G., Evans D.G., Jones D.A., Gear M.W.L., Cunliffe A.C. & Barr, Mollie (1963) Simultaneous active and passive immunization against tetanus. *Brit. med. J.* **i,** 237.

Te Punga W.A. & Preston N.W. (1958) Intratracheal infection of mice with *Haemophilus pertussis*. *J. Path. Bact.* **76,** 275.

Thomas C.G., Keleher M.F. & McKee A. (1951) Botulism, a complication of *Clostridium botulinum* wound infection. *Archs Path.* **51,** 623.

Toomey J.A. (1949) Reactions to pertussis vaccine. *J. Amer. med. Ass.* **139,** 448.

Wilson G.S. & Miles A.A. (1955a) *Principles of Bacteriology and Immunity*, 4th edn., vol. 2, p. 1671, ed. Topley W.W.C. & Wilson G.S. London: Edward Arnold.

Wilson G.S. & Miles A.A. (1955b) *Principles of Bacteriology and Immunity*, 4th edn., vol. 2, p. 1749. ed. Topley W.W.C. & Wilson G.S. London: Edward Arnold.

Woodruff M.F. & Lennox B. (1959) Reciprocal skin grafts in a pair of twins showing blood chimaerism. *Lancet* **ii,** 476.

Wright E.A., Morgan R.S. & Wright G.P. (1950) Tetanus intoxication of the brain stem in rabbits. *J. Path. Bact.* **62,** 569.

CHAPTER 45

PROPHYLAXIS OF VIRAL DISEASES

P.T.PERKINS AND C.KAPLAN

INTRODUCTION

That antibodies play a major role in protection against bacterial diseases is undisputed and indeed there is little to indicate that the viral diseases should be insusceptible to such a powerful defence mechanism. Evidence that immunity to virus diseases is effected by humoral mechanisms is given by several workers (Bedson & Crawford 1928; Andrewes 1929a and b; Andrewes 1930b; Morgan, Schlesinger & Olitsky 1942). Viruses are neutralized by antisera and the only reason for divergence in the parallelism of protection against the two types of infectious agents is in the necessity for the virus to grow inside the living cell where it may be protected from contact with antibody; some viral agents even spread from cell to cell without ever emerging from the cellular material.

Studies on hypogammaglobulinaemic subjects, however, have shown that other mechanisms may also play a part in immunity. These individuals who have little or no γ-globulin are very susceptible to recurrent attacks of bacterial infections although they recover from attacks of measles, varicella, mumps and poliomyelitis in a normal manner and are left with solid immunity to the diseases. When these subjects recover from the virus infections they show a normal hypersensitivity to the virus antigen.

The Specificity of Animals as virus Hosts

Some viruses are specific for the host; e.g. zoster and chickenpox for man and fowl leucosis for the domestic fowl. It is well recognized that the fight against many diseases has been seriously hampered by the lack of an experimental animal in which to study the pathogenicity of and immunity to the specific infection. Such was the case with yellow fever until it was shown that the monkey (Stokes, Bauer & Hudson 1928) and later the mouse (Theiler 1930) could be infected. Similarly, infection of the ferret helped influenza research (Smith, Andrewes & Laidlaw 1933); and so it was with poliomyelitis until the chimpanzee, rhesus monkey (Landsteiner & Popper 1909) and finally primate cell cultures (Enders, Weller & Robbins 1949) were shown to be susceptible. This cycle of events has been repeated many times and even today there are constant appeals for volunteers to aid researchers into the common cold since every species of laboratory animal has proved unsuccessful in replacing man. Although some viruses are host specific we also have diseases in which the virus has been isolated from every species of animal so far studied. The recent work on rabies has shown this virus to infect many animal species in several countries and stringent precautions to prevent it entering a country are certainly justified for both public health and economic reasons.

Cell Specificity

Just as we have a host specificity of the virus in the intact animal so we have many examples of virus specificity in cell cultures in which antibody production plays no part. Poliomyelitis virus infects simian and human tissue whether in primary or continuous culture, whereas rabbit tissue is completely insusceptible. On the other hand, the common cold viruses were isolated in cell cultures by altering the conditions of temperature and pH for cell growth. An even more subtle differentiation is shown by the simian virus SV 40 which causes no cytopathic degeneration in tissue from rhesus monkeys, in which it may be a contaminant, but the same virus passed to kidney tissue of vervet monkeys causes complete degeneration. Undoubtedly virus diagnostic laboratories require a number of different cell cultures for the examination of pathological specimens and the human diploid cell strains with their wide range of virus susceptibility are of considerable importance (Hayflick & Moorhead 1961) since some strains of rhinovirus will grow only in these cells.

Immunity after Infection

The immunity remaining after infection by viruses can vary from transient to permanent. After an attack of measles, smallpox or yellow fever, for example, second attacks are very rare, whereas multiple attacks of influenza and the common cold are well known. This may be due, in part, to the many different

serotypes causing the syndrome and a series of colds may be caused by immunologically unrelated agents. Thus, against antigenically stable viruses antibodies are generally effective but when the antigenic structure alters (influenza in man, foot-and-mouth in animals) the immunity, based on antibody, may fail.

In the early days of virology it was a common belief that infection with a virus altered the cell susceptibility, thus having a marked effect on reinfection, but the work of the last 20 years has given much more weight to the importance of protection by specific antibodies. In many careful studies with herpes, measles and poliomyelitis, immunity to the disease coincides with the appearance of antibodies and passive immunity can be effected by injecting convalescent serum which can reduce the severity or completely abort the disease. This is the basis of γ-globulin therapy practised here and on a much larger scale in America where injections of γ-globulin to measles contacts are routine. The injection of γ-globulin to women in the first trimester of pregnancy who have been in contact with rubella is now common in the United Kingdom.

It is not yet clear why immunity to some virus infections is permanent or indeed why high antibody titres are maintained for years. In studies with bacterial infections a high antibody response is obtained to a reinforcing stimulus by the antigen. It has been postulated, therefore, that the antibody titres after measles and yellow fever could only be maintained by a continuous antigenic stimulation but its source is not always evident. After the majority of virus infections or injection of a virus vaccine, however, the initial high antibody titre falls but there is a rapid response to a booster dose.

Action of Humoral Immunity

Antibodies, whether antitoxic or antibacterial, play a major role in immunity to bacterial infections; in the one the toxin is neutralized whereas in the other the bacterium is rendered more susceptible to phagocytosis. The serological techniques developed in virology have followed those in bacteriology. It is now common practice to measure responses of man to virus diseases and vaccine by neutralization tests in which a constant test dose of virus is mixed with serum dilutions and, after a suitable incubation time, inoculated into susceptible animals or cell cultures. Infection or cytopathic effect occurs when the serum has been diluted beyond the point of neutralization. Neutralization is not the only serological test; agglutination, precipitation of virus by antiserum and complement fixation are other serological techniques by which immunity may sometimes be measured. Combination of virus and antibody undoubtedly occurs, making the virus non-infectious. Immediately after mixing the virus and antiserum the majority of the virus particles can be recovered as infectious entities. This has been shown both by diluting the mixtures to decrease the neutralizing power of the serum (Bedson 1928; Todd 1928; Andrewes 1928) and by centrifuging the

virus particles out of such mixtures (Sabin 1935). With many viruses a period of hours and sometimes days at temperatures of 25°C or higher is required before complete neutralization occurs (Bedson 1928; Andrewes 1930a).

Interaction of virus and antibody has been studied in cell cultures and it has been shown that adding antiserum to cell cultures before the virus, or mixing the virus with antiserum before adding to cell cultures, will prevent infection of the cells, but no amount of antiserum added after the virus has been in contact with the cells will protect the cells (Andrewes 1930a). The antibody, in combining with the virus particle, in some way prevents entry into the cell. This combination is most effectively demonstrated in the haemagglutination test with influenza virus which has the property of attaching itself to mucoprotein receptors of red blood cells; aggregates are formed and the red cells are agglutinated. Addition of specific antiserum to the virus prevents this haemagglutination since the coating of the virus blocks the initial combination of virus and red cells.

In general it is clear that whatever the protective mechanism against disease may be, antibodies play a most significant part and attempts at prophylaxis are concerned with obtaining the greatest antibody response and maintaining circulating antibody throughout life.

VACCINATION AGAINST VIRUS DISEASES

Studies with Infants

Immunization against any disease should be started before the age of maximum risk and attempts have been made in this country (Perkins, Yetts & Gaisford 1958) to immunize infants in the first 3 months of life. Their early studies with poliomyelitis vaccine in 1958 in which infants were less than 10 weeks of age at the time of receiving the first two doses of vaccine for primary immunization were most disappointing (Perkins, Yetts & Gaisford 1958, 1959b, 1961). A typical picture of the responses of 6-week-old infants to the Type 1 antigen, the one giving the most unsatisfactory results and the most important one to which immunity must be achieved, is shown in Fig. 45.1. Clearly, the majority of infants were born with maternal antibody; 25% had levels of 1024 or more. Only three showed an antibody response after two doses of vaccine and these had no maternal antibody. The majority of infants with maternal antibody levels of 1024 or more not only failed to respond but the antibody levels after vaccination were no higher than that expected by natural decay of maternal antibody which was found to be halved every 21 days. It was not possible to know at this time, however, whether the higher levels of maternal antibody simply masked or inhibited active response to the vaccine. Recalling the infants 10–12 months later and giving them a third (booster) dose clarified the picture

and these responses are also shown in Fig. 45.1 (Perkins, Yetts & Gaisford 1959a). It is interesting to note that the majority of the infants giving a low response to the booster dose were born with levels of maternal antibody of 1024 or more, which seem to inhibit active immunization. The three infants responding to the primary doses gave responses to the booster dose shown as open circles. Some infants with maternal antibody levels between 64 and 1024, however, who gave no apparent antibody response to the primary stimulus gave a booster response to the third dose and in these infants the maternal antibody simply masked the primary response. In spite of the finding that more infants had responded to the primary stimulus than was apparent by a rise in antibody level at this time, the two-dose schedule could not be regarded as successful for the Type 1 antigen in the neonatal period.

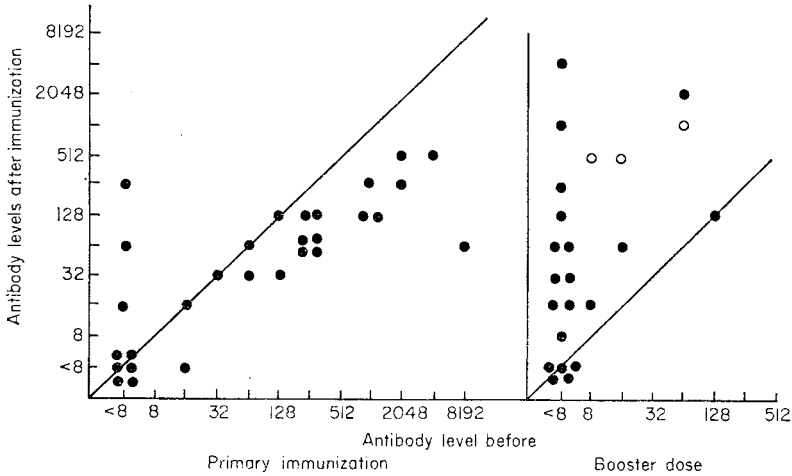


FIG. 45.1. Primary and booster response of 6-week-old infants to Type 1 poliomyelitis antigen (see text).

A further difficulty contributing to the poor responses was the inability of such young infants to respond to this small dose of antigen. In groups of older infants 4–9 months of age, who were either born with no maternal antibody or in whom the maternal antibody had fallen to undetectable levels, it was apparent that both the percentage responding and the geometric mean titre for the group increased with age (Fig. 45.2). In further attempts to obtain satisfactory responses in the newborn the antigenic stimulus was increased not only by giving a third dose in the primary immunization course but also by using a vaccine with concentrated antigens, especially of the Type 1 component. The antibody levels after both two and three doses of vaccine are shown in Fig. 45.3 in which the interrupted

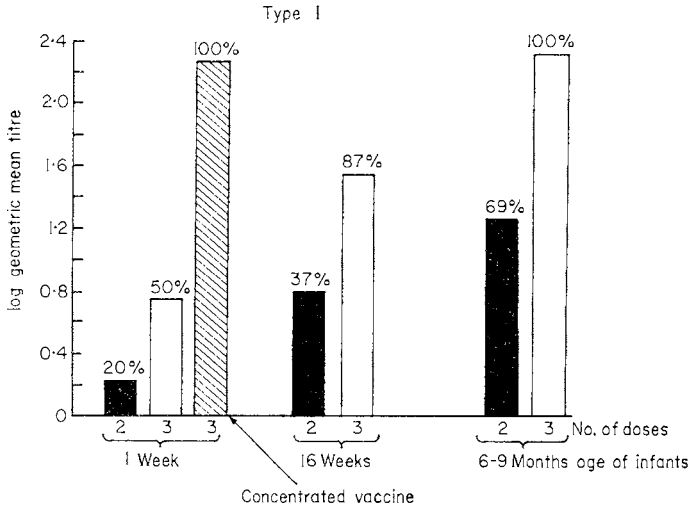


FIG. 45.2. The effect of age and number of doses on the response of infants to Type 1 poliomyelitis antigen (see text).

■—infants receiving two doses of vaccine.

▭—infants receiving three doses of vaccine.

▨—infants receiving three doses of concentrated vaccine.

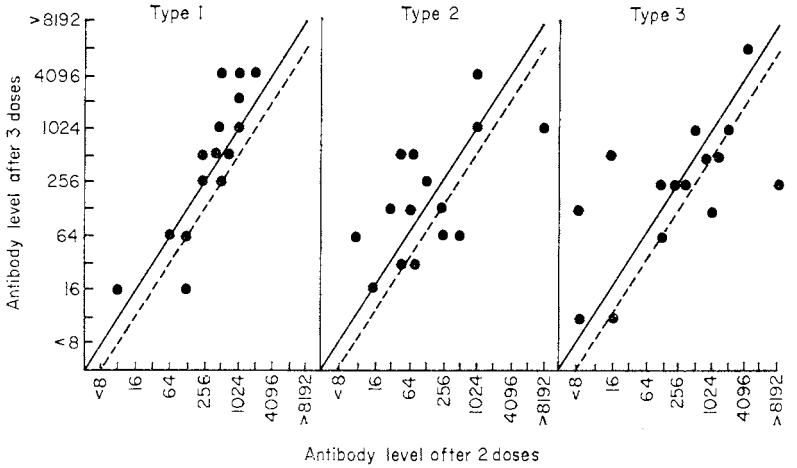


FIG. 45.3. Comparison of antibody response of 1-week-old infants after two and three doses of concentrated poliomyelitis vaccine (see text). The interrupted line indicates the expected levels of antibody in the absence of response between the second and third dose.

line shows the expected levels given by the decay of maternal antibody. Since the majority of the points lie above this line there is clear indication of response even in the presence of high levels of maternal antibody. Recalling the infants 9 months later and giving them a reinforcing dose confirmed the earlier observation that even with the concentrated vaccine three doses given at 4–6 week intervals are necessary to ensure satisfactory responses in the newborn. Increasing the antigenic stimulus successfully overcame both the inhibitory effect of high levels of maternal antibody and the relative inability of the newborn to produce antibodies. The whole picture is summarized in Fig. 45.4 which shows that the more concentrated antigens are able to break through the 'maternal antibody barrier' and that only when this is achieved are satisfactory booster responses obtained.

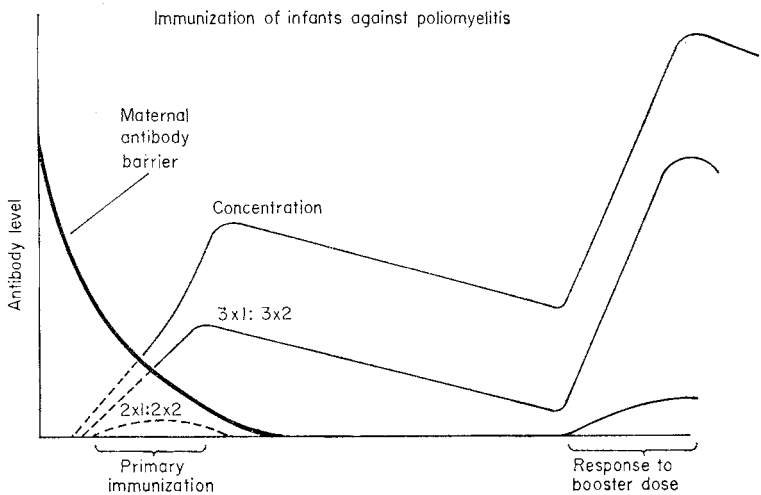


FIG. 45.4. The inhibitory effect of maternal antibody on the response of newborn infants to poliomyelitis vaccine (see text).

These findings are not unique to poliomyelitis, for the same has been shown to be true for pertussis vaccine and diphtheria toxoid and the same will also be true for any other antigen to which the mother has high levels of antibody at the time of parturition.

LIVE OR DEAD VACCINES?

Bacterial antigens, especially the exotoxins, are highly antigenic, stable and relatively easy to prepare. Experience with these antigens was of little help in preparing a killed virus vaccine, as in the case of poliomyelitis. All attempts to kill this virus and maintain its antigenicity were unsuccessful for many years until

Salk used a 1:10,000 dilution of formaldehyde and incubated the virus suspension for 10–13 days at 37°C (Salk, *et al* 1953; Salk 1956). This proved to be the optimal concentration for virus inactivation, killing the virus whilst retaining antigenicity, and subjects inoculated with killed vaccine gave high antibody responses. This concentration of formaldehyde although satisfactory for poliomyelitis cannot be taken as being satisfactory for all viruses and many attempts have been made to make a killed virus B vaccine without success. Formalin has been used successfully, however, to kill the viruses in making vaccines against adeno, equine encephalomyelitis, influenza, Japanese (B) encephalitis, mumps, measles, rabies and Russian spring-summer encephalitis virus infections; but the concentration used has been a matter of trial and error.

For some virus diseases attempts have been made to prepare live attenuated vaccines which have lost their virulence but maintained their ability to stimulate immunity against the virulent wild strains. One of the first living virus vaccines was that used by Jenner when in 1797 he used cowpox to protect a child against smallpox (Jenner 1798). Jenner believed cowpox to be smallpox of cattle, but did not clearly understand why its pathogenicity for man was reduced. Attenuated viruses have probably undergone a loss mutation; but vaccinia virus strains may have originated as hybrids of cowpox and variola viruses (Bedson & Dumbell 1964). Whatever the mechanism, virulence differences do not seem to be necessarily related to antigenicity and it is therefore not only possible to find avirulent mutants and strains by chance, but also to seek them consciously. Another example of an attenuated vaccine was discovered fortuitously when the now famous 17D strain of yellow fever was isolated (Lloyd, Theiler & Ricci 1936). Theiler and his associates propagated the Asibi strain of yellow fever in minced chick embryo tissue without brain and cord and found that after about 100 passages the virus had become attenuated. This strain when inoculated by extraneural routes in monkeys no longer caused illness and only small amounts of virus circulated in the blood. Furthermore, the infection could not be transmitted from monkey to monkey by the bite of the mosquito *Aëdes aegypti*. The virus had lost its virulence and today many millions of doses of this live virus vaccine have been used successfully against yellow fever. Virulent strains of poliomyelitis virus were attenuated by serial passage in cell cultures at low temperatures (33°–35°C) until they lost their virulence but maintained their ability to infect the human intestinal tract (Koprowski, Jervis & Norton 1952; Koprowski 1957; Sabin 1953, 1955, 1956, 1957, 1959). These strains were considered suitable for use in a vaccine and today after the world-wide administration of millions of doses we have ample proof of their value. The pattern is clearly becoming stereotyped; a wild virus able to infect experimental animals is passaged in cell cultures during which time the results of animal inoculation give an index of the degree of attenuation and at a suitable passage level a vaccine is made for use in humans. This series of events could well describe the attempts to

produce live measles vaccine (Enders, Katz, Milovanovic & Holloway 1960). The virus was isolated from the blood of a case of measles and many passages in human and monkey cell cultures were unable to alter its ability to cause viraemia or infect the tonsillar region of monkeys. Further passages in chick embryo cell cultures, however, decreased the virulence of the virus and viraemias were no longer obtained after animal inoculation. At this stage the live virus vaccine was used in man and many millions of doses have been given. The vaccine gives some mild reactions and in order to decrease the incidence and severity of reactions further attenuation was effected by propagating the virus in chick embryo cell cultures an additional 70 to 80 times. The further attenuated virus is still able to infect man and causes far less reaction (Schwarz 1961).

Many believe that live virus vaccines carry a greater risk and it is pertinent to ask the reasons for using a live vaccine when there is already a killed product available. In many countries millions of doses of killed poliomyelitis vaccine have been used and the incidence of the disease has been markedly reduced. In the outbreaks of poliomyelitis in England in 1961 more than 88% of the paralytic cases had been either inadequately vaccinated or had no vaccine at all. It must be concluded that the killed vaccine given in a schedule known to induce high antibody titres is able to protect against paralysis. Unfortunately, in the first 6 years after vaccination began in Britain only 75% of subjects under 18 years of age were vaccinated. Even more serious were the short sharp outbreaks of paralytic poliomyelitis that still occurred. From Sweden it has been reported that more than 90% of younger subjects have been adequately vaccinated with potent killed vaccine and outbreaks of paralytic poliomyelitis no longer occur. Perhaps the most important factor for the success in Sweden is the widespread use of vaccine reaching almost all subjects. Although killed vaccine has been successful in drastically reducing or even eliminating outbreaks of paralytic poliomyelitis it has not been able to prevent all vaccinated subjects from being reinfected and thus shedding the virus. The normal cycle of transmission of the virus in the community is not broken and the vaccinated subject may be a carrier of the virus. It is known that a subject naturally infected with poliomyelitis virus becomes resistant to reinfection by the specific virus type and similarly infections with the attenuated virus vaccine strains leave the subject refractory to reinfection. The mechanism of this gut immunity is not entirely clear, for whilst some believe it to be due to a tissue immunity it could equally well be due to the local production of antibody, but from the practical standpoint the result is the same. An advantage of the oral poliomyelitis is that it can be used in the face of an epidemic resulting in its collapse.

There are very few virus infections which lend themselves so ideally to prophylaxis by an oral vaccine since with poliomyelitis a natural infection by an attenuated strain can be induced so easily. The other live virus vaccines which are injected have an advantage over killed prophylactics because of the relatively

large quantity of antigen produced in the body which must greatly exceed that injected in a killed vaccine. Inducing an infection by an attenuated virus in a healthy body is likely to give a more lasting immunity and is considered by many to be the prophylactic of choice. The live virus vaccines already established (vaccinia, yellow fever, etc.) were accepted long ago and killed prophylactics would have to go through extensive trials before replacing them.

PROTECTION (ACTIVE AND PASSIVE IMMUNIZATION) AGAINST VIRUS DISEASES

For many virus diseases the first means of prophylaxis was passive immunization with pooled adult serum or preferably convalescent serum. Extraction of γ-globulin from serum provided us with much more concentrated antibody and it is natural that its use has met with much more success. With the successful development of vaccines the use of serum therapy is almost eliminated, but there are virus diseases today, notably rubella, for which our only defence weapon is γ-globulin.

Although serum therapy is useful as an emergency measure in preventing or attenuating a disease in a contact subject the passive immunity is transient. Active immunization against all the virus diseases to which the community is at risk and for which there is an effective prophylactic should be the aim for every member of the community. The virus diseases for which there are therapeutic or prophylactic measures available or under consideration are discussed below.

Respiratory Disease Vaccines

Influenza

Infection with influenza virus remains localized to the respiratory tract which may be one reason for the relatively poor immune response by the host. Both killed and live attenuated vaccines have been used to immunize against influenza, but the assessment of their value has been complicated by the appearance of antigenic variants to the different virus strains. The first influenza A strain was isolated in 1933 and antigenic variation within this strain was recognized when viruses recovered from different epidemics were not antigenically identical. In 1946 an antigenic variant of influenza A appeared in Australia. This spread throughout the world and all epidemics for the next few years were caused by the A1 (A prime) strain but in 1956 another new A variant appeared named A2 or Asian virus which was responsible for the pandemic of 1957. The antigenic differences between the influenza A viruses are small compared with the marked difference in the antigenic properties of a virus which caused influenza in New York in 1940 and, to differentiate it, this was called Type B. Antigenic variants

of the Type B have also been found since 1940 although the differences between them are not so marked as those between the Type A strains. In 1949 a virus from a sporadic case of influenza was isolated and found to be antigenically distinct from Types A and B and was termed influenza Type C; but this strain has only been found in minor respiratory cases and has not been involved in any epidemic.

The production of an effective prophylactic therefore requires the inclusion of strains of both Types A and B isolated during the most recent epidemic and unless a new variant appears in the current epidemic there is much evidence to show that the vaccine will be effective. The killed vaccine is prepared by harvesting the embryo material or extra-embryonic fluids at a suitable time after inoculation of the allantoic fluid with the virus. The harvested material is purified, killed with formalin and usually concentrated by centrifugation or adsorption on to red cells. The type A and Type B components are standardized by haemagglutination titrations before dilution and blending into a vaccine. Two adequately spaced doses of killed vaccine are recommended for primary immunization although only one dose may be given when an epidemic is imminent. Provided the strain composition and the timing of the single dose in relation to the appearance of the infection are both correct the protective effect may amount to 75–90%. In mass campaigns, however, much lower protection rates have been reported and more effective vaccines are needed. For optimal protection, a single booster dose should be given in the autumn of each year. In some vaccines a mineral carrier or oily adjuvant is incorporated and although higher antibody responses have been obtained they have not received general acceptance.

A second method of immunization is that developed in the U.S.S.R. where living attenuated virus is sprayed into the nose. The vaccine is an egg or tissue culture preparation of a strain passaged in eggs or cell cultures and selected because of its ability to multiply in the nose and appear in nasal secretions. Unlike the killed vaccine this live prophylactic is said to be effective even when used in the face of an influenza epidemic. Clinical reactions are minimal in adults but may be severe in young children. The same successes with live attenuated vaccines have not been obtained in Australia or England.

Recent findings show that the isolated haemagglutinin of the virus is immunogenic and does not give rise to the febrile and toxic reactions seen after giving the whole virus. At the moment, however, the procedure for preparing such vaccine is not economically practical.

Para-Influenza

This virus is the cause of some respiratory diseases in children. Experimental killed alum-adsorbed vaccines have been found to give good antibody responses in man but their protective efficacy has not been evaluated.

Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is a cause of both upper and lower respiratory tract illnesses in young children. Alum-adsorbed killed vaccines give an antibody response in man but their protective value is not yet known.

Adenovirus

Both killed and live vaccines have been used in America on a large scale, particularly in military units, and shown to give good protection. Recently, however, it has been shown that some serotypes of this group (including 3, 7, 12, 18 and 31) are oncogenic. Although an enteric coated capsule of adeno 4 living virus vaccine is still being used with success, the other vaccines may have to await purification of virus components.

Rhinovirus

Rhinoviruses are the main cause of common colds in adults and of both upper and lower respiratory tract disorders in children. Since there are already over fifty distinct serotypes isolated and with the numbers increasing rapidly, the possibility of controlling the infections with a vaccine is remote.

Smallpox Vaccine

Variola Major and Variola Minor

Passive immunization with adequate doses of γ-globulin prepared from the plasma of recently vaccinated donors may be expected to protect unvaccinated household contacts of smallpox or persons last vaccinated many years previously. Generalized vaccinia and vaccinial infections of the eye may also be controlled by γ-globulin: the dose varies from 500 mg to 2 g depending upon the age of the patient.

Smallpox vaccine is generally prepared from the vesicular eruption scraped from the skin of animals, e.g. calves, water-buffaloes or sheep. To each gram of pulp 1 ml of trifluorotrichloroethane (Arcton 113) is added and a 10% (w/v) suspension of the vaccinial material is made by mechanical homogenization in dilute buffer solution (pH = 7.4) containing 0.5% (w/v) phenol. During subsequent slow centrifugation up to 70% of the non-virus material is sedimented in the Arcton fraction (S.G. = 1.5). The supernatant fluid is incubated at 25°C for 24 hr to reduce the bacterial count and when the bacterial contamination of the extract is within the permitted limits glycerol and peptone are added to concentrations of 40% and 1% respectively. The bulk vaccine is assayed for potency and stored at −15°C. Before issue it is assayed again and the potency adjusted to 5×10^8 pock-forming units per ml by dilution with a solution of glycerol and peptone, with the addition of Brilliant Green dye to a concentration of 5g/litre. The vaccine retains its potency for 12 months if stored continuously below

− 10°C. After its removal from cold storage the permitted life of the vaccine is 14 days if stored below + 10°C and not more than 7 days if stored above + 10°C.

Suspensions of vaccinia virus partially purified by the method described above may be freeze dried in the presence of 5% peptone. In the dry state smallpox vaccine may be stored at tropical room temperature for many months or even years; after reconstitution its stability is the same as that of glycerolated vaccine.

Vaccinia virus may also be grown in chick embryo chorioallantoic membranes or monolayer cultures of chick embryo cells. The resulting vaccine is bacteriologically sterile and equal in prophylactic value to the conventional product.

Except in areas where smallpox is endemic primary vaccination should be delayed until the second year of life because of the reduced risk at this age of encephalomyelitis and other complications. On the 3rd or 4th day after primary vaccination a red papule appears at the site of insertion of virus. Within the next day or two the papule develops into a vesicle which becomes umbilicated and surrounded by a clearly demarcated areola. By the 8th or 9th day the vesicle becomes pustular, the areola extends and there is usually a noticeable systemic reaction from about the 7th day. The patient is generally febrile for a day or two and there may be an axillary lymphadenopathy. The inflammation regresses and by the 12th or 14th day the lesion is dry and crusted. The crust generally separates by about the 20th day leaving a depressed pink scar which during the next few months shrinks and, in white skins, becomes less pigmented than the surrounding skin. In persons of negro ancestry there may be a tendency to keloid formation.

Revaccination may be followed by an accelerated reaction, a so-called 'immediate' reaction, or no reaction at all. In the accelerated reaction the vesicle forms by the 3rd or 4th day and heals rapidly. This reaction is generally thought to signify some residual immunity in the subject which will have been increased by the successful infection with vaccinia virus. The so-called immediate reaction is really a delayed hypersensitivity reaction. It is sometimes known as the 'reaction of immunity' but this is quite inadmissible since no indication of the immune state of the individual can be elicited by clinical examination of the lesion.

The World Health Organization's Expert Committee on Smallpox recommended that both primary and re-vaccinations should be examined after a week. 'A vesicular or pustular reaction *or* an area of definite palpable induration or congestion surrounding a central lesion which may be a scab or ulcer' is a 'major reaction'. Anything else is an 'equivocal reaction'. Only major reactions indicate virus multiplication with consequent development or reinforcement of immunity. The absence of a major reaction is always an indication for another attempt at vaccination or revaccination—possibly with a different batch of vaccine if there is any doubt about the potency of the original batch used.

After successful vaccination, immunity to variola major is probably complete for 3 years, although individuals may vary; thereafter the risk of infection on exposure increases with each year until there is virtually no protection 20 years after primary vaccination. The risk of death from smallpox, however, is greatly diminished even very many years after successful vaccination. The efficacy of the immunity probably depends in some measure on the degree of exposure to variola virus. Continued heavy exposure of e.g. medical personnel in endemic foci is an indication for frequent (perhaps annual) revaccination. The general population in endemic areas should be revaccinated every 3 years and preferably not less seldom than every 5 years. In non-endemic areas adequate immunity will probably be maintained by revaccination at intervals of about 7 years.

Any vaccination procedure using infectious virus must be expected to produce some complications, not all of which will be preventable. The most dreaded complication is post-vaccinial encephalitis which, in the United Kingdom, has a very low incidence. The most dangerous complication is eczema vaccinatum—generalized vaccinia following the vaccination of a patient suffering from atopic eczema. This is largely preventable; eczematous subjects should not be vaccinated, nor put at risk by the vaccination of other members of the household. Eczema and other skin lesions clearly contraindicate vaccination except when there is a real risk of exposure to smallpox.

Herpes Group Virus Vaccines

Chickenpox (Varicella)

Although the virus has been grown in cell cultures it will not grow in cultures acceptable for vaccine production. A vaccine, therefore, is not available.

Convalescent serum has been used in the prevention of varicella but the results have been conflicting. Good results have been claimed with use of γ-globulin but controlled trials have not substantiated these claims.

Herpes Simplex (Febrilis)

There is some indication especially from America that in cases of disseminated herpes γ-globulin is effective but in England it is thought that no specific preventive measures are practicable.

Neurotropic Virus Vaccines

A. Arbovirus Vaccines

Yellow fever

As already mentioned, during the passage of the Asibi strain of yellow fever virus in tissue culture an attenuated variant, 17D, was isolated. The vaccine is

prepared by grinding up chick embryos infected with the living attenuated 17D strain and finally drying from the frozen state. The vaccine is reconstituted in sterile normal saline and a single dose, usually 0.5 ml, is injected subcutaneously. All reconstituted vaccine not used within 30 min should be discarded because of the instability of the virus. Only one injection is necessary and if it is absolutely essential a full dose may be given to infants in the first week of life. Where infants have to be inoculated against yellow fever and smallpox it is generally agreed that an interval of at least 21 days should elapse between the two inoculations but the W.H.O. Expert Committee on Yellow Fever Vaccine considered that the use of combined yellow fever and smallpox vaccine (by scarification) should not be condemned. Immunity following a satisfactory yellow fever inoculation is present for at least 10 years. Administration of the 17D vaccine by scarification although possible is not as effective as by subcutaneous injection. Two scratch vaccinations separated by an interval of 14 days using a mouse brain 17D vaccine have been shown to give 98% protection and may soon be acceptable for general use.

Western equine encephalomyelitis (WEE) and eastern equine encephalomyelitis (EEE)

These viruses are found in the west and east of America, Venezuela and the Caribbean region.

Vaccines against western equine encephalitis and eastern equine encephalitis have been prepared by formalin treatment of the virus grown in chick embryo tissue. Although these vaccines, containing one or both strains, are mainly used to prevent the disease in horses, two injections being given annually, they have also been used for laboratory workers in contact with the viruses. The incidence in outbreaks is so low, however, that general use of these vaccines is not indicated. The causal agent of the sporadic encephalitis of Great Britain is unknown.

Japanese B encephalitis (JBE)

This disease is the new form of encephalitis occurring in Japan so-called to distinguish it from von Economo's disease, or epidemic encephalitis lethargica which was called Japanese A encephalitis. A vaccine consisting of a 10% suspension of infected mouse brain in normal saline with the virus killed by 0.2% formalin at 4°C was given to hundreds of thousands of American troops in the Far East between 1942 and 1945. In 1942 the Russians used a similar vaccine which was effective against Russian autumn (Japanese B) encephalitis. A chick embryo vaccine was used over a 3-year period in children in an endemic area in Japan and a reduced incidence of illness resulted in the vaccinated group.

The use of this vaccine has been abandoned by the U.S. Armed Forces and a more purified mouse-brain preparation (protamine-treated) is being used for the vaccination of children in Japan and some other countries. Recently the virus has

been attenuated by workers in Japan and in the U.S.A.; the attenuated strain is grown in hamster kidney cells in a serum-free medium and the killed vaccine prepared from it is believed by some to be safer and more antigenic.

Russian spring-summer (RSSE) or tick-borne encephalitis (TBE)

This tick-borne disease was first described in 1953 occurring in forest areas of the far eastern U.S.S.R. and later in Siberia and western U.S.S.R. A formalin-treated vaccine prepared from a suspension of infected mouse brains has been used successfully in both man and animals. In man, incidence of disease amongst the vaccinated was reduced to 10% and there were no fatal cases in this group.

Recently an aluminium adsorbed killed vaccine grown in chick fibroblast cell cultures has been used on a large scale. A primary course of three injections followed by a fourth dose 4–6 months later stimulates production of antibody in 90–95% of those vaccinated. Annual booster doses for 3 years are indicated.

Dengue

Low passage mouse-adapted strains of dengue virus Type I and II have produced a modified disease in man giving solid homologous immunity and an immunity lasting a few months against heterologous strains. A more attenuated Type I strain has also provided protection. No data are available on the transmissibility of the vaccine strain from man to man by *Aëdes aegypti*.

Epidemic haemorrhagic fever of unknown aetiology has occurred in North Korea and Manchuria and the acute disease was treated with convalescent serum. Recent epidemics in Bangkok, Singapore and Calcutta were associated with dengue viruses; and in South India Chikungunya virus was isolated from all but a few cases (Myers *et al* 1965). Attenuated vaccines of the type mentioned above may possibly be useful in prophylaxis of this disease in the future.

Rift Valley fever

A killed vaccine grown on monkey kidney or hamster kidney cell cultures and killed with formaldehyde has been shown to be safe and immunogenic, apparently giving protection to workers with this disease.

Louping-ill

Formalin treatment of infected mouse brains has produced a vaccine which gives a high titre of neutralizing antibodies in immunized laboratory workers. Propagation of the virus in tissue culture and inactivation by ultraviolet light and β-propiolactone suggest that it may be possible to produce a suitable vaccine.

B. POLIOMYELITIS

Passive immunization against this disease is certainly not the method of choice. When a paralytic case occurs in a family it is more than probable that the family

contacts were exposed to the disease simultaneously or are already well into the incubation period and γ-globulin may not be effective. There are data, however, to suggest that when administered in the early stages of the disease, before the virus has infected the nerve cells, γ-globulin may help to neutralize the viraemia. There are special cases where γ-globulin may be used; children who have recently undergone tonsilectomy are at special risk, as are nurses and medical students entering an infectious area. A dose of 500 mg of γ-globulin for infants under 1 year, 1 g for children 1–6 years and 1.5 g for children 7 years and older should be used.

Poliomyelitis is now a preventable disease and the advantages and disadvantages of the killed and live attenuated prophylactics have already been discussed earlier in this chapter. These vaccines have been subjected to more stringent controls than any other prophylactic and both vaccines are undoubtedly safe.

The killed vaccine is produced by infecting monkey kidney cell cultures separately with each type of poliomyelitis virus (Brunenders Type 1, MEF-1 type 2, Saukett Type 3). The virus suspensions are harvested 3 days later, killed with formalin and blended to make a trivalent vaccine. A full course of immunization consists of two doses given by the intramuscular route at an interval of 4 weeks followed by a third similar dose given 9–12 months later. A fourth dose is recommended 5–6 years later but nothing is known of the necessity for further booster doses to maintain lifelong immunity. The killed vaccine can be incorporated with diphtheria, tetanus and pertussis prophylactics into a quadruple vaccine.

The live vaccine is also made by infecting monkey kidney cell cultures but with attenuated virus strains shown to be safe by large-scale clinical trials. The virus suspensions are harvested 3 days later and stringent control procedures ensure, as far as possible, that no changes have taken place from the original strains during the course of production. In many countries the vaccine is given orally as monovalent virus in the order of Type 1 followed by Type 3 and finally Type 2 with an interval of 6 weeks between each dose. In this country, in Canada and in Russia three doses of trivalent vaccine are given at 6–8 week intervals and a reinforcing dose is given at school entry. In Russia a dose of vaccine is given each year to children between 2 months and 3 years and little is known about the period of immunity following primary immunization.

Some of the other enteroviruses have caused sporadic cases and outbreaks of disease resembling poliomyelitis, with or without paralysis, notably the Karaganda B4 strain of Coxsackie virus Type A7.

c. Rabies (Hydrophobia)

Clinical rabies (hydrophobia) is inevitably fatal in man. The infection usually has a long incubation period after the introduction of the virus by the bite of a rabid animal. If a high level of immunity can be stimulated while the virus is slowly

travelling centrally via peripheral nerves its establishment in the brain may be prevented. Modern practice combines passive and active immunity to attain a high concentration of antibody as rapidly as possible. The majority of rabies vaccines used today consist of suspensions of virus-infected sheep or rabbit brain tissue (usually 5% w/v) and the virus may be inactivated by exposure to phenol (Semple type), β-propiolactone or ultraviolet irradiation. In the wet state rabies vaccine has a limited life even when stored between 2°C and 5°C. Lyophilized vaccine is more stable, but only a very small proportion of the total output of rabies vaccine is dried. The presence of nervous tissue in the vaccine makes it potentially dangerous since it may induce demyelination in the patient analogous to the 'allergic' experimental encephalomyelitis which can be produced in experimental animals by the injection of either homologous or heterologous brain tissue. The reported incidence of this complication of anti-rabic treatment varies from country to country, but is probably between 1/4000 and 1/10,000 of patients treated. In any case, even a 'light' course of treatment produces extremely painful local reactions to the later injections.

Anti-rabic treatment, therefore, must not be embarked on without careful consideration; but if it is decided that treatment is necessary, then every effort must be made to ensure that it is successful. Bites and other wounds should be given local treatment by thorough washing with soap or detergent and water. Bites by wild vectors (wolves, foxes, skunks, bats, mongooses, etc.) or severe exposure (multiple or face, head, neck or finger bites) call for the immediate application of antirabies serum, some of which should be infiltrated around and beneath the wound if this is possible. The recommended dose of serum is not less than 40 International Units/kg body weight. At least 14 daily doses of vaccine should be given (up to 21 after severe exposure); and where serum is given, supplemental doses of vaccine should be given 10 and 20 days after the completion of the treatment. If possible the supplemental vaccine should be non-encephalitogenic.

Vaccine prepared from non-neural tissue should be free from encephalitogenic material. To this end rabies virus strains have been adapted to growth in chick and duck embryos. Live avianized vaccines of attenuated virulence are not widely used because of the difficulties in producing batches of uniformly adequate potency. However, virus grown in duck embryos and inactivated by β-propiolactone does appear to be reasonably effective. A different approach to the preparation of safe rabies vaccine is to grow the virus in the brains of very young animals. Infected brains harvested before the start of myelination are a rich source of fixed rabies virus, but the brains appear devoid of encephalitogen when tested in guinea-pigs. Vaccines prepared in Chile, the U.S.S.R. and Holland from the brains of suckling mice, rats and rabbits have had very satisfactory potencies by the usual tests; and human subjects immunized with them have developed higher concentrations of neutralizing antibody than controls

immunized with conventional nervous tissue vaccines. However, the large numbers of immature animals needed and the difficulties associated with their care make it improbable that this type of vaccine will displace the more usual Semple type except for limited use in special circumstances.

Rabies antiserum is usually produced in horses. The animals are primed by a course of injections of inactivated fixed virus in nervous tissue and subsequently hyperimmunized by repeated courses of fully infectious fixed virus. Plasma from hyperimmune horses may be concentrated and refined by the same methods used for antitoxic sera, including peptic digestion. In practice, reactions to even refined globulins have about the same incidence as reactions to other refined antisera of animal origin, and the usual precautions should be taken to avoid them.

Active immunity to rabies will remain in an unsatisfactory state until highly potent, safe, inactivated vaccines are available. Current research on the suitability of human diploid cells, and other types of cell culture, as substrates for the growth of high concentrations of rabies virus may lead, in the foreseeable future, to that goal.

Other Virus Vaccines

Hepatitis

Epidemics of infectious hepatitis (viral hepatitis A) have been successfully controlled by injection of γ-globulin prepared from normal adult serum although this has been relatively ineffective against serum transfusion hepatitis (viral hepatitis B). Prophylactic γ-globulin is recommended to all contacts of infectious hepatitis, especially pregnant and post-menopausal women, as early as possible after exposure and confers protection even if given as late as 6 days before the onset of symptoms. The dosage is 250–750 mg for subjects under 10 years of age and 500–1500 mg for older subjects. Modern production methods ensure that the γ-globulin is virus free; in the absence of γ-globulin, plasma or serum should on no account be substituted.

Although there is no vaccine available today to control this disease the virus has been isolated in cell culture and attempts are being made to develop a prophylactic.

Measles (Rubeola)

This highly infectious disease can be attenuated or completely prevented with γ-globulin, prepared from pooled adult serum. Complete protection is desirable for children under 2 years of age or in poor health but for those in good heath an attenuated attack is preferable since a more permanent immunity is achieved. The γ-globulin should be given by the intramuscular route and 250 mg will probably attenuate the disease in all children over 1 year of age and prevent it in babies under 1 year. To prevent the disease in children between 1 and 3 years

500 mg should be given. The dose for older children is 750 mg. In children given γ-globulin to attenuate an attack, contact should be close to ensure infection.

Early attempts to produce a killed vaccine by formalin treatment of virus grown in either monkey kidney or dog kidney cell cultures met with little success. The antibody responses to such a vaccine were very poor although a single dose of killed vaccine even in the absence of antibody response was able to protect against the rash and febrile reaction caused by the injection of a live attenuated vaccine. Concentration of the killed vaccine and especially the addition of a mineral carrier has greatly improved the antibody responses but the duration of immunity is short.

The first vaccine produced from a live attenuated strain was grown in chick embryo cell cultures and clinical trials showed it to be insufficiently attenuated; rashes appeared in 45% of the infants and the average temperature was 102.6°F with some subjects having Koplik's spots. In spite of these reactions studies of encephalograms after injection of this vaccine have revealed little or no disturbance in the brain whereas brain changes occur in as many as 45% of subjects infected by the natural disease. Later studies with this vaccine have shown that a simultaneous injection of a small dose of γ-globulin at a different site from the vaccine injection greatly minimizes the rash and febrile reaction without decreasing the antibody response. Similarly a single dose of killed vaccine given 4–6 weeks before the live vaccine is also able to decrease the incidence of reactions without affecting the antibody response.

Several further attenuated strains of reduced pathogenicity have also been developed. These vaccines, though provoking a lower incidence of reaction, give antibody responses in as high a proportion of vaccines as the less attenuated strains. The duration of immunity given by the vaccines, however, is not known, but it is at least 2–4 years.

German Measles (Rubella)

There is a well-recognized connection between maternal rubella during the first 3 months of pregnancy and miscarriages, stillbirths and congenital defects such as mental retardation, deaf mutism, cardiac abnormalities and cataract. Until an effective vaccine is available young girls should be exposed to infection by contact so that they become immune before child-bearing age. Although γ-globulin is useless in treatment it has been used successfully to prevent infection in contacts. The best results were obtained with 1.5 g given within a week of exposure. Delay should be avoided and where there is more than one exposure at intervals of longer than 2 or 3 weeks a further dose should be given at each contact. Administration of γ-globulin is unnecessary after the first 3 months of pregnancy as rubella does not appear to infect the foetus in the later months.

In 1966 Parkman and Meyer in America announced the successful attenuation of the rubella virus after 77 passages in African green monkey kidney cell

cultures. The attenuated virus was given to children in a nursery with some uninoculated children serving as controls. Antibody appeared in all inoculated children but in none of the controls. Although the virus could be isolated from some of the inoculated children all specimens from the control children were negative. This attenuated strain is being used for the preparation of vaccine for clinical trials on a wider scale.

Mumps (Infective Parotitis)

Gamma-globulin prepared from adult donors is valueless. Gamma-globulin prepared from mumps convalescent serum given in 20 ml doses within 24 hr after the onset of illness has reduced the incidence of orchitis to 7.7% as compared with 27.4% in controls.

A killed vaccine has been prepared by formalin treatment or ultra-violet irradiation of virus grown in chick embryos. Although the early results are encouraging, the efficacy of the vaccine has not been assessed. Only a really effective and lasting immunity to mumps would justify the use of a mumps vaccine in children, since a transient or partial immunity would leave them susceptible to the disease after puberty when complications such as orchitis would be harmful.

Parenteral administration of an attenuated virus has recently been reported to give antibody responses and immunity. This vaccine is being tested in large-scale trials apparently with encouraging results.

Trachoma

Trachoma is the greatest single cause of progressive loss of sight in the world. The agents causing trachoma and inclusion conjunctivitis (TRIC) are closely related and belong to the genus *Bedsonia*. Vaccines so far tested in man have been prepared in yolk sacs of chick embryo. In most trials killed vaccines have been used, but there are indications that live vaccines may be more effective. The latest conclusions are that TRIC agents are poor antigens and a fully effective vaccine is not available.

FUTURE TRENDS

We have seen how serum prophylaxis is regarded as an emergency measure giving only transient immunity and if serum or plasma is used there is the danger of serum sickness or hepatitis which is eliminated by the use of γ-globulins. Active immunization is the method of choice and where safe inactivated and live attenuated prophylactics are both available the vaccination policy for each country should be based on careful consideration of all medical, economic, administrative and biological factors involved.

Research in the field of virus vaccines is most active and it is quite possible that the diseases caused by the respiratory and enteric (other than poliomyelitis) viruses will be brought under control by effective prophylactics. Not only new vaccines will appear but new methods of production on more carefully controlled tissue cell strains are being actively pursued. Looking to the future we must wonder how long it will be before cancer agents are controlled prophylactically.

SUGGESTIONS FOR FURTHER READING

BEDSON S.P., DOWNIE A.W., MACCALLUM F.O. & STUART-HARRIS C.H., *Viruses and Rickettsial Diseases of Man*, 3rd edn. London: Edward Arnold.

BURNET F.M. *Viruses and Man*. London: Penguin Books.

PARISH H.J. & CANNON D.A., *Antisera, Toxoids, Vaccines and Tuberculins in Prophylaxis and Treatment*, 5th edn. Edinburgh: E. & S. Livingstone.

RHODES A.J. & VAN ROOYEN C.E., *Textbook of Virology*, 3rd edn. Baltimore: Williams & Wilkins.

WORLD HEALTH ORGANIZATION, *Technical Report Series No. 325 (1966)*. "Human Viral and Rickettsial Vaccines".

REFERENCES

ANDREWES C.H. (1928) The action of immune serum on vaccinia and virus III *in vitro*. *J. Path. Bact.* **31,** 671.

ANDREWES C.H. (1929a) Virus III in tissue culture. 1. The appearance of intranuclear inclusions *in vitro*. *Brit. J. exp. Path.* **10,** 188.

ANDREWES C.H. (1929b) Virus III in tissue culture. 2. Further observations on the formation of inclusion bodies. 3. Experiments bearing on immunity. *Brit. J. exp. Path.* **10,** 273.

ANDREWES C.H. (1930a) Antivaccinial serum. 3. Evidence for slow union with virus *in vitro*. *J. Path. Bact.* **33,** 265.

ANDREWES C.H. (1930b) Immunity to the salivary virus of guinea-pigs studied in the living animal, and in tissue cultures. *Brit. J. exp. Path.* **11,** 23.

BEDSON H.S. & DUMBELL K.R. (1964) Hybrids derived from the viruses of variola major and cowpox. *J. Hyg., Camb.* **62,** 147.

BEDSON S.P. (1928) Observations on the mode of action of a viricidal serum. *Brit. J. exp. Path.* **9,** 235.

BEDSON S.P. & CRAWFORD G.J. (1928) Immunity in experimental herpes. *Brit. J. exp. Path.* **8,** 138.

ENDERS J.F., KATZ S.L., MILOVANOVIC M.V. & HOLLOWAY A. (1960) Studies on an attenuated measles virus vaccine. I. Development and preparation of the vaccine; techniques for assay of effects of vaccination. *New Engl. J. Med.* **263,** 153.

ENDERS J.F., WELLER T.H. & ROBBINS F.C. (1949) Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. *Science* **109,** 85.

HAYFLICK L. & MOORHEAD P.S. (1961) The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585.

JENNER E. (1798) *An Inquiry into the Causes and Effects of the Variolae Vaccinae*. S. Low, 1798. Republished 1896 by Cassell & Co.

KOPROWSKI H. (1957) In discussion on Sabin's paper entitled 'Properties of attenuated polio. viruses and their behaviour in human beings'. *Spec. Publ. N.Y. Acad. Sci.* **5,** 128.

KOPROWSKI H., JERVIS G.A. & NORTON T.W. (1952) Immune responses in human volunteers upon oral administration of a rodent-adapted strain of poliomyelitis virus. *Amer. J. Hyg.* **55,** 108.

LANDSTEINER K. & POPPER E. (1909) Vebertragung der Poliomyelitis acuta auf Affen. *Z. Immun.-Forsch.* **2,** 377.

LLOYD W., THEILER M. & RICCI N.I. (1936) Modification of the virulence of yellow fever virus by cultivation in tissues *in vitro*. *Trans. roy. Soc. trop. Med. Hyg.* **29,** 481.

MORGAN I.M., SCHLESINGER R.W. & OLITSKY P.K. (1942) Induced resistance of the central nervous system to experimental infection with equine encephalomyelitis virus. I. Neutralizing antibody in the central nervous system in relation to cerebral resistance. *J. exp. Med.* **76,** 357.

MYERS R.M., CAREY D.E., REUBEN R., JESUDASS E., RANTZ C.D.S. & JADHAV M. (1965) The 1964 epidemic of dengue-like fever in South India: isolation of Chikungunya virus from human sera and from mosquitoes. *Ind. J. Med. Res.* **53,** 694.

PARKMAN P.D. & MEYER H.M. (1966) *Development and Characterization of a Live Attenuated Rubella Virus. New Eng. J. Med.* **275,** 569.

PERKINS F.T., YETTS R. & GAISFORD W.F. (1958) Serological response of infants to poliomyelitis vaccine. *Brit. med. J.* **ii,** 68.

PERKINS F.T., YETTS R. & GAISFORD W.F. (1959a) Poliomyelitis immunization in infants in the presence of maternally transmitted antibody. *Brit. med. J.* **i,** 404.

PERKINS F.T., YETTS R. & GAISFORD W.F. (1959b) A comparison of the responses of 100 infants to primary poliomyelitis immunization with two and with three doses of vaccine. *Brit. med. J.* **i,** 1083.

PERKINS F.T., YETTS R. & GAISFORD W.F. (1961) Response of infants to a third dose of poliomyelitis vaccine given 10 to 12 months after primary immunization. *Brit. med. J.* **i,** 680.

SABIN A.B. (1935) The mechanism of immunity to filterable viruses. I, II, III, IV. *Brit. J. exp. Path.* **16,** 70, 84, 158, 169.

SABIN A.B. (1953) Present status and future possibilities of vaccine for control of poliomyelitis. *Amer. J. Dis. Child.* **86,** 301.

SABIN A.B. (1955) Behaviour of the chimpanzee-avirulent poliomyelitis viruses in experimentally infected human volunteers. *Amer. J. med. Sci.* **230,** 1.

SABIN A.B. (1956) Present status of attenuated live-virus poliomyelitis vaccine. *J. Amer. med. Ass.* **162,** 1589.

SABIN A.B. (1957) Properties of attenuated polioviruses and their behaviour in human beings. *Spec. Publ. N.Y. Acad. Sci.*, **5,** 113.

SABIN, A.B. (1959) Present position of immunization against poliomyelitis with live virus vaccines. *Brit. med. J.* **i,** 663.

SALK J.E. (1956) Requirements for persistent immunity to poliomyelitis. *Amer. J. med. Sci.* **232,** 369.

SALK J.E., BENNETT B.L., LEWIS L.J., WARD E.N. & YOUNGER J.S. (1953) Studies in human subjects on active immunization against poliomyelitis. I. A preliminary report of experiments in progress. *J. Amer. med. Ass.* **151,** 1081.

SCHWARZ A.J.F. (1961) Preliminary tests of a highly attenuated measles vaccine. *Amer. J. Dis. Child.* **103,** 216.

SMITH W., ANDREWES C.H. & LAIDLAW P.P. (1933) A virus obtained from influenza patients. *Lancet* **ii,** 66.

STOKES A. BAUER J.H. & HUDSON N.P. (1928) Experimental transmission of yellow fever to laboratory animals. *Amer. J. trop. Med.* **8,** 103.

THEILER M. (1930) Studies on the action of yellow fever virus in mice. *Amer. trop. Med. Parasit.* **24,** 249.

TODD C. (1928) On a dilution phenomenon observed in the titration of the serum of fowls immunized against the virus of fowl plague. *Brit. J. exp. Path.* **9,** 244.

CHAPTER 46

IMMUNOPROPHYLAXIS OF PROTOZOAL DISEASES

P.E.C.MANSON-BAHR AND B.WEITZ

INTRODUCTION

The complexity of the host-parasite relationships in infections of man and animals with protozoa affects the development of immunity of the host in a variety of ways. The peculiar and still undetermined nature of the antigens of protozoa and the specific affinity of the parasites for certain organs or tissues during their development results in a very variable immune response which depends largely on the intimacy of the contact of the antigens with the antibody-producing centres in the host.

The diverse methods of entry of the parasite into the host (i.e. cyclical or mechanical transmission by arthropods; ingestion; venereal infection or even contact infection), may either delay or minimize the immune response of the host and give the invading organisms a chance to establish themselves in some tissues not affected by the antibody response. The development of some pro-

tozoa, such as coccidia, in the gut or the intestinal cells is not conducive to a generalized antibody response although the susceptible cells may eventually develop an intolerance to the organism. The characteristic periodicity of haematogenous protozoal infections (piroplasmosis, malaria, trypanosomiasis) is partly determined by the host's specific defence mechanisms and partly by the reaction of the parasites to the host's defences.

All these factors affect the final protection of the host against infection or against the development of the parasites in the body to a very variable extent. It is therefore not surprising that vaccination of the host against such protozoal infections is generally not possible against the natural disease.

Host Susceptibility

Protozoa, as many other organisms, show a marked host specificity. Some species of animals sometimes contain specific antibodies against the parasites to which they are not susceptible and this has favoured the idea of an innate immunity in the host (Terry 1957). It is very doubtful if the intolerance of certain animals to a particular protozoal infection is due to a specific genetic immunity. The susceptibility of a host to infection is a complex state concerned largely with the micrometabolic requirements of the parasite and the invasive nature of the organism. For example, in man the presence of abnormal haemoglobins is known to affect susceptibility to malaria. A normally non-susceptible host can be experimentally infected by adapting the protozoon to its new environment, while leaving the state of the host unchanged, showing that the infection depends more on the nature of the parasite than on antibody defences of the host (Desowitz & Watson 1951, 1953).

A passive immunity transmitted by an immune mother to her offspring may give rise to a temporary 'natural immunity' which is converted to an acquired and permanent immunity by repeated challenge with the parasite.

Splenectomy is known to remove innate insusceptibility in certain instances by allowing an existing infection to proliferate more effectively or in exceptional cases by rendering the splenecticized host susceptible to an infection with a protozoon which is otherwise not tolerated. For example, a case of babesia infection in a splenectomized man has been reported (Bray 1960). However, the functions of the spleen are numerous and are not solely concerned with immunity.

Acquired Immunity

Acquired immunity may be a true sterilizing immunity with a clinical and parasitological cure when humoral antibodies develop or it may be a state of partial immunity or premunition (Sergent, *et al* 1924). This is characterized by the persistence of organisms which prevent superinfection with the homologous protozoon. The concept of premunition is a very debatable one.

As the antigens of many protozoa vary during infection, relapses occur at intervals caused by an organism with a different antigenic character from the original infecting strain. During the latent or occult infection which persists in between the relapses, the organisms proliferate and a new variant not susceptible to antibodies already present in the host may be selected naturally and proliferate. The antibody to the original infecting variant persists for the duration of infection and thus reinfection with the original strain may be unsuccessful although the persistence of infection is due to a variant differing from the infecting strain. Under these conditions it is difficult to be sure of the experimental validity of the premune state. It may be useful and convenient to accept the term 'premunition' to describe the clinical manifestation of these complex phenomena. Premunition in these terms is thus a selective resistance of the host to reinfection with the same antigenic variant: the host, however, remains a carrier capable of transmitting the antigenic variant which it carries at any time to another susceptible host. A premune host, however, remains susceptible to strains or species of the organism antigenically different from the original infecting strain and from the subsequent antigenic variants which have been produced in the host.

Immunoprophylaxis

The practical prophylaxis of protozoal diseases is, however, mainly restricted by the difficulty of obtaining antigens from the organisms. Very few organisms can be grown *in vivo* in sufficient amounts to make vaccines a real possibility. Even when cultivation of protozoa is possible the antigenic nature of the organisms in culture is most frequently different from the blood-infecting organisms, rendering them useless as a source of antigens. Until the proper cultivation of protozoa is realized on a large scale the discussion of artificially induced immunity is limited to the use of live strains of antigens obtained from organisms harvested from a suitable host.

In general the use of prophylactic or therapeutic chemotherapy in many protozoal diseases may produce a resistance of variable duration in some hosts. There is some evidence that chemotherapy in the face of natural infection is more effective than the treatment given in the absence of infection, indicating that the constant contact with the organism favours development of immunity under such conditions.

MALARIA

Host Susceptibility

In the truly susceptible host all stages of the parasite are found. In less susceptible hosts only the tissue stages may be found and only a low parasitaemia of the

blood occurs, as in *Plasmodium bastianelli* in man. In even less susceptible hosts tissue stages occur but only an occult infection of the blood is found as in *P. vivax* infection in chimpanzees (Bray 1957). In the completely immune host no tissue stages develop as in avian malaria in man or *P. vivax* infection in rhesus monkeys.

It has long been known that native peoples in highly malarious areas do not suffer from overt malaria to the same extent as immigrants. This may be due to the development of premunition in the indigenous people, but certain groups show a natural immunity to natural and artificial infection. In these groups the infection is held to a subclinical level and an acquired immunity may be superimposed. The relative insusceptibility of American negroes to *P. vivax* infection has been shown by Young, Eyles, Burgess & Jeffery (1955).

Allison (1954) noted that in areas where the sickle-cell trait was common there was much less malaria and postulated that possession of this gene conferred protection against malaria. It is now known that sickling is accompanied by some protection against *P. falciparum* infection in early life, thus allowing sicklers an advantage over non-sicklers which maintains the high rate of this gene (Allison 1957). It is suspected that possession of the genes responsible for thalassaemia, and haemoglobins C and F may similarly protect against malaria (Allison & Clyde 1961).

Nature of the Immune Response to Natural Infection

The malaria parasite liberates many antigens which stimulate the formation of antibodies in the host. Zuckerman (1964) has shown six to eight separate antigens in extracts of *P. vinckei* and *berghei*, several of which were shared, though the two plasmodia do not cross-immunize. These antigens could be responsible for group-specific but non-protective serological reactions described below.

The frequent antigenic variation in blood-induced *P. knowlesi* infections gives rise to a specific immune response for each relapse variant but some immunity, however, transcends the antigenic variation (Brown & Brown 1965). Complement-fixing antibodies and precipitins are genus specific and have been used in diagnostic tests (see Chapter 4). They are unimportant in protective immunity which is strain specific.

The allergic or immune response in malaria is predominantly cellular. Macrophages phagocytose parasitized erythrocytes, isolated malaria parasites and malarial pigment and increase progressively from a hyperplasia of the reticuloendothelial system and spleen.

Splenectomy and blocking of the reticuloendothelial system have the same effect since these procedures remove the greatest concentration of macrophages from the system. Following splenectomy a relapse of the blood infection occurs in *P. malariae* infections and an innate immunity may be weakened to allow

blood as well as tissue forms to develop, e.g. *P. vivax* infection in chimpanzees (Bray 1957).

Antibodies which are closely associated with IgG are active against the late-stage intracellular asexual parasites and against the liberated extracellular parasites but do not appear to have any obvious effect on the gametocytes. The antibodies show a surprising degree of common reactivity against different species of mammalian plasmodia (McGregor 1964). These antibodies can be transferred passively and provide a passive immunity (McGregor *et al* 1963), and are responsible for the high γ-globulin levels associated with a high incidence of malaria (Holmes, Stanier & Thompson 1955). The levels of antibody against plasmodia measured by the fluorescent antibody test are high at birth in a holoendemic malarious area, fall and remain low for a year and then rise gradually until adult life (Voller & Bray 1962). This pattern follows the same course as the development of premunition, and suggests that the fluorescent antibody test measures the protective antibodies responsible for premunition.

After sporozoite-induced malaria the protective antibodies develop rapidly and may persist for 20 months (Collins *et al* 1964). They thus indicate the degree of premunition in populations and the changes which may take place after control measures have been applied.

Acquired Immunity

There is no evidence in naturally acquired malaria of any immunity against sporozoites or exo-erythrocytic forms of the parasite. Sporozoite invasion, pre-erythrocytic development, subsequent primary infection of red blood cells, and even gametogenesis may not be directly inhibited in the immune subject (McGregor 1965). Premunition is the commonest form of immunity in malaria and develops as the result of trophozoite activity. In holoendemic areas where the most intense transmission occurs, infants acquire a transplacentally transmitted passive immunity against the sporozoite transmission of natural infection (Edozien *et al* 1962), for the first 3 months of life which is followed by acute infestation for up to 3 years. The greater degree of protection experienced up to 6 months may be the result of the protective effect of a milk diet which has been shown to have a suppressive effect on blood-induced *P. berghei* infection in rats (Maegraith, Deegan & Jones 1952) and in *P. cynomolgi* infections in rhesus monkeys (Bray & Garnham 1953).

A state of premunition follows for the duration of life. True holoendemicity is recognized by a high spleen rate of 70% or more in children, decreasing later in life (Wilson, Garnham & Swellengrebel 1950). Infants suffer severely from overt malaria and there is an appreciable mortality. Adults do not suffer from malaria and are apparently quite healthy. There are varying degrees of endemicity and in less endemic areas the acquisition of immunity is delayed until after puberty and adults may suffer from actual fever. Various forms of partial or

unsuccessful control of transmission may alter the immunological state of people so that the premunition may be lessened with the development of much malaria.

Premunition can be lost by residence in a non-malarious area, and severe attacks may be expected after return to the former malarious area (Colbourne 1955). Premunition is also found in *P. vivax* malaria; Blackburn (1948) records repeated artificial infections producing a state of solid clinical immunity in which perfectly fit individuals showed no splenomegaly and in whom *P. vivax* parasites could be demonstrated in the blood at any time.

Immunoprophylaxis

The state of premunition naturally prevents the disease and protects populations against the worst effects of malarial infection. In the absence of any plan to eradicate the disease completely this form of immunity cannot be bettered. Protection of the people is obtained at the cost of high infantile mortality. A combination of premunition and limited chemotherapy is the practice in most holoendemic areas of the tropics. Complete cure is not attempted and antimalarial drugs are used in just sufficient quantities to keep symptoms down without disturbing the state of premunition of the population. In recent years the appearance of drug-resistant parasites and insecticide-resistant mosquitoes has made the search for some form of immunoprophylaxis urgent. The use of artificially induced immunity has been confined in man to passive immunization, although active immunization has been achieved experimentally in birds.

Passive Immunization

Immune serum may be used in the treatment of malaria. The protective effects of immune serum against the erythrocytic stages of *P. knowlesi* infection was demonstrated in monkeys (Coggleshall & Kumm 1937), and in man (Coggleshall 1940). Immune γ-globulin from West African adults was used to treat young children infected with the West African strain of *P. falciparum*. The globulin was effective in reducing the fever and parasitaemia and was active against the dividing schizonts and merozoites. There was no action against gametocytes and relapses were not prevented (Cohen, McGregor & Carrington 1961; McGregor, Carrington & Cohen 1963).

This action of immune γ-globulin is probably only against the homologous strain of *P. falciparum*, since pooled immune γ-globulin from West Africa had a suppressive and prophylactic effect in splenectomized chimpanzees only when they were infected with a West African strain of *P. falciparum*. No action was found against a drug-resistant South-east Asian strain (Sadun *et al* 1966).

In view of the development of drug-resistant strains of malaria in some areas of the world it is possible that a combination of antimalarial immunoglobulin with chemotherapy may be useful in the treatment of resistant strains of *falciparum* malaria.

Active Immunization

Active immunity could be induced against either the erythrocytic or sporozoite forms of the parasite, but studies in man and monkeys have been limited to investigations with preparations containing erythrocytic forms of the parasite.

Active immunization against the erythrocytic stages and hence against blood-induced but not mosquito-induced malaria has been achieved in monkeys and ducks (Targett & Fulton 1965; Thompson, Freund, Sommer & Walter 1947). This active immunity is likely to be only strain and not species specific since a West African strain of *P. falciparum* did not protect splenectomized chimpanzees against subsequent infection with a South-east Asian strain (Sadun *et al* 1966). An active immunity to the erythrocytic stages of malaria would not prevent the development of pre-erythrocytic forms in naturally acquired sporozoite-induced infections. Any immunity would have to last long enough to allow the pre-erythrocytic forms to die out. This could only happen with *P. falciparum* infections and reinfections would not be prevented.

Anti-sporozoite immunity using inactivated sporozoites has been successfully induced in fowls (Russel *et al* 1942) and the protection was against mosquito-induced infections. Better protection was provided when injections of immune serum and inactivated sporozoites were employed in combination (Russel & Moham 1942).

The potential effectiveness of vaccines containing inactivated sporozoites with or without adjuvants has not been tested in man. Difficulties to overcome will be that such immunity may be only against the homologous strains (Taliaferro 1949; Brown & Brown 1965), and that the duration may be short lived. Since the amount and rate of development of immunity will depend upon the amount and duration of antigenic stimulus, the use of suitable adjuvants may be important.

LEISHMANIASIS

Host Susceptibility

Many animals are susceptible to infection in the laboratory with human leishmania, and even *Leishmania adleri* from lizards can cause a transient skin infection in man (Manson-Bahr & Heisch 1961). All races of man are susceptible to cutaneous and visceral leishmaniasis; no racial, individual or innate resistance is known and freedom from infection depends mainly upon epidemiological factors.

Nature of the Immune Response to Infection

Kala-azar is primarily an infection of the reticuloendothelial system; proliferation of the cells of this system in which the parasite lives is the chief characteristic

of the disease. The chief clinical features of kala-azar such as splenomegaly and leucopenia are due to a blockage of the reticuloendothelial system with parasites. Since the leishmania live in the phagocytes it is difficult to know whether these cells play any part in the defence of the body. In most parts of the world 98% of cases of kala-azar will die if they are not treated. In some cases resistant to treatment with all drugs, splenectomy cures the disease (Napier 1949; Manson-Bahr 1959) suggesting that in fact removal of the parasitized reticuloendothelial system is of benefit and that cellular immunity plays little part in man in providing immunity against infection with *L. donovani*.

Another immune mechanism in kala-azar is the skin sensitivity which develops in cured cases of kala-azar and in abortive infections with rodent strains of *L. donovani*. This immunity prevents the development of a primary skin lesion and thus the development of a visceral infection (Manson-Bahr 1959, 1961). A similar immune phenomenon is seen in the development of post-kala-azar dermal leishmanoid, when a nodular rash appears during recovery from the disease which may disappear with relapses, only to reappear again with final recovery (Manson-Bahr 1959).

It has long been known that famine, war and epidemic diseases are followed by outbreaks of kala-azar and this has been ascribed by Corkill (1949) to the activation of latent cases of kala-azar due to insufficient protein being available for the formation of antibody. Sati (1958) has described patients from the Sudan in whom enlarged lymph glands containing leishmania are the only signs of kala-azar; there is no visceral infection. He regards these subjects as being in a state of premunition since they are well and do not suffer from disease. Many ambulant cases of kala-azar with a true visceral infection may be found on surveys (Manson-Bahr 1957) but these people if left untreated will eventually become sick and die.

Dostrovsky (1934) has described a variety of *L. tropica* infection which he has called leishmaniasis recidiva. This is a chronic relapsing lesion with a tuberculoid histology. When superinfection with *L. tropica* takes place the second lesion assumes the same histological appearances as the first and both lesions fade together (Dostrovsky, Zuckerman & Sagher 1952).

Acquired Immunity

Acquired immunity in leishmanial infections is nearly always a true sterilizing immunity. In oriental sore due to *L. tropica* a solid lifelong immunity is acquired when the lesions are allowed to run their natural course. No antibodies have been found in the serum of people infected with *L. tropica*; the immunity in this infection is probably a local cellular immunity in the skin activated by the parasites which live and thrive in reticuloendothelial cells in the skin.

In visceral leishmaniasis due to *L. donovani* a solid immunity is acquired after an attack of kala-azar independent of the stage in the infection when drug

treatment is given; no true second attack of kala-azar has ever been described (Napier 1946). Humoral antibodies develop and can be demonstrated in the serum by complement fixation tests; they are not alone responsible for the high γ-globulin values found in kala-azar. Experiments in hamsters have shown that the increase in γ-globulin is the result of parasite activity and not renal disease (Ada & Fulton 1948), and is caused by an abnormal parasitized reticulo-endothelial system.

There is no evidence that these antibodies can be transferred passively or that they provide a successful defence mechanism. In *L. braziliensis* infections an attack also confers an immunity for life against the homologous strain.

Immunoprophylaxis

Leishmaniasis is the protozoal infection in which active immunization has been used on the largest scale. Infection with a live parasite to produce a permanent active sterile immunity is the method employed.

'L. tropica.' Old World Cutaneous Leishmaniasis

Active immunization against oriental sore due to *L. tropica* infection is an established method of control. Following the ancient practice of inoculating children with material from sores on a covered portion of the body to prevent unsightly scars, artificial lesions have been produced using material from sores or cultures of *L. tropica*. In these lesions, nodules and not ulcers form at the site of inoculation (Katzenellenbogen 1944; Adler & Katzenellenbogen 1952).

The development of immunity to cutaneous leishmaniasis from vaccination is a slow and gradual process. Vaccination conveys immunity only provided the disease is allowed to run its course; if the lesion is excised the immunity does not develop (Mosshkovsky 1942). Superinfection can take place during the development of immunity; the new lesions are similar in structure to the original lesion and disappear at the same time. The cellular infiltration has a tuberculoid structure, with few or no parasites. This is an allergic reaction on the part of the host which appears with the development of immunity (Kolesnikov & Djafarov 1941).

This immunity is not complete until the lesion has completely healed and no parasites are present, a period of usually 4–6 months (Berberian 1944). The immunity is solid and lifelong and is a true sterilizing immunity since leishmania cannot be found in the skin; in only one out of 167 vaccinations was reinfection proved (Katzenellenbogen 1942), and this was the only one in 17 years' experience in Palestine. A reaction of hypersensitivity with immunity to reinfection has been clearly demonstrated in individuals immune to *L. tropica* (Berberian 1944; Adler & Katzenellenbogen 1952; Ansari & Mofidi 1950).

Vaccination is a useful means of prophylaxis in the field and has considerably reduced the incidence of oriental sore in hyperendemic areas (Katzenellenbogen

1944). It is widely used in the Central Asian republics of the U.S.S.R. against the zoonotic forms of *L. tropica* infection (Sokolova 1940; Mosshkovsky 1942). The zoonotic strain of *L. tropica* var. *major* is used for vaccination since it protects against the urban strains of *L. tropica* var. *minor*, but not vice versa (Kozhevnikov 1959).

'L. BRAZILIENSIS.' 'L. MEXICANA.' NEW WORLD CUTANEOUS LEISHMANIASIS

Immunity in New World cutaneous leishmaniasis is complex, since there are at least two sub-species of parasite *L. mexicana* and *L. braziliensis*.

No second attacks of cutaneous leishmaniasis occur with the homologous strains. There is some cross-immunity also between *L. mexicana* and *L. tropica* since it was not possible to infect an individual with *L. mexicana* who had had *L. tropica* infection previously (Adler & Gunders 1964).

A positive leishmanin test denotes resistance to superinfection and Pifano (1962) found that individuals who were leishmanin positive could not be infected with living *Leishman donovani* bodies or cultures of the local strain of *L. braziliensis*.

Attempts have been made to induce an active immunity in South American leishmaniasis using killed cultures of *L. braziliensis* and some protection was shown (Pessoa & Barretto 1944). More than one injection was necessary. If a true cross-immunity does in fact exist between *L. mexicana* and *L. braziliensis* then it has been suggested that in areas where the more severe metastasizing form of the disease is found, people should be immunized with the relatively harmless non-metastasizing *L. mexicana*.

'L. DONOVANI.' VISCERAL LEISHMANIASIS, KALA-AZAR

Manson-Bahr (1959) demonstrated that a primary skin lesion which occurs in kala-azar could be prevented if a skin immunity was first established. This immunity could be produced by the intradermal inoculation of leishmania isolated from wild rodents which caused only dermal and not visceral infections in man. From 6 to 8 weeks after the establishment of a skin nodule, the intradermal skin reaction to leishmanin became positive and an immunity to subsequent challenge from human strains of *L. donovani* was demonstrated, although unprotected controls developed kala-azar. Subsequent trials on a large number of volunteers showed that these rodent strains were quite safe and did not cause visceral disease (Manson-Bahr 1961).

However, field trials of the vaccine did not show any protection probably owing to a loss of virulence on the part of the culture used. It was shown, however, that a positive leishmanin test indicated a considerable degree of protection against naturally acquired kala-azar (Manson-Bahr unpublished).

AFRICAN TRYPANOSOMIASIS

Host Susceptibility and Epidemiology

Human trypanosomiasis

Man is susceptible to natural infection with *T. rhodesiense* and *T. gambiense* subgenus Trypanozoon (Hoare 1964). A third species of this subgenus, *T. brucei*, infects a large number of species of animals but not man. These three species of Trypanozoon are indistinguishable morphologically and represent clinical and ecological entities. Animal reservoir infections of *T. rhodesiense* have been suggested on epidemiological evidence (Jackson 1955), and confirmed by the isolation of strains capable of infecting man from bushbuck (Heisch, McMahon and Manson-Bahr 1958) and from cattle (Onyanga, van Hoeve and de Raadt 1966). An animal reservoir of *T. gambiense* so far has not been established although it would seem likely to exist on epidemiological grounds. Inapparent infections with *T. gambiense* lasting for many years may be important in the spread of this disease (Ceccaldi 1941).

Recently the two human infecting species have been distinguished immunologically by the presence of heterophile antibodies which occur only in persons infected with *T. rhodesiense* (Houba & Allison 1966). This finding suggests that this trypanosome perhaps may contain Forsmann-like antigens which may help to identify this organism.

Animal trypanosomiasis

The most important clinical infections of sheep and cattle are due to *T. vivax* and *T. congolense* and although cattle are commonly infected by *T. brucei* this organism does not cause noticeable clinical symptoms. Pigs are highly susceptible to *T. simiae* and die very soon after infection. All these trypanosomes are transmitted cyclically by tsetse flies (*Glossina*) but mechanical transmission by other biting flies may occur. *T. evansi* which causes Surra in camels is transmitted by mechanical vectors only and is not able to infect tsetse flies. Horses become infected venereally by *T. equiperdum*. Wild game animals are susceptible to many species of trypanosomes to variable degrees and they form the most important reservoir of the trypanosomes of cattle and sheep and to some extent of human trypanosomiasis with *T. rhodesiense*. Most wild animals show a marked tolerance to infection and thus facilitate the transmission of the parasites by carrying the organisms in the blood stream without apparently being affected by them.

Nature of the Immune Response to Infection

The immune response to infection is very rapid and humoral antibodies (agglutinins, precipitins, complement-fixing antibodies and lysins) reacting

with the infecting strain persist for the duration of infection in experimental animals. Antigenicity of crude extracts of *T. rhodesiense* was associated mainly with 1s and 4s proteins, but mainly with the latter, which are mostly of cytoplasmic origin (Brown & Williamson 1962, 1964).

The marked ability of the organism to vary its antigenic character considerably complicates the nature of the immune response. Infection is characterized by successive waves of different antigenic variants which in turn induce the production of variant specific antibodies. The serum of an infected animal in the clinical stages of the disease thus contains antibodies reacting with all the variant antigens which have been produced during infection, but in the early stages of the disease the serum contains antibodies only to the infecting strain. It seems that the formation of antigenic variants is provoked by the antibody response of the host and this mechanism ensures the persistence of the infection of the host (see Gray 1962).

The trypanosomes release certain antigens in the blood during their multiplication in the host. These soluble antigens, called 'exo-antigens' (Weitz 1960), induce protection only against homologous antigenic variants. Antigens which are obtained from mechanically homogenized trypanosomes recovered from the blood give rise to humoral antibodies cross-reacting with other species or strains but induce active protection only against the homologous variant (Weitz 1963 a, b; Miller 1965). If a variant strain is transmitted to another host by a mechanical transmission it usually causes initially an infection with the same variant antigen, but when the strain is transmitted cyclically through tsetse flies the antigenic nature of the infection reverts to that of the original strain (Gray 1965). These findings have important bearings on the development of immunity in trypanosomiasis because a host, although immune to a number of variants from a previous infection, may not be protected against infection with other variants which would proliferate immediately after transmission (Weitz 1964).

Although these complex mechanisms have been studied mostly with *T. brucei* strains in laboratory animals the pattern appears to be similar with other trypanosomes. The exo-antigens of *T. congolense*, *T. rhodesiense*, *T. vivax* or *T. gambiense* also have been described. The pattern of antigenic variants in goats naturally infected with *T. brucei* is similar and variants of *T. congolense* and *T. vivax* have been established. It thus seems that the same mechanisms operate in the natural disease to some extent at least, and is largely responsible for the characteristic periodicity of the disease in many hosts.

Acquired Immunity

In human trypanosomiasis there is no evidence of an acquired immunity. Patients who have been cured by any treatment have relapses sometimes many years later, but it is not always clear if these are due to reinfections or to the recurrence of latent infection. Antibodies which may be present as the result

of infection may thus be merely of diagnostic and epidemiological significance.

For the reasons already stated vaccines are not likely to be of much value except on a theoretical basis in a rapid epidemic when a given strain spreads before antigenic variation has time to occur. However, to date, due to the difficulty of cultivating suitable organisms *in vitro*, there is no hope of any form of practicable vaccination in man.

In animals some evidence of immunity, acquired following the treatment of cattle with drugs, has been described (Bevan 1936; Browning & Calver 1943; Soltys 1955; Whiteside 1962). The time interval between each treatment with the drug and reinfection in the field becomes progressively longer. When the cattle were removed from the infected area the time interval reverted very soon to the original short incubation period. These findings indicate an acquired resistance which was reinforced by natural infection. The same principle has been described (Chandler 1952, 1958; Desowitz 1959) in N'Dama cattle, a naturally resistant breed, in Nigeria. Such cattle were normally insusceptible to challenge with *T. vivax* but they suffered from acute trypanosomiasis with eventual self-cure when they had been born and bred outside tsetse fly infested country. The virulence of the disease depended on the intensity of the natural challenge before the experimental infection was given. In contrast, domestic pigs, which are completely susceptible to infection with *T. simiae*, die before any immunity can develop.

Immunoprophylaxis

It is evident that no form of vaccination treatment is possible against trypanosomiasis. However, prophylactic drug therapy combined with subsequent therapeutic treatment when drug resistance develops, confers immunity in some hosts lasting up to 6 months. The disease therefore is controlled by a combination of drug therapy, tsetse fly control and by preventing access to infected areas.

American Trypanosomiasis (Chagas' Disease)

T. cruzi introduced by the triatomid bug enter cells and multiply intra-cellularly as the leishmanial form; the parasite may be finally destroyed by phagocytosis (Pizzi 1957). Trypanosomes occur but do not multiply in the blood as in African trypanosomiasis. Cultures of *T. cruzi* are infective and may be used to assay experimental immunity in laboratory animals.

An antiserum prepared from a horse immunized with *T. cruzi* failed to protect mice (Voller & Shaw 1965). Killed cultures are not efficient immunizing agents although specific humoral antibodies may be produced (Seneca *et al* 1964; Muniz *et al* 1946; Hauschka *et al* 1950; Johnson *et al* 1963).

Trypanosome cultures disintegrated by sonic oscillation or other mechanical means protect mice for about 4 months (Goble *et al* 1964). The antigens of *T.*

cruzi cultures are complex but the immunogenic activity occurs mostly in a toxic fraction extracted with 88% phenol (Seneca & Peer 1966).

OTHER PROTOZOAL DISEASES

The serological aspects of the following protozoal diseases are discussed fully in Chapter 5 on Sero-diagnosis. Practical immunoprophylaxis in these diseases has either not been fully developed, or is still in the experimental stages. The present knowledge on the mechanisms of immunity is summarized below.

Coccidiosis

The differences in the immune response to infection depends on the species of coccidia concerned and on the nature of the tissues which are invaded. Thus *Eimeria stiedae*, infecting the epithelial cells of the bile ducts of rabbits, promotes a more rapid and more prolonged immune response than the infection of fowls by *E. tenella* which invades the cells of the intestine only. Rabbits given a single large but subclinical dose of oöcysts of *E. stiedae* develop a complete resistance to reinfection lasting for several years. The same treatment of susceptible fowl with *E. tenella* provides only a partial immunity and several doses of oöcysts are necessary to produce a complete but transient resistance to reinfection (Horton-Smith, Beattie & Long 1961).

The sera of animals thus made resistant contain precipitins and complement-fixing antibodies which are, however, not increased by a large experimental challenge with oöcysts (Rose 1961; Rose & Long 1962). Evidence of a humoral transfer of immunity in which these antibodies were not concerned was obtained by Horton-Smith, Beattie & Long (1961). One of the paired caeca of susceptible fowls after being ligated and severed was infected with sporozoites. Subsequent challenge by mouth with oöcysts, or intracaecally with sporozoites failed to infect *either* caecum. The passive transfer of the serum or its globulin fractions failed to protect other birds, showing that the transfer of immunity from one caecum to the other was probably effected by cellular elements infiltrating the site of infection. Immunoprophylaxis of the disease is thus possible experimentally but has not been applied to livestock in the field. As young animals are mostly susceptible the disease is controlled during early life by suitable coccidiocidal drugs in the food or water. Such animals usually develop a resistance under the stimulus of natural infection thus controlled.

Babesiasis

The immunity of cattle to infection with various species of *Babesia* is mainly acquired. Cattle reared in non-enzootic areas are highly susceptible but newborn calves are naturally tolerant and show little or no clinical symptoms. The protection of young calves in endemic areas is transferred by the colostrum from

immune mothers (Hall 1960, 1963). Although complement-fixing antibodies are present in the colostrum and serum of immune animals their significance in relation to immunity is not clear (Riek 1963; Mahoney 1962). Immunity in adults results from previous infection. When clinical symptoms disappear, whether spontaneously or after drug treatment, a non-sterile immunity of variable duration persists.

In endemic areas the premunity appears to be strain-specific and when cattle are moved to a different locality, even within an enzootic area, they may develop the disease (Neitz 1956).

Susceptible cattle before entering an enzootic area are vaccinated by an injection of blood from cattle infected with the species of *Babesia* present in the infected area. This form of live vaccination may cause clinical symptoms and if these are severe drug treatment may be necessary. On recovery the animals remain premune for variable periods but at least for a few months.

The effect of such vaccination varies depending on a large number of factors, e.g. the age of the vaccinated animals, the species of *Babesia* employed and the weight of infection which vaccinated animals may subsequently have to bear. In Sweden, Bodin & Hlidar (1963) estimated that the mortality of vaccinated cows in endemic areas was 0.12% compared with 6–12% in unvaccinated animals, but in Peru, premunity does not develop (Seifert 1962).

Toxoplasmosis

The numerous serological studies on man and animals infected with *Toxoplasma gondii* are essentially of epidemiological or diagnostic interest. No effective form of immunization is available but infected hosts appear to develop a lasting premunity.

Trichomoniasis

The immune reactions to *Trichomonas foetus* in cattle are peculiar due to the local nature of the infection in the genital tract. Local muco-antibodies are produced by cells in the vaginal tract independently of the other antibody reactions (Pierce 1953, 1959) and these can be measured by a specific agglutination test with the vaginal mucus (Kerr & Robertson 1941; Pierce 1947). When *T. foetus* or its antigenic extracts are present in the uterus antibody is produced in the uterine mucus (Kerr & Robertson 1947).

Hypersensitivity of the uterine tissues appears after repeated introduction of the antigen or following passive sensitization of the uterine wall with immune serum. Following the local development of antibody, circulating antibodies appear in the blood stream which eventually become fixed in skin tissues and provoke an anaphylactic type of reaction on the intradermal injection of a polysaccharide extract of the cultivated organism (Feinberg & Morgan 1953).

This complex but perhaps unique immune reaction to infection is of immunological interest but does not appear to play any part in altering the course of the disease.

THEILERIASIS

The natural but variable susceptibility of different races of cattle may be due to a local selection of resistant animals. Calves are apparently less susceptible than adults and although passive immunity is acquired from the colostrum, immunity is passed from non-susceptible dams by other means. Vaccination with live strains of *Theileria annulata* from infected animals has been successful in reducing the mortality of the disease (Sturman 1959). Artificial immunity against *Th. parva* is more difficult as injections can only be successful by tick passage and only a strain of *Th. parva* of mild virulence such as isolated by Barnet & Brocklesby (1961) could be used for active immunization of cattle in East Africa with or without the use of drugs.

REFERENCES

ADA G. & FULTON J.D. (1948) Electrophoretic studies on the serum of golden hamsters infected with *Leishmania donovani*. *Brit. J. exp. Path.* **29,** 524.

ADLER S. & GUNDERS A.E. (1964) Immunity to *Leishmania mexicana* following spontaneous recovery from oriental sore. *Trans. R. Soc. trop. Med. Hyg.* **58,** 274.

ADLER S. & KATZENELLENBOGEN I. (1952) The problem of the association between particular strains of *Leishmania tropica* and the clinical manifestations produced by them. *Ann. trop. Med. Parasit.* **46,** 25.

ALLISON A.C. (1954) Protection afforded by sickle cell trait against subtertian malarial infection. *Brit. med. J.* **1,** 290.

ALLISON A.C. (1957) Malaria in carriers of the sickle cell trait and in newborn children. *Expl. Parasit. (N.Y.)* **6,** 418.

ALLISON A.C. & CLYDE D.F. (1961) Malaria in African children with deficient erythrocyte glucose-6-phosphate dehydrogenase. *Brit. med. J.* **1,** 1346.

ANSARI N. & MOFIDI C. (1950) A contribution to the study of 'moist forms' of cutaneous leishmaniasis. *Bull. Soc. Path. exot.* **43,** 601.

BARNET S.F. & BROCKLESBY D.W. (1961) A mild form of East Coast fever. *Vet. Rec.* **73,** 43.

BERBERIAN D.A. (1944) Cutaneous leishmaniasis (oriental sore). II. Incubation period. *Arch. Derm. Syph.* **50,** 236.

BEVAN LL.E.W. (1936) Notes on immunity in trypanosomiasis. *Trans. R. Soc. trop. Med. Hyg.* **30,** 199.

BLACKBURN C.R.B. (1948) Observations on the development of resistance to vivax malaria. *Trans. R. Soc. trop. Med. Hyg.* **42,** 117.

BODIN S. & HILDAR G. (1963) Immunization of Swedish cattle against Piroplasmosis. *Proc. 9th Nordic vet. Congr., Copenhagen* **1,** 328.

BRAY R.S. (1957) Plasmodium ovale in Liberia. *Am. J. trop. Med. Hyg.* **6,** 961.

BRAY R.S. (1960) Personal communication.

BRAY R.S. & GARNHAM P.C.C. (1953) Effect of milk diet on *P. cynomolgi* infections in monkeys. *Brit. med. J.* **i,** 1200.

BROWN K.N. & BROWN I.N. (1965) Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature* (*Lond.*) **208,** 1286.

BROWN K.N. & WILLIAMSON J. (1962) Antigens of *Brucei* trypanosomes. *Nature* (*Lond.*) **194,** 1253.

BROWNING C.H. & CALVER K.M. (1943) The effect of the stage of infection on the chemotherapeutic response of *T. congolense* and the immunity following cure. *J. Path. Bact.* **55,** 393.

CECCALDI J. (1941) *Brazzaville* (*Afrique francaise libre*): *Rapport sur le Fonctionnement Technique de L'Institut Pasteur en 1940*, p. 67. (Sleeping sickness service.)

CHANDLER R.L. (1952) Comparative tolerance of West African N'Dama cattle to trypanosomiasis. *Ann. trop. Med. Parasit.*, **46,** 127

CHANDLER, R.L. (1958) Studies on the tolerance of N'Dama cattle to trypanosomiasis. *J. Comp. Path. Ther.* **68,** 2, 253.

COGGLESHALL L.T. (1940) The occurrence of malaria antibodies in human serum following induced infections with *Plasmodium knowlesi*. *J. exp. Med.* **72,** 21.

COGGLESHALL L.T. & KUMM H.W. (1937) Demonstration of passive immunity in experimental monkey malaria. *J. exp. Med.* **66,** 177.

COHEN S., MCGREGOR I.A. & CARRINGTON S. (1961) Gamma globulin and acquired immunity to human malaria. *Nature* (*Lond.*) **192,** 733.

COLBOURNE M.J. (1955) Malaria in Gold Coast students on their return from the United Kingdom. *Trans. R. Soc. trop. Med. Hyg.* **49,** 483.

COLLINS W.E., JEFFERY G.M. & SKINNER J.C. (1964) Fluorescent antibody studies in human malaria. II. Development and persistence of antibodies to *Plasmodium falciparum*. *Am. J. trop. Med. Hyg.* **13,** 2, 256.

CORKHILL N.L. (1949) The activation of latent kala azar in relation to protein metabolism. *Ann. trop. Med. Parasit.* **43,** 291.

DESOWITZ R.S. (1959) Studies on immunity and host-parasite relationships. I. The immunological response of resistant and susceptible breeds of cattle to trypanosomal challenge. *Ann. trop. Med. Parasit.* **53,** 293.

DESOWITZ R.S. & WATSON H.J.C. (1951) Studies on *Trypsanosoma vivax*. I. Susceptibility of white rats to infection. *Ann. trop. Med. Parasit.* **45,** 207.

DESOWITZ R.S. & WATSON H.J.C. (1953) Studies on *Trypanosoma vivax*. IV. The maintenance of a strain in rats without sheep serum supplement. *Ann. trop. Med. Parasit.* **47,** 62.

DOSTROVSKY A. (1934) Leishmaniasis recidiva of the skin. *Harefuah* **8,** 118.

DOSTROVSKY A., ZUCKERMAN A. & SAGHER F. (1952) Isophasic reaction following experimental superinfection of *Leishmania tropica*. *Arch. Derm. Syph.* **66,** 665.

EDOZIEN J.C., GILLES H.M. & UDEOZO I.O.K. (1962) Adult and cord blood gamma globulin and immunity to malaria in Nigerians. *Lancet* **951,** 5.

FEINBERG J.G. & MORGAN W.T.J. (1953) The isolation of a specific substance and a glycogen-like polysaccharida from *Trichomonas foetus* (var. Manley). *Brit. J. exp. Path.* **34,** 104.

GOBLE F.C., BOYD J.L., GRIMM WEHNER M. & KONRATH M. (1964) Vaccination against experimental Chagas' disease with homogenates of culture forms of *Trypanosoma cruzi*. *J. Parasit.* **50,** No. 3, Section 2, 19.

GRAY A.R. (1962) The influence of antibody on serological variation in *Trypanosoma brucei*. *Ann. trop. Med. Parasit.* **56,** 4.

GRAY A.R. (1965) Antigenic variation in a strain of *Trypanosoma brucei* transmitted by *Glossina morsitans* and *G. palpalis*. *J. gen. Microbiol.* **41,** 195.

HALL W.T.K. (1960) The immunity of calves to *Babesia argentina* infection. *Aust. vet. J.* **36,** 361.

HALL W.T.K. (1963) The immunity of calves to tick-transmitted *Babesia argentina* infection *Aust. vet. J.* **39,** 386.

HAUSCHKA T.S., GOODWIN M.B., PALMQUIST J. & BROWN E. (1950) Immunological relationship between seven strains of *Trypanosoma cruzi* and its application in the diagnosis of Chagas' disease. *Am. J. trop. Med.* **30,** 1.

HEISCH R.B., MCMAHON J.P. & MANSON-BAHR P.E.C. (1958) The isolation of *Trypanosoma rhodesiense* from a bushbuck. *Brit. med. J.* **2,** 1203.

HOARE C.A. (1964) Morphological and taxonomic studies on mammalian trypanosomes. X. Revision of the systematics. *J. Protozool.* **11,** 200.

HOLMES E.G., STANIER M.W. & THOMPSON M.B. (1955) The serum protein pattern of Africans in Uganda: relation to diet and malaria. *Trans. R. Soc. trop. Med. Hyg.* **49,** 376.

HORTON-SMITH C., BEATTIE J. & LONG P.L. (1961) Resistance to *Eimeria tenella* and its transference from one caecum to the other in individual fowls. *Immunology* **4,** 111.

HOUBA V. & ALLISON A.C. (1966) M-antiglobulins (rheumatoid-factor-like globulins) and other gamma globulins in relation to tropical parasitic infections. *Lancet* **1,** 848.

JACKSON C.H.N. (1955) The natural reservoir of *Trypanosoma rhodesiense*. *Trans. R. Soc. trop. Med. Hyg.* **47,** 582.

JOHNSON R.A., NEAL R.A. & GALL D. (1963) Protective effect of killed trypanosome vaccines with incorporated adjuvants. *Nature (Lond.)* **200,** 83.

KATZENELLENBOGEN I. (1942) Vaccination against Jericho boil. *Ann. trop. Med. Parasit.* **36,** 28.

KATZENELLENBOGEN I. (1944) Vaccination against oriental sore. Reports of five hundred and fifty-five inoculations. *Arch. Derm. Syph.* **50,** 239.

KERR W.R. & ROBERTSON M. (1941) An investigation into the infection of cows with *Trichomonas foetus* by means of the agglutination reaction. *Vet. J.* **97,** 351.

KERR W.R. & ROBERTSON M. (1947) A study of re-exposure to *Trichomonas foetus* of animals already exposed to the infection as virgin heifers, with some observations on the localization of antibody in the genital tract. *J. comp. Path. Ther.* **57,** 301.

KOLESNIKOV K. & DJAFAROV K. (1941) The pathogenesis and pathological anatomy of cutaneous leishmaniasis. Problems of cutaneous leishmaniasis. *Ashkahabad*, p. 265.

KOZHEVNIKOV P.V. (1959) *Med. Parasit. (Mosk.)* **28,** 695. Personal communication. HOARE C.A.

MAEGRAITH B.G., DEEGAN T. & JONES E.S. (1952) Suppression of malaria (*P. berghei*) by milk. *Brit. Med. J.*, **11,** 1382.

MAHONEY D.F. (1962) Bovine babesiosis: diagnosis of infection by a complement-fixation test. *Aust. vet. J.* **38,** 48.

MANSON-BAHR P.E.C. (1957) Leishmaniasis south of the Sahara. *Cent. Afr. J. Med.* **3,** 189.

MANSON-BAHR P.E.C. (1959) East African kala azar with special reference to the pathology prophylaxis and treatment. *Trans. R. Soc. trop. Med. Hyg.* **53,** 123.

MANSON-BAHR P.E.C. (1961) Immunity in kala azar. *Trans. R. Soc. trop. Med. Hyg.* **55,** 550.

MANSON-BAHR P.E.C. & HEISCH R.B. (1961) Transient infection of man with a leishmania (*L. adleri*) from a lizard. *Ann. trop. Med. Parasit.* **55,** 381.

MCGREGOR I.A. (1964) Studies in the acquisition of immunity to *Plasmodium falciparus* infections in Africa. *Trans. R. Soc. trop. Med. Hyg.* **58,** 80.

MCGREGOR I.A. (1965) Considerations of some aspects of human malaria. *Trans. R. Soc. trop. Med. Hyg.* **59,** 125.

MCGREGOR I.A., CARRINGTON S.P. & COHEN S. (1963) Treatment of East African *P. falciparum* malaria with West African human gamma globulin. *Trans. R. Soc. trop. Med. Hyg.* **57,** 170.

MILLER J.K. (1965) Variation of the soluble antigens of *Trypanosoma brucei*. *Immunology* **9,** 521.

MOSSHKOVSKY D.C. (1942) The principle of reinoculation in oriental sore in relation to prophylactic vaccination. *Medskaya Parasit. (Mosk,)* **10,** 101.

MUNIZ J., NOBREGA G. & CUNHA M. (1946) Attempts to vaccinate against *T. cruzi* infections. *Mem. Inst. Oswaldo Cruz.* **44,** 529.

NAPIER L.E. (1946) *The Principles and Practice of Tropical Medicine*, p. 180. New York: Macmillan.

NAPIER L.E. (1949) Splenectomy in the treatment of kala azar. *J. trop. Med. Hyg.* **52,** 243.

NEITZ W.O. (1956) Classification, transmission and biology of piroplasmas of domestic animals. *Ann. N.Y. Acad. Sci.* **64,** 56.

ONYANGA R.J., VAN HOEVE K. & DE RAADT P. (1966) The epidemiology of *Trypanosoma rhodesiense* sleeping sickness in Alego location, Central Nyanza, Kenya. I. Evidence that cattle may act as reservoir hosts of trypanosomes infective to man. *Trans. R. Soc. trop. Med. Hyg.* **60,** 175.

PESSOA S.B. & BARRETTO M.P. (1944) Immunity in S. American cutaneous leishmaniasis. Reprinted from L. REUNAIO, *Anual des Dermato-Sifilografos Brasileiros, Rio de Janeiro*, p. 137.

PIERCE A.E. (1947) The serum agglutination reaction of *Trichomonas foetus*, Riedmuller (1928). *Lab. J.* **8,** 238.

PIERCE A.E. (1953) Specific antibodies at mucous surfaces. *Proc. R. Soc. Med.* **46,** 785.

PIERCE A.E. (1959) Specific antibodies at mucous surfaces. *Vet. Revs. Annot.* **5,** 17.

PIFANO O.C.F. (1962) An assessment of cutaneous leishmaniasis in the Aroa Valley Yaracuy State by means of an allergic index. *Archivos Venesolanos de Med. trop. y Parasit. Med.* **4,** 25

PIZZI JULIO (1957) *Immunology of Chagas' Disease.* Santiago: Centro de Publicaciences Biologicas, Universidad de Chile, Casilla 12967, Chile.

RIEK R.F. (1963) Immunity to babesiosis. In *Immunity to Protozoa*, pp. 160–79, eds. GARNHAM P.C.C., PIERCE A.E. & ROITT I. Oxford: Blackwell.

ROSE N.E. (1961) The complement fixation test in hepatic coccidiosis of rabbits. *Immunology* **4,** 346.

ROSE N.E. & LONG P.L. (1962) Immunity to four species of *Eimeria* in the fowl. *Immunology* **5,** 79.

RUSSELL P.F. & MOHAN B.N. (1942) Immunization of fowls against mosquito borne *P. gallinaceum* by injection of serum and inactivated homologous sporozoites. *J. exp. Med.* **76,** 477.

RUSSELL P.F., MULLIGAN H.W. & MOHAN B.N. (1942) Active immunization of fowls against sporozoites but not trophozoites of homologous sporozoites. *J. Mal. Inst. India*, **4,** 311.

SADUN E.H., HICKMAN R.L., WELLDE B.T., MOON A.P. & UDEOZO I.O.K. (1966) Active and passive immunization of chimpanzees infected with West African and Southeast Asian strains of *Plasmodium falciporum. Military Medicine* **131,** 1251.

SATI M.H. (1958) Kala azar in the Sudan and Tropical Africa. *Proc. 6th Int. Cong. trop. Med. Malar.* **3,** 651.

SEIFERT H. (1962) Bovine piroplasmosis on the coast and in the valleys of the Cordillera mountains of northern Peru, with special reference to the altering periods of drought and rain. (Transl.) *Zentbl. Vet. Med.* **9,** 989.

SENECA H. & PEER P. (1966) Immuno-biological properties of Chagas' toxin (lipopolysaccharide). *Trans. R. Soc. trop. Med. Hyg.* **60,** 610.

SENECA H., PEER P. & REGAN J.W. (1964) Chemotherapy of experimental *Trypanosoma cruzi* infection in mice with L-furaltadone. *Expl. Parasit.* **15,** 479.

SERGENT E., DONATIEN A.L., PARROT C.F., PLANTUREUX E. & ROUGEBIEF H. (1924) Études experimentales sur les piroplasmoses bovines d'Algeria. *Annls. Inst. Pasteur, Paris* **38,** 273.

SOKOLOVA A.N. (1940) Preventive vaccination with the living parasites of cutaneous leishmaniasis. (*Trans. Turkmen, Cutan. Venereol. Inst. Ashkahabad.* pp. 11–441.) Abstract in *Problems of Cutaneous Leishmaniasis*, Ashkahabad, 1941. p. 240.

SOLTYS M.A. (1955) Studies on resistance to *Trypanosoma congolense* developed by zebu cattle treated prophylactically with antricide pro-salt in an euzoatic area of East Africa. *Ann. trop. Med. Parasit.* **49,** 1.

STURMAN M. (1959) *Tick Fever in Israel.* Symposium for Veterinarians. Rehovoth.

TALIAFERRO W.H. (1949) Immunity to the malaria infections. In *Malariology,* **11,** 935, ed. BOYD M.F. Philadelphia: W.B.Saunders Co.

TARGETT C.A.T. & FULTON J.D. (1965) Immunization of rhesus monkeys against *Plasmodium knowlesi* malaria. *Expl. Parasit., N.Y.* **17,** 180.

TERRY, R.J.(1957) Antibody against *Trypanosoma vivax* present in normal cotton rat serum. *Expl. Parasit.* **6,** 404.

THOMSON K.J., FREUND J., SOMMER H.E. & WALTER A.W. (1947) Immunization of ducks against malaria by means of killed parasites with or without adjuvants. *Am. J. trop. Med.* **27,** 79.

VOLLER A. & BRAY R.S. (1962) Fluorescent antibody staining as a measure of malarial antibody. *Proc. Soc. exp. Biol. Med.* **110,** 907.

VOLLER A., & SHAW J.J. (1965) Immunological observations on an antiserum to *Trypanosoma cruzi. Z. Tropenmed. Parasit., Stuttgart* **16,** 181.

WEITZ B. (1960) The properties of some antigens of *Trypanosoma brucei. J. gen. Microbiol.* **23,** 589.

WEITZ B. (1963a) The antigenicity of some African trypanosomes. In *Immunity to Protozoa,* pp. 196–203, eds. GARNHAM P.C.C., PIERCE A.E. & ROITT I. Oxford: Blackwell.

WEITZ B. (1963b) The specificity of trypanosomal antigens by immunofluorescence. *J. gen. Microbiol.* **32,** 145.

WEITZ B. (1964) The reactions of trypanosomes to their environment. In *Symposia of Soc. Gen. Microbiol.* **14,** 112. *Microbial Behaviour 'in vivo' and 'in vitro'*, eds. SMITH H. & TAYLOR J. Cambridge.

WHITESIDE E.F. (1962) Interactions between drugs, trypanosomes and cattle in the field. In *Drugs, Parasites and Hosts, Biological Council Symposium,* pp. 116–41, eds. GOODWIN L.G. & NIMMO-SMITH R.H. London: Churchill.

WILLIAMSON J.B. & BROWN K.N. (1964) The chemical composition of trypanosomes. III. Antigenic constituents of *Brucei* trypanosomes. *Expl. Parasit., N.Y.* **15,** 44.

WILSON D.B., GARNHAM P.C.C. & SWELLENGREBEL N.H. (1950) A review of hyperendemic malaria. *Trop. Dis. Bull.* **47,** 677.

YOUNG M.D., EYLES D.E., BURGESS R.W., and JEFFERY G.H. (1955) Experimental testing of the immunity of negroes to *Plasmodium vivax. J. Parasit.* **41,** 315.

ZUCKERMAN A. (1964) Antigenic analysis of *Plasmodia. Am. J. trop. Med.* **13,** 209.

CHAPTER 47

IMMUNOPROPHYLAXIS OF HELMINTH DISEASES

E.J.L. SOULSBY

INTRODUCTION

Though the incidence of helminth infection in man is low in Great Britain, elsewhere, particularly in tropical areas, it is rampant and brings ill health and early death to millions every year with unrelenting persistence. In these areas it is of some importance that diseases such as schistosomiasis, filariasis, ascariasis and hookworm be controlled since they lead to a stagnation of economy and populations with a morale which is moribund.

Of no less importance are the helminth diseases of animals since they represent a serious drain on the food supply in areas where it can be least afforded. The control of such disease is of as much importance as the urgency to grow more crops and raise more animals. Previously, control depended and still depends mainly on the use of antihelminthic drugs and hygiene. Prophylactic measures are urgently needed and immunological methods, as in other infection, may offer the best means for long-term control.

In a large number of helminth infections it is known that the host develops an acquired immunity and within recent years there has been much interest in the production of immunity artificially for control measures. This is best exemplified by the development of a commercially produced X-irradiated larval vaccine for the control of cattle lungworm disease (Jarrett *et al* 1958).

This vaccine has now been used on several hundred thousand or more cattle with very satisfactory results.

GENERAL PRINCIPLES OF IMMUNITY TO HELMINTHS

The immunological response of a host to helminth infection does not appear to differ from that which occurs with any other type of infectious agent. The physical and chemical properties of antibodies appear to be the same and the cellular response is comparable except that the eosinophil is frequently present in large numbers in lesions associated with helminth infection. The immune response probably follows more closely that applicable to an invasive infectious agent rather than an infectious agent producing its ill effects by intoxication.

In some infections (e.g. the larval stages of several cestodes, the adult stages of some hymenolipid cestodes, the nematodes *Trichinella spiralis*, *Nippostrongylus brasiliensis*, *Ascaris suum* and *Dictyocaulus viviparus*) the passive transfer of immunity by serum has been possible, though in some cases large amounts of serum were necessary to accomplish this. With *D. viviparus*, for example, the equivalent of 4.5 litres of immune serum was transferred.

In many other infections, however, it has not been possible to demonstrate any satisfactory transfer of immunity either artificially or under natural conditions. Thus, sheep produce antibodies to their gastro-intestinal nematodes and these antibodies can be detected in the colostrum of ewes and in the sera of lambs which suckle them. No protection is afforded the lambs by this passive transfer of antibody. In fact the role of circulating antibody in immunity to helminths is by no means clear, and it may be merely an accompanying phenomenon of the immune reaction in some instances. A limited number of experiments have demonstrated successful transfer of immunity by lymphoid cells, and this has raised the possibility that immunity to helminths may be based on 'delayed hypersensitivity' mechanisms.

A wide range of serological cross-reactions occurs between parasites of various families and even certain orders, and these have been used for diagnostic tests (see Chapter 6). Protective immunity, on the other hand, does not show this wide range of cross-reactions, and it appears to be fairly specific. There are examples where species within a genus will immunize against one another, but at the generic level it is more usual to find little or no cross-immunity. For example sera from sheep immune to either *Trichostrongylus* spp. or *Haemonchus contortus* will react with antigens from either species, but protective immunity to *Trichostrongylus* spp. is distinct from that to *H. contortus* and vice versa. Again, *T. spiralis* gives serological cross-reactions with *Schistosoma mansoni*, but animals immunized against *T. spiralis* are not protected against *S. mansoni* infection. If the antigens which are responsible for these serological cross-reactions were also

responsible for protective immunity, then it would be expected that one parasite would induce resistance to another which shared common antigens. This does not occur. It is probable, therefore, that the antigens responsible for serological cross-reactions are not concerned in protective immunity but others, of a more specific nature, associated with the living parasite, induce it.

Protective immunity is best induced by a living parasite. Not only must the living parasite be present in the host, but also a certain degree of development of the parasite must occur before protective antigens are produced. In several nematode infections this degree of development appears to include at least one moulting phase, this apparently being the time when the protective antigens are released. It is also the time when the parasite is adversely affected by the host. Experimental work with *Ascaris suum* in guinea-pigs and rabbits has shown that infective larvae behave for the first few days of an infection in an immune animal as if they were in a normal animal. Such larvae are able to maintain themselves and grow, but their numbers very rapidly decrease when they reach the moulting period.

The effects of immunity on helminths are varied and depend to a great extent on the degree of immunity that is present. These effects range from a complete refractoriness to infection, through inhibition of larval development to the inhibition of egg production by adult worms. When egg production is inhibited, the adult worm remains in the body and may, when immunity falls, recommence egg production. Similarly, this reversibility of the reaction is seen in inhibited larvae. Inhibition frequently occurs just before or just after a moulting period, and larvae remain 'dormant' so long as the immune status remains high. If it falls or if the larvae are transferred to a normal animal, then the inhibited larvae recommence development. With many helminths, immunity is short lived and can wane comparatively rapidly in the absence of stimulating antigen.

Little is known regarding the nature or the source of the protection-inducing antigens. A variety of reactions between immune serum and various developmental stages of helminths can be demonstrated *in vitro*, but these do not necessarily indicate that a comparable reaction operates, or is important, *in vivo*.

The demonstration of immune precipitates at the various external orifices of nematode larvae by Sarles (1938) led to the postulate that the 'metabolic products' (excretory and secretory materials) which diffuse from these orifices were the protective antigens. Further support for this view came from the fact that circumlarval precipitates were demonstrated *in vitro*. It was envisaged by some workers that in an immune animal the 'metabolic products' are precipitated by antibody as they diffuse from the various natural orifices of the parasite such as the mouth, excretory pore and anus. It was suggested that this results in the parasite being immobilized or retarded in development, and it is then surrounded by inflammatory cells which act as scavengers when the worm dies.

If the metabolic products are of outstanding importance in immunity to

helminths, it would be expected that vaccination of animals with them would lead to a high degree of resistance. Some success in this direction has been achieved with antigens obtained from parasites cultured *in vitro* (Soulsby 1963a), but when 'metabolic products' are obtained from short-term incubates of helminths, the immunity induced by these is usually of a very much lower order than that produced by natural infections. However, it is generally difficult to collect metabolic products in large amounts, and usually they are available only from a limited phase of development of the parasite. In addition, it is likely that such materials contain a large variety of enzymes, and the collection of them may result in their deterioration by the action of the enzymes contained therein. No satisfactory work exists to date to indicate the nature or origin of these substances though their amino-acid composition has been studied by certain workers. It is difficult to define exactly what is meant by the 'metabolic products' of worms, and in the final analysis possibly all helminth antigens can be regarded as such.

Several years ago, Chandler (1953) suggested that protective antigens might be enzymatic in nature. Undoubtedly numerous enzyme systems exist in a helminth, and these could function as antigens. However, it remains to be determined whether such enzymes become available to the host during the life cycle of the helminth. It may well be that there are special periods, such as the moulting phase, when they immunize the host and are also affected by the immune mechanisms. A malic dehydrogenase from the pig *Ascaris suum* has been shown to induce specific antibodies which inhibit the enzyme activity and also precipitate with the purified enzyme (Rhodes *et al* 1965). Antibodies to malic dehydrogenase could not be stimulated in guinea-pigs by crude extracts of *A. suum* or by vaccinating guinea-pigs with infective eggs; however, guinea-pigs immunized with purified malic dehydrogenase showed some protection against *A. suum* infection as compared with control animals.

Any method of active prophylaxis must obviously provide the protective antigens to the animal, and the methods which might be used to induce functional immunity artificially are as follows:

1. The use of standardized doses or normal infective larvae.
2. The use of related species of parasites or those with naturally reduced virulence.
3. The use of artificially attenuated infective stages.
4. The use of materials from parasites cultivated *in vitro*.

The use of immune serum as a prophylactic in helminth disease is of little value. In certain experimental trematode and cestode infections, passive transfer of immunity gives satisfactory immunity (e.g. *Cysticercus fasciolaris* and *Hymenolepis nana* var. *fraterna*). In other helminth infections, however, and particularly with the nematodes, passive transfer of immune serum results in but a low degree of immunity and is certainly not applicable on a therapeutic level.

PROPHYLAXIS

Use of Standardized Doses of Normal Infective Larvae

It is generally inapplicable, on a wide-scale basis, to consider the use of such a system. In nature, however, this is the means whereby a host is immunized against its helminth burden and a parasite undergoes normal development in the host, the necessary protective antigens being produced by the various developmental stages. One major drawback is that if living larvae are used as immunizing agents, they may give rise to the disease against which protection is desired. It would be difficult to standardize a dose for all individuals and particularly the young, which may be overwhelmed by the infection. In addition, since normal infective stages would produce a patent infection, the use of such a system may result in the initiation of infection in an otherwise normal population of hosts and lead to the introduction of the disease rather than to the control of it.

Nevertheless, it may be possible to use this system if the normal infection is terminated at an appropriate point by an anti-helminthic with a marked larvicidal effect. This has been accomplished experimentally with *Ascaris suum* in the rat, infection being terminated after the first few days by high doses of thiabendazole. Subsequently, such rats were highly resistant to a challenge infection (Campbell & Timinski, 1965). Work with other species of nematodes has indicated the feasibility of this, and the technique has been applied to the field control of parasitic bronchitis of cattle (*Dictyocaulus viviparus*) in that calves are treated with diethylcarbanazine as soon as clinical signs of parasitic bronchitis appear. This drug kills the larval stages of the parasite without interfering with the acquisition of immunity.

In domestic animals, the use of a carefully controlled pasture management system can do much to immunize animals satisfactorily against helminths. This allows animals to acquire a limited number of infective larval stages which could be regarded as a number of standardized doses. Under conditions of good management, a satisfactory immunity to gastro-intestinal nematodes is produced which not only equips the individual animals with an ability to withstand severe challenge but also leads to the control of gastro-intestinal nematodes in the herd or flock as a whole.

It is unlikely that immunization with standardized doses of normal infective larvae will be applicable to the parasites of man. Nevertheless, an increasing resistance to helminths is seen in different age groups of population, and under endemic conditions it appears that helminth-host parasite relationships of man are not dissimilar to those of domestic animals.

In natural immunization the infective stage enters the body by the natural

route, normally the mouth, or by skin penetration in the case of hookworms and schistosomes. During development a certain amount of pathological change may occur in various tissues or organs through which the developmental stages pass. Indeed, in some cases it is likely that enough antigen will be produced only by numbers of migratory stages which in their turn produce an equivalent amount of pathological change as they develop. In order to avoid this, and at the same time provide an animal with larger numbers of parasites than it would withstand normally, attempts have been made to stimulate immunity by introducing infective stages by an abnormal route. For example, Soulsby (1957) has shown that a satisfactory immunity to *Ascaris suum* can be produced if infective eggs of this parasite are injected subcutaneously. A number of the eggs hatch at the subcutaneous site and undergo a local migration in the tissues, but a minimal number also undergo a delayed migration to the lungs of the animal. By this method, doses in excess of the lethal dose can be given, the larvae undergo development without affecting the animal in an adverse clinical manner, and yet the animal is provided with the necessary protective antigens to induce a good immunity.

A similar technique has been employed by Gemmell (1966) to produce immunity to the larval stages of *Echinococcus granulosus*, *Echinococcus multilocularis* and other cestodes of dogs in sheep and rabbits. Eggs, or the embryos from the eggs activated by artificial digestive mixtures, of these parasites were injected into a foreign site. Preliminary studies also indicated that the intravenous or intra-muscular injection of artificially activated embryos of *E. granulosus* may produce a degree of protection against the adult phase of *E. granulosus* in the intestine of dogs.

Use of Related Species of Helminths or Species with Reduced Virulence

This method of vaccination has been used widely in other infections. However, in helminth diseases only a limited amount of information is available concerning the value of related species as immunizing agents.

Gemmell (1964, 1966) has been able to demonstrate cross-protection between the two sheep metacestodes *Taenia hydatigena* and *Taenia ovis* and in addition he has found the activated embryos of these species protected sheep against the 'post-encystment' phase of *Echinococcus granulosus*. Though it might be expected that the closely related forms, *E. granulosus* and *E. multilocularis* (of wolf and microtine rodents), would share common protective antigens, this is not so (Rausch & Gemmell 1966), rather the degree of protective antigen sharing between taeniid cestodes seems more related to the closeness of the hosts parasitized than to the generic relationships of them. Thus, dog-sheep related forms showed more protective antigenic relationship to sheep metacestodes than dog-rodent related forms. Though much work still remains to be done on these species

there seems a real possibility of a 'vaccine' for echinococcosis at some time in the future.

There has been little investigation of the use of strains of parasites with reduced virulence for the production of immunity. There are, however, some indications that such forms may be of value in immunization.

The use of helminths of lower animals to stimulate immunity to related species in man has received attention recently. Infections with animal schistosomes in man (e.g. *Schistosoma bovis*, *Schistosoma indicum*, etc.) are uncommon and usually abortive. Nevertheless, continued exposure to the cercariae of these non-human species may possibly influence the severity of natural infections with the more pathogenic human species (e.g. *Schistosoma haematobium*, *S. mansoni*). The term 'Zooprophylaxis' has been introduced to describe this phenomenon (Nelson, Teesdale & Highton 1962), and experimental studies have shown that sensitization with heterologous species may induce some degree of immunity against the more pathogenic species.

The Formosan (non-human) strain of *Schistosoma japonicum* will induce immunity in monkeys and other animals against the Chinese and Japanese (human) forms of *S. japonicum* (Sadun, Yamaki, Lin & Burke 1961). The Formosan strain can induce immunity without the presence of eggs in the tissues, and this is of importance since in using methods in this category, it is essential that the variant which is used to produce immunity does not produce clinical disease and does not initiate disease in an area where the parasite has not previously existed.

A report by Allen & Samson (1961) indicated that the *Haemonchus* from the pronghorn antelope, which is relatively non-pathogenic to domestic sheep, was capable of inducing significant resistance to a challenge with the sheep strain of *Haemonchus*. Though the resistance was not as marked as with the homologous strain, other variants from wild ruminants may give better protection with the same lack of pathogenicity. There may well exist in the wild fauna of the world strains of parasites that would stimulate a good protective immunity of the more pathogenic forms of the species. However, these have not been looked for with any vigour.

Use of Artificially Attenuated Infective Stages

The most popular method of attenuation of infective larvae for the production of a vaccine is the exposure of larvae to ionizing radiation such as X-rays. As a result of irradiation, the ability of infective stages to reach patency in the animal is greatly reduced or even eliminated, but at the same time a certain degree of development does occur, thus ensuring that the protective antigens are produced and are made available to the animal. Some of the earliest work on the effect of ionizing radiation on nematodes was carried out in 1916 by Tyzzer and Honeij,

who exposed encysted *Trichinella spiralis* larvae to radium and rendered them non-infective to mice. Later it was found that when *T. spiralis*-infected pork which had been exposed to X-irradiation was fed to rats they developed infections with the parasite but the X-rays had had a selective and destructive effect on the gonadic tissue of the adult *Trichinella*. Relatively large doses of X-rays prevented maturation of larvae to adult worms, but smaller doses permitted larvae to develop to structural, but not sexual, maturity. Later, Levine & Evans (1942) induced immunity in rats to reinfection with *T. spiralis* by feeding to them larvae that had been irradiated with 3000–3750 r of X-rays. This amount of X-irradiation allowed the larvae to develop to maturity, but the majority of adult worms in such an infection were sterile. Subsequent work in the United States showed that rats fed larvae of *T. spiralis* which had been exposed to 10,000 r from a cobalt-60 source developed a definite degree of immunity to reinfection with non-irradiated larvae. However, they found that if the dose of radiation was increased so as to prevent the majority of the larvae developing to the adult form, then little or no immunity resulted. In the case of *T. spiralis*, therefore, it appears that it is necessary for the infective larvae to develop through to the adult stage before immunity is produced.

In other nematode infections, development to the adult stage is not necessary for an adequate immunity response. In the case of *Dictyocaulus viviparus* and *Haemonchus contortus* irradiated larval vaccines, it is necessary only for the early larval stages to undergo some development (Jarrett *et al* 1959, 1960). Since the adult stages of these parasites are responsible for a considerable amount of pathological damage, it is most advantageous that the adult stages are not necessary for a good immune response. Too much irradiation is to be avoided since, with increasing doses of irradiation, the ability of the larval stages to develop and migrate is increasingly affected and a point is reached when little immunity results. Work with both *D. viviparus* and *H. contortus* has shown that over-irradiation of larvae results in a loss of immunizing power. As well as providing a satisfactory method of immunization, irradiated vaccines can also be subject to strict control in production and can be standardized before issue for general use. Generally, there is little danger that a batch of vaccine will cause a patent infection and hence clinical disease in hosts. However, this eventuality should be recognized and guarded against. Where helminth disease is endemic, the patent infection resulting from irradiated larval vaccines would be of little significance because the degree of patency produced is always of a much lower order than that produced by a normal infection. On the other hand, where helminth disease is sporadic and depends to some extent on climatic conditions or on management, an insufficiently attenuated vaccine capable of developing to an adult and patent infection may lead to outbreaks of disease rather than control.

Nevertheless, despite such difficulties a highly successful vaccine for the prevention of the lungworm disease of cattle has been produced. In this disease

infective larvae are acquired from pasture by grazing animals. After being swallowed the larvae penetrate the gut wall and migrate via the lymphatics or blood to the lungs where the immature and adult worm cause a severe pneumonia. If X-irradiated larvae are administered, it is found that there is an inverse relationship between the dose of X-rays and the number of worms found in the lungs. A vaccine consisting of two oral doses, at a month's interval, of 1000 infective larvae exposed to 40,000 r has given very satisfactory immunity and in this form is available commercially.

As a result of the successful development of a vaccine against lungworm infection in cattle, many studies have been carried out on the effect of X-rays and γ-rays on the infective stages of other helminth parasites. A commercial vaccine for the lungworm of sheep, *Dictyocaulus filaria*, has been developed in Yugoslavia and is used successfully for practical control (Sokolić, Jovanović, Cuperlović & Movsesijan 1965). An indication that this method of immunization may be of value in hookworm in humans comes from comparable work on the hookworm (*Ancylostoma caninum*) of dogs. Irradiated larvae, given subcutaneously on two occasions to dogs, successfully protects them from the clinical effects of a pathogenic challenge infection (Miller 1965). This form of immunization is also practical in puppies which may acquire their infection prenatally from the pregnant dog. Experiments have shown that if an existing prenatal infection is removed by appropriate therapeutic treatment, the puppy can then be immunized successfully with the vaccine. It appears, therefore, that prenatal infection and exposure to the antigens of this parasite at an early age do not induce any prolonged immunological unresponsiveness.

However, this is not necessarily the case with all parasitic infections, and a condition of immunological unresponsiveness has been induced experimentaly in *Cysticercus bovis* infection in calves (Soulsby, 1963b) and is seen naturally in young lambs to their gastro-intestinal nematodes (Soulsby 1966). Special measures will have to be adopted to overcome such immunological unresponsiveness especially since clinical disease is most frequently seen in the young animal. This is well exemplified with the abomasal parasite, *Haemonchus contortus*, which can cause severe losses in young sheep. An X-irradiated larval vaccine will induce a high level of immunity in sheep aged 7 months or more, but it fails completely to induce protection in lambs aged 3 months (Urquhart *et al* 1966).

The possibility for an irradiated larval vaccine for human hookworm infection has, until recently, been hindered by the lack of a suitable host in which to produce infective stages for irradiation. However, the major human hookworm, *Necator americanus* has been shown to infect dogs which have been given heavy doses of corticosteroids (Miller 1966), and it may now be possible to produce large numbers of infective larvae of the parasite for irradiation work.

In the field of schistosomiasis, great encouragement for practical immuniza-

tion against the human infection has derived from the work of the Hsüs. These workers found that irradiated cercariae of *Schistosoma japonicum* were able, following several immunizing exposures, to protect monkeys against challenge infection of *S. japonicum* (Hsü, Hsü & Osborne 1963). Even so, such results should be interpreted with caution since the immune response to schistosomiasis varies greatly with the host for the parasites. In monkeys (*Macacus mullata*), for example, after a year or more of infection a fairly sterile immunity is produced which may persist for 34 months, even following chemotherapeutic cure of *Schistosoma mansoni* infections. Some degree of protection has been observed in most hosts for schistosomes, and it appears that *S. japonicum* is a more effective immunizing species than *S. mansoni*. There is a vast body of evidence now available which indicates that following infection with schistosomes, man develops some degree of acquired immunity to subsequent exposures, but this is not of a high order.

Some indication of this variability of the immune response to schistosomes is given by the work of Sadun, Bruce & Macomber (1964), who were unable to induce immunity in monkeys with irradiated cercariae of *S. mansoni* and by that of Hsü, Hsü & Osborne (1965), who failed to induce immunity in mice with irradiated cercariae of *S. japonicum*.

In several studies with irradiated infective stages of helminths, it has been noted that irradiated forms may be better stimulators of immunity than the normal infective stages. The reason for this is as yet unclear; it may be due to a prolongation of the important developmental stages, thus providing greater contact with the host, to the death of developmental stages in the host tissues and thus a greater availability of protective antigens, or to the helminth antigens becoming denatured so as to make them more foreign, and more antigenic, to the host.

Use of Materials from Parasites Cultured *in vitro*

Though no vaccine of this nature is available for use, the method of vaccination offers the greatest promise for the future. Materials prepared by such a technique should be capable of being standardized and subjected to careful control. There is no danger that a patent infection will be produced and little danger that other infectious agents will be transferred by the vaccine.

It is anticipated that the infective stages of a parasite will be cultured to a stage where they produce the protective antigens which would have been produced in the host if the larvae had been given as a normal infection. However, the situation may turn out to be that the provision of enough antigen even from the early stages would be satisfactory in producing immunity, whereas in normal infections the larval stages must progress to a certain degree of development before a sufficient amount of antigen is available. Using *in vitro* cultivation

techniques it is likely that substantially larger amounts of protective antigen can be produced and presented to an animal than would be able to occur under conditions of natural infections. If such an amount of antigen were to be derived from a natural infection, it is probable that the animal would be overwhelmed by it.

There have been many attempts to induce protective immunity with antigens prepared from non-viable helminths, but, with few exceptions, they have been unsuccessful. Many reasons have been given for these failures, but the principal one is that the protective antigens are associated with the living parasites and are possibly the excretory or secretory materials produced by the worms. Since it has been suggested that the protective antigens are enzymes, it would be expected that only a limited amount of enzymatic protein would be available if an antigen was prepared from a dead parasite. In the past, however, physical conditions and techniques were employed which are now known to be deleterious to many macromolecular systems, and it may be that the protective antigens were indeed present in these preparations initially but were destroyed by the technique used in handling them. A few reports exist which indicate the successful use of antigenic materials from dead parasites for the induction of resistance. These include the parasitic tapeworms of laboratory animals and immunity has been induced to *Trichinella spiralis* by the intraperitoneal injection of heat-killed larvae or dried, powdered adult worm material.

Despite the obvious advantages of *in vitro* produced antigens for immunization, there are only a few encouraging reports about it. Silverman, Poynter & Podger (1962) were able to prepare antigens from the *in vitro* cultured larvae of *Dictyocaulus viviparus*, *Trichostrongylus colubriformis* and *Strongyloides papillosus* which were highly effective in protecting guinea-pigs and rabbits against infection and mortality from these species. Infective larvae were cultured until 60% of them had reached the fourth larval stage of development; then the whole culture was lyophilized and injected intraperitoneally with adjuvant. The antigens were relatively stable and could be stored in sealed ampoules at room temperature for several months.

Comparable studies were reported by Soulsby (1963a) for the larval stages of *Ascaris suum*. Third-stage *A. suum* larvae, harvested from the lungs of rabbits 7 days after infection, were cultured *in vitro* for 4 days. Guinea-pigs, which were vaccinated twice subcutaneously with various antigens prepared from these cultures, along with Freund's complete adjuvant, showed good protection against a challenge dose of *A. suum* eggs. Protection was good with whole culture, culture fluid alone or larval bodies alone, which indicated that the protective antigens were not solely secretory or excretory in nature. However, the *A. suum* antigens were quite labile, losing much of their immunizing power if they were heated, frozen at −20°C or lyophilized.

The nature of the protective antigens which are produced by the *in vitro*

techniques is unknown and no information is available as to the best methods of preserving them. It is possible, for example, that the antigens of helminths are particularly susceptible to physical agencies, and if this is so then special methods of preservation will be necessary.

Recent studies on the basic aspects of the immunology of helminth infections have indicated that delayed hypersensitivity, reagin-like antibodies and lymphoid cells are very likely to play a substantial role in the immune response to these organisms. If this turns out to be so, special adjuvants may be necessary to elicit the correct immune response.

REFERENCES

ALLEN R.W. & SAMSON K.S. (1961) Preliminary report on the immunization of sheep with a relatively non-pathogenic strain of *Haemonchus* from pronghorn antelope. *J. Parasit.* **47,** Sect. 2, 22.

CAMPBELL W.C. & TIMINSKI S.F. (1965) Immunization of rats against *Ascaris suum* by means of nonpulmonary larval infections. *J. Parasit.* **51,** 712.

CHANDLER A.C. (1953) Immunity in parasitic diseases. *J. Egypt. med. Ass.* **36,** 811.

GEMMELL M.A. (1964) Species specificity of the immunogenic complexes of the tapeworm hexacanth embryo. *Nature (Lond.)* **204,** 705.

GEMMELL M.A. (1966) Species specificity and cross protective functional antigens of the tapeworm embryos. *Nature (Lond.)* **213,** 500

HSÜ H.F., HSÜ S.Y.LI & OSBORNE J.W. (1963) Further studies on rhesus monkeys immunized against *Schistosoma japonicum* by administration of X-irradiated cercariae. *Z. Tropenmed. Parasit.* **14,** 402.

HSÜ S.Y.LI, HSÜ H.F. & OSBORNE J.W. (1965) Immunizing effect of X-irradiated cercariae of *Schistosoma japonicum* in albino mice. *Z. Tropenmed. Parasit.* **16,** 83.

JARRETT W.F.H., JENNINGS F.W., MCINTYRE W.I.M., MULLIGAN W., SHARP N.C.C. & URQUHART G.M. (1958) A field trial of a parasitic bronchitis vaccine. *Vet. Rec.* **70,** 451.

JARRETT W.F.H., JENNINGS F.W., MCINTYRE W.I.M., MULLIGAN W., SHARP N.C.C. & URQUHART G.M. (1959) Studies on immunity to *Haemonchus contortus* infection—vaccination of sheep using a single dose of X-irradiated larvae. *Amer. J. Vet. Res.* **20,** 527.

JARRETT W.F.H., JENNINGS F.W., MCINTYRE W.I.M., MULLIGAN W. & URQUHART G.M. (1960) Immunological studies on *Dictyocaulus viviparus* infection. Immunity produced by the administration of irradiated larvae. *Immunology* **3,** 145.

LEVINE A.J. & EVANS T.C. (1942) Use of roentgen radiation in locating an origin of host resistance to *Trichinella spiralis* infection. *J. Parasit.* **28,** 477.

MILLER T.A. (1965) Studies on canine ancylostomiasis-double vaccination with X-irradiated *Ancylostoma caninum* larvae. *J. Amer. Vet. Med. Ass.* **146,** 41.

MILLER T.A. (1966) The effect of a synthetic corticosteroid upon the susceptibility of dogs to infection with the human hookworm *Necator americanus*. *J. Parasit.* **52,** 285.

NELSON G.S., TEESDALE C. & HIGHTON R.B. (1962) The role of animals as reservoirs of bilharziasis in Africa. In Ciba Foundation Symposium, *Bilharziasis*, 1962, pp. 127–49. London: J. & A. Churchill.

RAUSCH R.L. & GEMMELL M.A. (1966). Personal communication of unpublished work.

RHODES M.B., NAYAK D.P., KELLY G.W. JR. & MARSH C.L. (1965) Studies in helminth enzymology. IV. Immune responses to malic dehydrogenase from *Ascaris suum*. *Exptl. Parasit.* **16,** 373.

SADUN E.H., BRUCE J.I. & MACOMBER P.B. (1964) Parasitologic, pathologic and serologic reactions to *Schistosoma mansoni* in monkeys exposed to irradiated cercariae. *Amer. J. trop. Med. Hyg.* **13,** 548.

SADUN E.H., YAMAKI A., LIN S.S. & BURKE J.C. (1961) Resistance induced in mice and in monkeys with the Japanese and Formosan strains of *Schistosoma japonicum*. *J. Parasit.* **47,** Sect. 2, 48.

SARLES M.P. (1938) *In vitro* action of immune rat serum on the nematode *Nippostrongylus muris*. *J. infect. Dis.* **62,** 337.

SILVERMAN P.H., POYNTER D. & PODGER K.R. (1962) Studies on larval antigens derived by cultivation of some parasitic nematodes in simple media: protection tests in laboratory animals. *J. Parasit.* **48,** 562.

SOKOLIĆ A., JOVANOVIĆ M., CUPERLOVIĆ K. & MOVSESIJAN M. (1965) Vaccination against *Dictyocaulus filaria* with irradiated larvae. *Brit. Vet. J.* **121,** 212.

SOULSBY E.J.L. (1957) Immunization against *Ascaris lumbricoides* in the guinea pig. *Nature (Lond.)* **179,** 783.

SOULSBY E.J.L. (1963a) The nature and origin of the functional antigens in helminth infections. *Ann. N.Y. Acad. Sciences* **113,** 492.

SOULSBY E.J.L. (1963b) Immunological unresponsiveness to helminth infections in animals. *Proc. 17th Wld Vet. Congr. Hannover* **1,** 761.

SOULSBY E.J.L. (1966) The mechanism of immunity to gastro-intestinal nematodes. In *Biology of Parasites*, pp. 255–76. New York and London: Academic Press.

TYZZER E.E. & HONEIJ J.A. (1916) The effects of radiation on the development of *Trichinella spiralis* with respect to its application to the treatment of other parasitic diseases. *J. Parasit.* **3,** 43.

URQUHART G.M., JARRETT W.F.H., JENNINGS F.W., MCINTYRE W.I.M., MULLIGAN W. & SHARP N.C.C. (1966) Immunity to *Haemonchus contortus* infection: failure of X-irradiated larvae to immunize young lambs. *Amer. J. Vet. Res.* **27,** 1641.

CHAPTER 48

IMMUNOPROPHYLAXIS IN VETERINARY MEDICINE

M. STERNE AND IRENE BATTY

IMMUNOTHERAPY

IMMUNOPROPHYLAXIS
Passive immunization: Active immunization with bacterial, viral, protozoal and metazoal vaccines: Adjuvants: Inoculation schedules

The principles governing the use of immunological procedures in the veterinary field are the same as those in the human field. However, there are important factors in the application of these principles and these, rather than similarities, will be emphasized.

IMMUNOTHERAPY

Except in a few instances, treatment with antisera has been neither important nor effective. One of the chief reasons is the difficulty of administering them in time to be of any value. For example, although anti-anthrax sera are very effective in treatment, the course of the disease may be so rapid that the animal is found dead before it can be helped; similarly with anti-gas gangrene sera such as anti-chauvoei or anti-malignant oedema sera which can be useful if administered in time. In diseases caused by toxins such as tetanus, the symptoms, which may be evident a considerable time before death, indicate that toxin is already fixed to the tissues. Once fixed it is not usually possible to neutralize its effects with antisera.

The British Veterinary Codex (1965) lists sixteen antibacterial or antitoxic sera. Only two are recommended for therapy alone: anti-snake venom and *Corynebacterium pyogenes* antiserum—the latter a somewhat questionable recommendation. Two antisera, swine erysipelas and anti-anthrax, are recommended for both therapy and prophylaxis: seven are recommended mainly for prophylaxis with a proviso that they may be used therapeutically if given very early in the disease: five—and these include the most widely used antisera such as lamb dysentery and white scour—are only recommended for prevention. Many other

antisera have been used therapeutically by the less critical amongst clinicians. In a number of cases anti-bacterial drugs have made the use of antisera superfluous.

TABLE 48. 1

Recommendations for the use of antisera listed in the British Veterinary Codex

Antiserum	Recommended for	
	Therapy	Prophylaxis
C. pyogenes	T	—
Venom	T	—
Anthrax	T	P
Swine erysipelas	T	P
Black disease (*C. novyi*)	t	P
Blackleg (*C. chauvoei*)	t	P
Canine distemper	t	P
Canine hepatitis	t	P
Leptospirosis	t	P
Rinderpest	t	P
Swine fever	t	P
Tetanus	—	P
Lamb dysentery (*C. perfringens* Type B)	—	P
Pulpy kidney (*C. perfringens* Type D)	—	P
Past. multocida	—	P
White scour (*E. coli*)	—	P

P = for prophylaxis.
T = recommended for therapy
t = may be used for therapy if given early.

IMMUNOPROPHYLAXIS

Since Pasteur's demonstration of the possibilities of artificial immunization, both antisera and vaccines have been widely used for the prevention of disease in animals. In man, vaccines may be used only where the risks attending vaccination are practically nil or when the threat of disease to life far exceeds the risks attending vaccination. There is virtually no limit to the amount which can be spent on technical improvements which lessen the risks of reactions and morbidity. The criteria for the employment of immunoprophylactics in animals are far less stringent. They may be used so long as they save more animals than they kill. Reactions are only important if they cause serious physical blemishes which

affect the value of the animal either as meat or as leather, or for show purposes. It would not, of course, be possible to use agents causing obvious and severe pain to pets.

As a rule, veterinary prophylactics are more severely challenged in the field than are prophylactics for human use. Consequently users are likely to demand the maximum practicable potency, and will accept some risk of severe reactions or even of occasional deaths. Thus, since the criteria for accepting a veterinary prophylactic are practical rather than sentimental, vaccines tend to be more potent than corresponding vaccines used in humans, and thus have a better chance to control or eradicate disease. On the other hand, the need to provide for human use prophylactics that are effective in small non-reactogenic doses has meant that far more research could be lavished on developing methods for refining such antisera and vaccines. Veterinary prophylactics have benefited indirectly from this work.

Because large doses of relatively crude preparations can be used in veterinary practice, problems of disease control tend to be solved practically before they become subjects for academic research. Thus they escape examination at a fundamental level. For example, about 12 years ago concern was felt because of the inefficacy of vaccines against bovine haemorrhagic septicaemia. The problem was soon solved by increasing the number of organisms per dose ten times, and incorporating these in an oily emulsion (Bain 1954).

A similar problem still exists with whooping-cough vaccines. There is an impression, almost certainly justified, that the protection they give is marginal. There is no doubt that this could be substantially increased by including more organisms in a dose. This is barred by regulations framed to protect children against the risks of severe reactions. Equally, the use of powerful but reactogenic adjuvants would not be tolerated. Therefore a very great deal of work is still being done to develop more highly immunizing but less reactive whooping-cough vaccines. Veterinary prophylactics—amongst them haemorrhagic septicaemia vaccine—will benefit eventually from this work; which could not be justified in a purely veterinary context.

Passive Immunization

Passive immunization has been far more widely practised in veterinary than in human medicine. A number of diseases of animals kill within the first days of life, before active immunity can be established. In man, such conditions would probably be controlled by hygienic measures. However, these may prove too impractical or too costly on the farm, so that immunological solutions to the problem are usually desired.

Lamb dysentery and enterotoxaemia, caused by *Clostridium perfringens* Type B and Type D respectively, are important killers of young lambs. Until recently neonatal animals were protected by the injection, soon after birth, of hyper-

immune sera prepared in horses. However, the inescapable need to administer this antiserum within the first 18 hr of life restricts the method to systems of husbandry where animals are continuously under the observation of shepherds or herdsmen. Since this is not practicable under extensive systems of husbandry, a programme of active immunization of the mothers has been devised to ensure that the colostrum would contain enough antibody to protect the offspring for the period of risk—from birth until they are marketed at about 5 months. Such active-passive systems of prophylaxis have now become integrated into farm programmes. Since it illustrates the application in the field of a number of classical immunological principles, a typical programme will be discussed later after other methods of prophylaxis have been considered. It is noteworthy that programmes have recently been devised (Rubbo 1966) to protect babies against tetanus with maternally transmitted antitoxin. This is required where hygiene is poor and where the risk of infection is maximal shortly after birth: in fact, where conditions approximate to those under which animals are kept.

Animals with high titres of homologous antibody acquired from the mother may prove difficult to immunize actively against the corresponding antigen. This complicates the problem of immunizing animals which are to be added to the flock or herd and kept until they are adult. Except in the case of neonates, active immunization is overwhelmingly more important than passive.

Active Immunization

Vaccines may be prepared from living or killed organisms: bacteria, mycobacteria, viruses, protozoa, fungi, or derivatives of these.

Bacterial Vaccines

These may be living or killed bacteria, extracts of bacteria, or products of bacteria which may or may not have been modified or detoxified.

Living Bacterial Vaccines

Historically, living organisms were the first to be used on a large scale to stimulate immunity to disease. The use of variolation in man long preceded Jennerian vaccination. Pasteur's anthrax vaccine was the first demonstrably successful vaccine against a bacterial infection. Nowadays, a large number of living bacterial, protozoal and viral vaccines are used and it is interesting to observe that wherever there exists a good living vaccine, strenuous efforts are made to devise a killed vaccine and vice versa.

All living bacterial vaccines are derived from stabilized attenuated strains. In the case of anthrax, the vaccine is prepared from spores whose resistance to adverse conditions makes a long shelf-life possible. However, in the case of vaccines prepared from non-sporing organisms, long shelf-life depends on lyophilization—which adds appreciably to their cost. At times, lyophilization is

undertaken for the convenience of the distributor who is thus enabled to keep stocks for long periods to cope with the fluctuating demands. Where manufacture and distribution are state monopolies, demand can be gauged accurately and vaccine used soon after preparation, so making lyophilization unnecessary. Liquid *Brucella abortus* and salmonella vaccines, for example, can retain efficiency for several months at room temperature.

Immunity stimulated by living vaccines is to a large extent dependent on the degree to which the organisms multiply in the host. The permissible limits for this multiplication are those which do not cause irreversible damage to more than an insignificant proportion of animals inoculated. The ability of living vaccines to multiply *in vivo* is often limited so that the amount of immunizing antigen the animal receives may—as in the case of anthrax—be insufficient to stimulate a long-lasting immunity. A further disadvantage is the impossibility of controlling the balance between antigens. All the antigens elaborated by the organism must be accepted with the consequent possibility of interference by response to irrelevant antigens. Possibly the worst defect is the difficulty of combining living vaccines in multi-component vaccines. These are of growing importance because of the undesirability of frequent injections and handling, and because of the increasing cost of the labour needed for assembling animals for inoculation.

The chief reason for using living vaccines is that some essential antigens may not be produced *in vitro* or not produced in sufficient amounts. This problem can usually be solved when the expense is justified, as when it becomes advisable to immunize man against anthrax (Wright 1965). Public health authorities have a lively apprehension that attenuated organisms may revert and become pathogenic for man. Veterinary authorities may therefore be placed in the invidious position of being asked for assurance that this could not happen—an impossible task. The discovery of the existence of transfer factors in bacteria which theoretically make it possible for attenuated strains to acquire missing factors from other organisms and so permitting the vaccine strain to revert to the wild type (Anderson 1966) has added scientific backing to an argument formerly based on vague apprehensions. Although the evidence for the occurrence of such reversions is scanty, public health authorities remain unhappy about the use of attenuated strains derived from organisms pathogenic for man.

Consider, for example, *Salmonella typhimurium* infection; since the greatest risk to humans is from infected animals, a reduction in their number would correspondingly reduce the risk for humans. Thus the hypothetical risk of reversion of the living vaccine should perhaps be accepted, since there is no evidence of its occurring in the field. It is interesting that the hazards of living oral polio vaccine were accepted despite some reports that vaccine strains passed through man might cause paralysis (Morse, Rubin & Blount 1966).

A somewhat intractable problem is the susceptibility of some living vaccines

to inactivation *in vivo* by antibiotics (Lee, Kidd & Sterne 1961). Although a warning is given against the use of these agents during the immunization period, this is often ignored. Moreover, many stockowners are not aware of the extent to which antibiotics are added to feeds, although this has to be stated on the label.

Inactivated bacterial vaccines

Inactivated vaccines depend for their efficacy on the amount and quality of antigen actually administered, since no multiplication *in vivo* occurs. This means that killed vaccines only become practicable when substantial amounts of antigen at a suitable concentration can be produced *in vitro* or when this can be obtained from infected animals and subsequently inactivated.

Toxoids. Where the agent responsible for the disease is a specific toxin it is often possible to administer sufficient detoxicated antigen to ensure a solid immunity of long duration; as in tetanus, botulism, and in various toxaemias due to *C. perfringens*, and, to a lesser extent, in various gas gangrene infections due to *C. septicum*, *C. novyi*, *C. sordellii*, etc. The principles are in no way different from those used to immunize man against diphtheria and tetanus, except that far larger doses are permissible in veterinary vaccines, thus simplifying the stimulation of a substantial enduring immunity. It is worth emphasizing once more that this ability of the veterinarian to use what would be—by human standards—massive doses, makes it possible to immunize animals to certain types of disease against which it would be difficult to protect man.

Killed organisms. Generally speaking, vaccines made from killed whole bacteria are less effective than those prepared from toxins specifically concerned in the pathogenesis of the disease. Pasteurella or salmonella vaccines do not approach the efficacy of tetanus or botulinum or *C. perfringens* vaccines. Most of the vaccines prepared from whole organisms are derived from Gram-negative bacteria. Since the protective antigen is usually confined to the cell, the number of organisms incorporated in the vaccine is of prime importance. For example, vaccines against haemorrhagic septicaemia of bovines had a poor reputation, but are now reasonably effective because of the tenfold increase in the number of organisms incorporated in a dose. Mice can usually be immunized without difficulty, but this protection may not parallel results from the field.

Classical immunology deals mainly with the reactions between toxic proteins —such as diphtheria, tetanus and botulinum toxins—and their antibodies. The endotoxins of the Gram-negative bacteria, however, are lipopolysaccharide complexes or complexes of lipopolysaccharides with proteins and lipids. They have a remarkable spectrum of pharmacological properties and very great stability to physical and chemical agencies. Their chemical and immunological properties are such that classical immunological principles only apply in a broad sense.

The dose of Gram-negative organisms that may be included in a vaccine is limited by the risk that the lipopolysaccharide of the cell wall may occasionally precipitate acute shock. This phenomenon is not well understood, because it is difficult to provoke at will and hence to study. Most vaccines prepared with Gram-negative bacteria will occasionally provoke this 'anaphylactoid' reaction. The symptoms are those of acute classical anaphylaxis, although there is no history of sensitization.

In a particular herd or flock, a subcutaneous dose of vaccine may provoke acute shock within 10–30 min of injection. A number of animals may die; some recover after seeming moribund. The degree of sensitivity is remarkable, and 1 or 2 ml of vaccine given *subcutaneously* may kill a large steer. It is quite usual for that particular batch of vaccine to have been used previously on a large number of animals without causing any ill effects. The sensitivity shown by the herd is ephemeral. Half the herd may be injected one day and be acutely sensitive; the remainder of the herd may be injected on the following day and show no ill effects.

No satisfying explanation of this syndrome has been given. It could be an example of a generalized Shwartzman reaction, since the history suggests a transient sensitization. Possibly a change of pasture or diet or even an attack of gastroenteritis might result in a change in the gut flora and a great increase of coliforms or other organisms that make endotoxin. Since the gut wall is normally permeable to small amounts of large molecular material (Bullen & Batty 1956, 1957), enough endotoxin might leak through to sensitize the animal to a vaccine containing toxic lipopolysaccharide, provided this were inoculated within a few hours. Such a hypothesis does not violate known immunological principles, and would explain the symptoms, the sudden onset and the evanescence of the sensitivity.

An important property of endotoxin is its ability to provoke a rapid increase in resistance to artificial infection—a resistance fully manifest within 24 hr. There have been attempts to harness this phenomenon to protecting young animals against gastro-intestinal conditions caused by coliform infections. Passive immunization with maternally transmitted antibodies is of limited value because the large number of different serotypes responsible for these conditions vary from outbreak to outbreak. Unfortunately, the precocious immunity provoked by endotoxin has proved to be markedly strain specific (Cameron, personal communication) so that the hope of being able to obtain by this simple means a group resistance to those diseases has had to be abandoned for the time being.

Viral Vaccines

Vaccines against viral infections have been far more widely used in veterinary than in human practice and for a far longer time, with, of course, the notable exception of vaccinia and, on a far more restricted scale, rabies vaccine. Living

virus has been used more often than inactivated virus, mainly because of the difficulty of incorporating enough inactivated virus in a reasonable dose. This difficulty is fast being resolved by modern methods of culture.

Immunization with living virus

Use of virulent virus. Immunization with living virulent virus modified by the simultaneous inoculation of immune serum was widely practised long before passaging viruses to reduce virulence for the natural host had become common. The so-called serum-virus method was used for vaccination against blue tongue, African horse sickness, rinderpest, swine fever and canine distemper, amongst others. The considerable risks attending this method were accepted because of great losses from the natural disease. It could not, however, be used in human practice. Before effective foot-and-mouth disease vaccines existed, virulent virus was often used during an outbreak in order to spread the disease rapidly through herds so as to shorten the duration of an epizootic, when other methods of control could not be applied.

Use of attenuated virus. Nowadays, a large number of pathogenic viruses have been attenuated by passaging strains in embryonated eggs, in tissue culture or in animals. The extent of attenuation can be controlled by varying the number of passages to yield vaccine strains of different degrees of attenuation and immunogenicity. Lower passage strains tend to be of higher virulence and immunogenicity than strains which have undergone many passages. In veterinary practice it is possible to use vaccines considerably more virulent than those tolerable for use in man. This has meant that a more effective use of viral vaccines has been possible in the veterinary than in the human field.

Immunization with inactivated virus

Before growth in embryonated eggs or in tissue culture had been perfected it was difficult to incorporate sufficient virus in killed vaccines to immunize satisfactorily. Sufficiently large harvests could only be obtained by removing organs or tissues from infected animals and treating these with virus-inactivating agents. Vaccines of this kind have been widely used, and some are still used. Examples are formolized rinderpest spleens and foot-and-mouth vaccines made from formolized infected tongue epithelium. The crudity of such preparations may surprise workers more familiar with vaccines and sera used in man. However, to keep matters in proportion, it should be remembered that the most widely used vaccine of all time—vaccinia—was, and often still is, a highly contaminated suspension of calf scabs.

The development of powerful adjuvants generally too reactogenic for use in man has improved the outlook for inactivated vaccines in veterinary practice, as in the case of enzootic abortion of sheep.

Protozoal Vaccines

The use of procedures for immunizing against protozoa is almost entirely confined to animals. These may be actively immunized with attenuated strains or they may be infected with virulent strains and subsequently treated with drugs to control the infection. It has been, and still is, a custom in the Middle East to infect humans with cutaneous leishmaniasis in order to obtain a better cosmetic result on healing than might follow natural implantation of the parasite.

Immunity to protozoal infections is, generally speaking, less than that following bacterial or viral infections. At times, resistance to protozoa seems to depend on the persistence of a low-grade infection either by virulent or by attenuated organisms, thus making the host resistant to superinfection. Such an animal remains a carrier of the disease. Artificial immunity of this type has been produced against *Anaplasma marginale* by infecting animals with the mild *Anaplasma centrale* strain. Mild strains of babesia have also been used to immunize cattle against redwater; but it has been advisable to control the infection with suitable drugs, since the state of attenuation of these strains has been somewhat unpredictable. Living vaccines have also been used against theileria, for example *T. annulata*. In the laboratory, inactivated vaccines have been prepared against *Trypanosoma cruzi*, but these have not been used in practice (Johnson, Neal & Gall 1963). Exo-antigens (soluble protective antigens) have been demonstrated in trypanosomiasis, babesiasis and anaplasmosis.

On the whole, vaccines against protozoal infections have had only a limited success. The plurality of strains within a species as well as the tendency of strains to mutate has made it difficult to prepare vaccines effective against a group of strains of the same species. This difficulty is not, of course, confined to protozoa.

Metazoal Vaccines

Parasitic infections are amongst the most costly and intractable problems that beset animal industry. The attraction of an immunological solution is that continually repeated treatments would no longer be required. Perhaps the best hope for success is against parasites which pass a stage of their existence in organs, muscles or tissues, so that antibodies could be raised against them. A moderately successful vaccine exists against lungworm of cattle (Jarrett *et al* 1958) which suggests that an immunological attack on helminth infections with a migratory larval stage is feasible. This makes sense in veterinary but not necessarily in human practice. Passive or active immunization against the migratory stage might result in tissue reactions to the parasite which could be more dangerous to the host than the parasite itself. A tissue reaction to *Toxocara canis* infection in the retina or brain of a child might be more disastrous than giving the larva a chance of leaving the site naturally. Some success has followed attempts to immunize laboratory animals against helminths infesting the gut, but it has not yet been possible to use these results in practice.

Reactions to Tissue Components

Research on auto-allergic diseases or on immunity to neoplasms plays a minor part in veterinary immunology. These conditions are too sporadic to justify expensive treatment or prevention. Moreover, the impression that hereditary factors may be concerned in susceptibility to such conditions discourages breeding from affected animals or those related to them. Most of the considerable amount of experimental work carried out in animals has been done to help solve such problems in man.

Blood groups of animals were of some interest when horses had to be transfused with large amounts of blood containing virus in the preparation of hyperimmune horse sickness serum. Deaths due to incompatibilities could be avoided by direct matching of the bloods of donors and recipients. Such matching is occasionally necessary in those cases where the value of an animal justifies transfusion. A renewed interest in this subject has been stimulated by the realization that haemolytic disease of the newborn is a significant cause of death in foals and that incompatibilities between foetal calves and their mothers may result in reduced fertility.

Adjuvants

The application of adjuvants to enhance the immunogenicity of vaccines began with the work of Glenny *et al* (1926) who found that the addition of compounds such as potash alum to the detoxified toxins used in the hyperimmunization of horses for the preparation of diphtheria and tetanus antisera increased the antitoxin response.

Originally it was thought that this increased response was due entirely to the effect of the potash alum in forming a depot in the animal from which the adsorbed toxoid was slowly released, giving rise to a prolonged antigenic stimulus. It was later realized that adjuvants also stimulated the proliferation of cells of the reticuloendothelial system, ensuring a more efficient transfer of the antigen to the sites of antibody production as well as increasing the number and efficiency of the immunologically competent cells of these sites. It is not surprising, therefore, that the most efficient adjuvants often give rise to undesirable reactions, and that a balance must be struck between maximum antibody response and the maximum acceptable reactions. The acceptability of reactions depends on the site of inoculation and on the type and species of animal inoculated. A small indurated nodule following the injection of an alum adjuvant may be undesirable in show cattle, acceptable in a dairy herd and pass completely unnoticed in ranch cattle or in sheep. In breeding animals in which the value of the carcasses as meat is of secondary importance, the highly efficient but particularly reactogenic oil emulsion adjuvants may be used to boost poorly antigenic vaccines such as that against enzootic abortion.

The depot function of an adjuvant is well exemplified by the most commonly used of adjuvants, the various compounds of aluminium; potash alum, aluminium phosphate, aluminium chloride and the preformed gels of aluminium hydroxide. However, the stimulatory function is better shown by water-in-oil emulsions. The addition of mycobacteria is avoided in veterinary practice, especially in vaccines intended for use in cattle, because the increased adjuvance is off-set by the disadvantage of sensitizing the animal to mycobacteria.

Sites of inoculation

In animals the choice of site is governed by many factors other than the immunological ones, and whereas inoculation in humans can usually be made under reasonably aseptic conditions, inoculations in animals have frequently to be made in wet, dirty surroundings through skin contaminated by faecal organisms. Sites prone to contamination, sites where reactions will result in lameness, and sites where nodules would result in 'carcass blemish' are to be avoided. There is a far greater likelihood of gas gangrene following dirty intramuscular than dirty subcutaneous injections.

Ideally, the sites should be chosen so that normal drainage would result in the antigenic material being spread to the greatest number of lymph nodes. The antigen could be contained in a relatively large volume to aid this spread and additional response may result from dividing the dose. Although it is commonly believed that the intravenous route is the most desirable for particulate antigens such as bacterial cells, this route is not used outside the laboratory.

Spacing of injections

The spacing of injections is governed mainly by immunological considerations, but attempts are made to conform to farming schedules as far as possible. The immunization of animals born of non-immune mothers can be commenced as soon after birth as practicable. A primary sensitizing course should consist of two doses, and so far as immunization against bacterial diseases caused wholly or in part by toxins is concerned, the interval between these two doses should not be less than 6 weeks.

Inoculation Schedules

These are based on the fundamental work of Glenny & Südmersen (1921) who drew attention to the profound effect which previous experience of an antigen had on the response to further stimulation with the antigen. They showed that primary stimulation was succeeded by a latent period of about 3 weeks during which little or no antibody was detectable in the circulation, followed by a slowly increasing immunity which reached its maximum in about 8 weeks, in

contradistinction to the responses to second or subsequent stimulation which had a short latent period followed by a rapid and great increase in antibody production, reaching its maximum in about 10 days.

Glenny and his co-workers (Barr & Glenny 1945) showed that these principles applied to a wide variety of animal species and to many kinds of toxoid. Later they showed that the latent period was shortened as the size of the primary stimulus was increased; that the size of the second inoculation had little effect on the magnitude of response, provided that the primary stimulus was adequate, and that a better secondary response occurred if the interval between the first and second injections was made longer than 3 or 4 weeks. The conversion of clinicians to using schedules based on second inoculations given a month and more after a primary stimulus was a gradual process. It will be remembered that the first recommendations for Salk polio vaccine prescribed a pre-Glenny schedule which was later amended.

Many immunization procedures in veterinary practice depend on a primary stimulus given at some convenient time, as when animals are gathered for some necessary manipulation. The second or 'booster' inoculation may thereafter be given shortly before a disease is expected to occur. For example, the greatest incidence of *C. septicum* infection (braxy) of sheep is in winter. Therefore the first inoculation of vaccine may be given early in the year and the second in autumn or in the beginning of winter. *C. novyi* infection (black disease) is often associated with infestation with liver fluke. Thus the booster inoculation should be given about 2–4 weeks before fluke infestation is expected. Similarly, if newborn animals are to be protected by maternal antibodies, the mother should ideally receive the booster dose of vaccine 2–4 weeks before parturition. The previous inoculation may have been a year or more earlier.

These principles and these intervals hold for most protein antigens such as diphtheria, tetanus, botulinum, septicum, novyi, perfringens toxoids as well as a number of others. The indications are less clear in the case of Gram-negative pathogens with their somewhat heterogeneous collection of antigens; lipopolysaccharides, and their complexes with proteins, lipids and polysaccharides. Living vaccines very often act as their own secondary stimulus by virtue of their continued presence. Indeed, someliving vaccines, because of their persistence, result in virtually life-long imm unity. This is less surprising than the failure of other living vaccines—such as anthrax—to provoke more than an ephemeral immunity. In such cases the amount of antigen produced during the infection may be too little to ensure a long-lasting response. On the other hand, further inoculations do not materially prolong immunity. Possibly, interference by the multiplicity of antigens produced by the living growing organisms is a factor. In other examples of limited response to living vaccines, over-attenuation and consequent paucity of immunizing antigen may be decisive. In some cases there may even be paralysis of the antibody-producing mechanism because of

over exposure to immunizing antigens, particularly in the case of polysaccharide antigens.

Hyperimmunization

There are perhaps lessons to be learnt from the techniques employed successfully in hyperimmunization for the production of therapeutic sera. This is a somewhat specialized field of applied immunology where the inoculation schedules are largely empirical but are nevertheless based on the principles of primary and secondary stimulation. Where possible, for example, in the production of staphylococcus or *C. perfringens* Type A antisera, naturally immune animals are chosen for hyperimmunization. When this is not possible, as is the case with tetanus or *C. perfringens* Types B and D, it is customary to give the primary stimulus either by the injection of adsorbed toxoid or by a short course of five or six injections of small doses of toxin followed by a period of rest. Although 4 weeks is a sufficient interval to produce a secondary response to another injection, it is not a sufficient interval between a primary stimulus and hyperimmunization. If hyperimmunization by repeated injections two or three times a week of high-value toxins is started too soon after a primary stimulus, the antitoxin produced is of low value and poor quality, that is, non-avid and combining so loosely with toxin that dilution is sufficient to free this from the complex. Such antitoxin would clearly be unsatisfactory used either therapeutically or prophylactically. The following figures given by Glenny, Pope, Waddington & Wallace (1925) show the effect of interval between injections on horses immunized against tetanus:

Length of rest after primary stimulation (months)	Average titre of ten or more horses (units per ml)
0	310
1–3	825
2–3	1090
4–6	1340
>6	1490

The consensus of workers engaged in hyperimmunization is that time is essential for the establishment of good basal immunity, and this is achieved naturally by repeated very small stimulation, or artificially by the system of rest.

An average horse will produce 50,000 g of specific immune globulin during its productive life. Throughout this period it suffers a loss of 3×10^{10} lymphocytes at each bleeding, which amounts to approximately 3×10^{12} for its working life. There are considerable variations between individual horses as regards the total amount of any given antibody that they make in response to hyperimmunization with the same batches of antigen. It is commonly found that each

toxin has its own pattern of responses—for example, in the production of diphtheria antitoxin the highest titre is usually obtained after the second course of hyperimmunization and this titre then declines slowly with each subsequent course. The same pattern of response is seen in the production of tetanus antitoxin if the type of toxin used is kept constant. However, if the toxin is changed with each course, a high titre can be maintained over many courses. Sooner or later the amount of a particular antibody produced begins to flag, and the animal can then be switched to producing another antibody. For example a horse no longer producing commercially adequate amounts of diphtheria antitoxin might still be able to make useful amounts of *C. septicum* antitoxin—provided it had been primed for this at some time in the past.

It is even possible to hyperimmunize a horse with more than one major antigen at the same time. For example, when large amounts of mixed gas gangrene antisera were required, the experiment was made of injecting horses with mixed culture filtrates of *C. perfringens* Type A, *C. septicum* and *C. novyi*. Although the titres to each component were not as high as the average titre when a single component was used, they were always higher than the average obtained by mixing equal volumes of serum from horses immunized with a single antigen. The method was abandoned largely because of the difficulty of blending such mixed antisera to give fixed titres of each component. This lower titre when two or more antigens are used for hyperimmunization might result from a diminished availability of antibody-producing cells because of the drain of the comparatively long-lived mononuclear cells by the repeated large-scale bleedings. It would be interesting to see whether replacing these cells (plasmaphoresis) would result in better responses to more than one antigen.

Programming Immunization Schedules

To illustrate the practical application of the principles considered earlier a comparatively simple programme of immunization against lamb dysentery and enterotoxaemia (*C. perfringens* Types C and D) will be outlined. Lamb dysentery kills during the first 2 weeks of life; enterotoxaemia kills throughout life. Inoculations must be kept to the minimum consistent with protection since the cost of gathering and treating animals is by no means negligible.

The following requirements must be met:

1. Lambs must be protected against lamb dysentery for the first week, and against enterotoxaemia throughout life.
2. In order to save handling and to avoid carcass blemish due to inoculations, lambs should receive sufficient antibody through the colostrum to protect them until they are killed at about 5 months.
3. Animals retained for wool production, or breeding, or marketing as adults must be actively protected against enterotoxaemia in such a way that no gap is left between waning passive immunity and a functioning active immunity.

An animal is protected if it has 2 units/ml of anti-B and 0.1 unit/ml anti-D. The former is required at 2 weeks and the latter at 20 weeks post-partum. Since the titre of homologous antibody is halved every 14 days, the titres at birth should be four of B and one hundred of D. The first is easily provided, but the latter would not usually be transmitted by first season ewes. However, there would be only a short gap of 2 or 3 weeks during which the lamb would not be fully protected.

To provide the antibody required, the ewe will need two inoculations with an interval of 6 weeks or more between them. The ewe itself will become solidly immune. To ensure adequate transmission to the lamb, it must receive a 'booster' about 2 weeks before lambing. In subsequent years only this one inoculation is required and it will ensure the protection of the ewe and lamb. Ewes in their second and subsequent seasons will usually provide sufficient anti-D for the lamb to protect it until it is marketed.

Animals which are to be retained for breeding should get their first dose of vaccine when their inherited immunity is tending to fall below an effective level. If they are inoculated earlier, the high level of circulating antibodies will interfere with the immune response. Two inoculations at 6 weeks' interval are required, followed by a single 'booster' before lambing in subsequent years.

Nowadays combinations of vaccines effective against six or more diseases are commonly used. The diseases may differ greatly in respect of seasonal incidence, severity of exposure, age susceptibility, and duration of immunity. Since 'boosters' should be given as close as possible to the time of severest challenge, these variables set quite intricate problems. It is clear that the compiling of schedules for complex mixtures of vaccines demands reliable epizootiological information as well as a good deal of ingenuity.

REFERENCES

ANDERSON E.S. (1966) Possible importance of transfer factors in bacterial evolution. *Nature* **209,** 637.

BAIN R.V.S. (1954) Immunisation contre les pasteurelloses des bovins. *Bull. off. int. Epiz.* **42,** 256.

BARR M. & GLENNY A.T. (1945) Some practical applications of immunological principles. *J. Hyg., Cambs.* **44,** 135.

BRITISH VETERINARY CODEX (1965) London: The Pharmaceutical Press.

BULLEN J.J. & BATTY I. (1956) The effect of *C. welchii* Type D culture filtrates on the permeability of the mouse intestine. *J. Path. Bact.* **71,** 311.

BULLEN J.J. & BATTY I. (1957) Experimental enterotoxaemia of sheep. The effect on the permeability of the intestine and the stimulation of antitoxin production in immune animals. *J. Path. Bact.* **73,** 511.

CAMERON, J. Personal communication.

GLENNY A.T. & SÜDMERSEN H.J. (1921) Notes on the production of immunity to diphtheria toxin. *J. Hyg., Cambs.* **20,** 176.

GLENNY A.T., POPE C.G., WADDINGTON H. & WALLACE U. (1925) Immunological note, XVI. A method of producing tetanus antitoxin serum of high potency. *J. Path. Bact.* **28,** 481.

GLENNY A.T., POPE C.G., WADDINGTON H. & WALLACE U. (1926) Immunological notes, XVII–XXIV. The antigenic value of the toxin-antitoxin precipitate of Ramon. *J. Path. Bact.* **29,** 31.

JARRETT W.F.H., JENNINGS F.W., MARTIN B., MCINTYRE W.I.M., MULLIGAN W., SHARP N.C.C. & URQUHART G.M. (1958) A field trial of a parasitic bronchitis vaccine. *Vet. Rec.* **70,** 451.

JOHNSON P., NEAL R.A. & GALL D. (1963) Protective effect of killed trypanosome vaccines with incorporated adjuvants. *Nature,* **200,** 83.

LEE L.A., KIDD A.R.M. & STERNE M. (1961) Antibiotic treatment and its effect on anthrax immunization. *Vet. Rec.* **73,** 1426.

MORSE L.J., RUBIN H.E. & BLOUNT R.E. (1966) Vaccine-acquired paralytic poliomyelitis in an unvaccinated mother. *J.A.M.A.* **197,** 1034.

RUBBO S.D. (1966) New approaches to tetanus prophylaxis. *Lancet* 7461 **ii,** 449.

WRIGHT G.G. (1965) The anthrax bacillus. In *Bacterial and Mycotic Infections of Man,* 4th edn., p. 538. London: Dubos & Hirsch.

CHAPTER 49

BIOLOGICAL STANDARDS

D.G.EVANS

PRINCIPLES OF BIOLOGICAL STANDARDIZATION

There are frequent occasions when the immunologist, wishing to measure the activity of biological substances, finds that physical and chemical methods are of little or no help. Such substances are those whose activity can be demonstrated only by their action on a biological system such as a whole animal or part of an animal and include toxins, antigens, antibodies, living virus suspensions and so on. There are many well-known examples. Diphtheria toxin kills guinea-pigs when injected intramuscularly and produces characteristic lesions when given intradermally. Other toxins, such as those obtained from pathogenic strains of streptococcus and staphylococcus produce haemolysis when added to a suspension of red blood cells. Antitoxins manifest their activity by neutralizing the lethal, dermonecrotic, haemolytic or other toxic properties of homologous toxins. Immunizing antigens, for example tetanus toxoid, on injection into animals induce an immunity which can be shown by the presence of antitoxin in the serum or by the resistance of the animals to a challenge with a fatal dose of toxin. Virus suspensions, such as vaccinia virus and poliovirus, produce cytopathic changes when added to tissue cultures.

Direct Measurement of Biological Activity

It is common practice in order to measure the activity of a biological substance to test a series of doses in the appropriate biological system and thereby determine an end-point of activity. Such a test, usually referred to as a titration, gives a sigmoid dose-response curve when the logarithm of the dose is plotted against the effect produced (Fig. 49.1). The effect may be the percentage of animals dying, the diameter of intradermal lesions or the degree of haemolysis if a toxin is being titrated; it may be the level of antibody produced if an antigen is being tested; or it may be the percentage of animals surviving a challenge infection when titrating an antibody. From this curve a number of convenient end-points

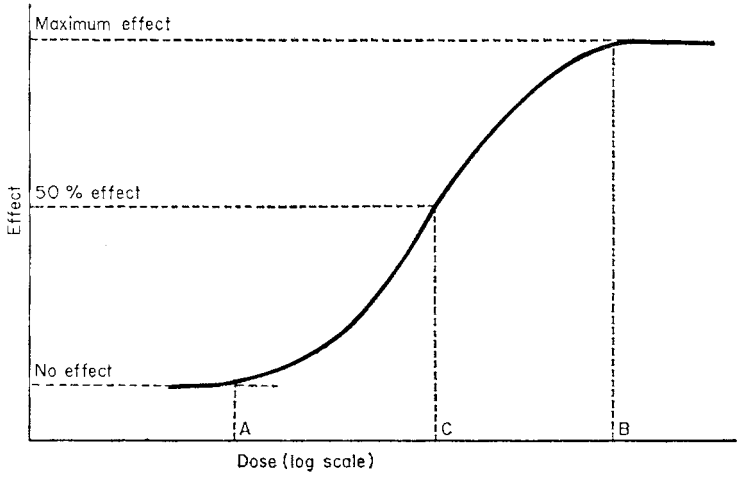


Fig. 49.1.

can be obtained, the main ones being (A) the largest dose which produces no effect, i.e. the maximum 'non-effective' dose; (B) the smallest dose which produces maximum effect, i.e. the minimal effective dose; and (C) the dose which produces 50% effect, i.e. the median effective dose or ED50. It is clear that end-points A and B lying on the lower and upper parts of the curve are imprecise measures of activity while the ED50 end-point at C lying on the steepest part gives the most precise measure.

These facts are now widely appreciated and the use of such terms as minimal lethal dose (MLD), minimal skin-reacting dose (MRD) and minimal haemolytic dose (MHD) are becoming less often used. Lethal doses are now expressed as LD50, immunizing doses as ImD50, haemolytic doses as HD50 and tissue

culture infecting doses as TCID50. It is not often realized, however, that no matter how accurate the determination of activity, the result obtained applies only to the particular experiment made. Thus the ImD50 end-point of a preparation of an immunizing antigen obtained using one batch of guinea-pigs, on one day and in one laboratory may be vastly different from that obtained when the same preparation is tested in another batch of animals, or on another day or in another laboratory. Moreover, the slope of the dose-response curve may vary considerably with such variations in the biological test system. Similarly the TCID50 of a suspension of poliovirus may vary with different batches of cell cultures and will depend on the sensitivity of the culture to infection with poliovirus. It is in fact quite impossible, because of the innate variability of a biological system, to specify the conditions that will give the same ED50 and the same dose-response slope whenever the system is used, and because this of we are thus unable to make a *direct* measurement of biological activity.

Comparative Measurement of Biological Activity

It is, however, possible to make accurate *comparative* measurements of the biological activity of two or more preparations. For example, if four different

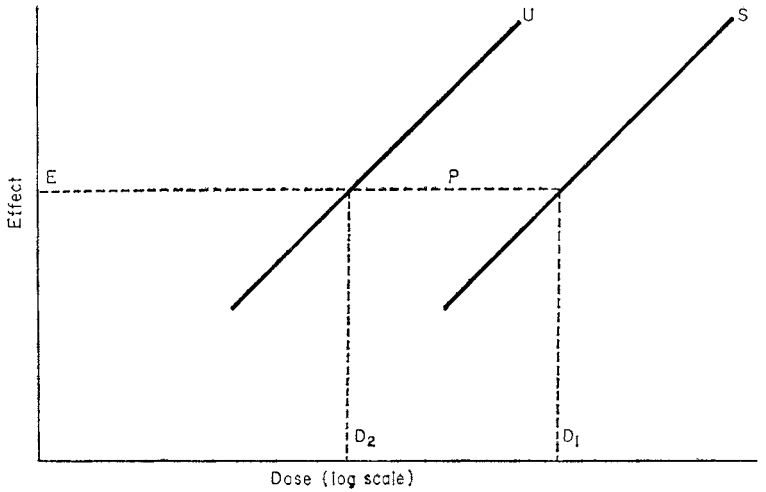


Fig. 49.2.

preparations of the same antigen were tested in an adequate number of guinea-pigs, at the same time, side by side and under uniform conditions, their relative potencies would be the same as those obtained when similar tests were made on another occasion or in another place. Herein lies the principle on which the

estimation of the activity of biological substances of unknown chemical composition is based. By choosing one preparation as a standard, the activity of other preparations of the same substance can then be determined relative to the standard by a parallel test using the same biological system. In order to ensure parallelism as far as possible, the test on the standard and the other preparations are made simultaneously using the same batch of animals randomly divided into groups, the same suspension of red blood cells, the same batch of cell cultures or as in tests involving skin reactions, the same animal.

Such comparative tests can be represented by Fig. 49.2 in which the activity of preparation U is compared with that of a standard preparation S of the same substance. In this figure an effect E is obtained by dose D_1 of the standard and also by dose D_2 of the preparation under test. The ratio of D_1 to D_2, or the distance P between the curves (since a log scale is used) represents the relative potency. Although it may be convenient to make the comparison using the dose levels which produce a 50% effect (ED50), this is not imperative as long as the comparison is made along the steep part of the curves.

From comparisons such as these we can, therefore, conclude that a preparation has the same potency, or is so many times as strong, or so many times as weak as the standard, and if we now assign a unitage to the standard the potency of the preparation can then be expressed in terms of units. It is on this basis that international standards for many biological substances have been established over the past 40 years and that classical biological assays in terms of standards have been developed.

INTERNATIONAL BIOLOGICAL STANDARDS

The First Biological Standard

The importance of comparative testing in determining the activity of biological substances was recognized by Ehrlich in his pioneer work on the standardization of diphtheria antitoxin. Ehrlich first tried to obtain uniform results by determining the potency of antitoxin in terms of its ability to neutralize 100 minimal lethal doses of diphtheria toxin when tested in 250 g guinea-pigs. The method, however, proved unsatisfactory mainly because of the variation in toxin preparations which, although similar in their toxic effects, could differ considerably in their ability to combine with antitoxin on account of the presence of varying amounts of toxoid. This led Ehrlich finally to choose as his reference point the antitoxin, as this could be preserved in a stable dry form. Thus in 1897 he established a unit for diphtheria antitoxin which was defined as the antitoxic activity of a certain weight of a particular preparation of dried antitoxin. Using this standard, the potency of other diphtheria antitoxin preparations could then be determined by a comparative test which was independent both of the biological system, namely the guinea-pig, and the toxic preparation used.

The Ehrlich standard diphtheria antitoxin was distributed internationally from his institute in Frankfurt until 1914. During the war those countries outside German domination adopted a subsidiary standard which had been prepared, in terms of the original Ehrlich unit, by the Hygienic Laboratory, Washington, U.S.A., and finally after the war in 1922 the Ehrlich unit was adopted as the international unit for diphtheria antitoxin by the Health Organization of the League of Nations.

Further Development of International Standards

Thereafter many more international standards were established by the League of Nations for other antitoxins and also for biological substances such as vitamins and hormones, which were rapidly being introduced. This work was mainly carried through by the initiative of Dr Thorvald Madsen, director of the State Serum Institute, Copenhagen, and Sir Henry Dale, director of the National Institute for Medical Research, London, and later by Sir Percival Hartley, the first director of the Department of Biological Standards at the National Institute. Since the Second World War the World Health Organization, through its Expert Committee on Biological Standardization, has continued the work and today there are more than 150 standards and reference preparations including 100 microbiological products (antigens and antibodies), thirty-three antibiotics, eleven hormones and twelve other miscellaneous preparations such as enzymes.

For each standard a sample of the preparation is chosen and, by international agreement through the World Health Organization, is established as an international standard. The strength of the standards is expressed in arbitrary units of activity referred to as 'international units (i.u.)'. For example, 1 i.u. of diphtheria antitoxin is the activity contained in 0.0628 mg, 1 i.u. of insulin in 0.04167 mg and 1 i.u. of penicillin in 0.0005988 mg of the respective international standards. The biological standards are unique samples of the different substances, each with its own arbitrary unit of activity and are in fact comparable to the physical standards of weight and length. They may be regarded as the 'yardsticks' in the *comparative* measurements of other preparations of the same substance which can then be given a strength in terms of international units. By this means, as the units become generally adopted, uniformity is ensured throughout the world in the designation of potency for those prophylactics and therapeutic substances which cannot be characterized adequately by chemical and physical methods. Table 49.1 gives a few examples from the current list of international standards.

Some standards have of course been discontinued—for example those for vitamins A, B, C and E, oestrone and progesterone—since it was found possible to measure their potency simply by chemical and physical tests. But despite this, the number of standards will undoubtedly increase during the next few years, mainly due to the continued developments in antibiotics, the introduction

of new viral vaccines, the extension of vaccines in the veterinary field and the use of enzymes of therapeutic activity. This was strikingly evident from the discussions during the Seventh International Congress on Biological Standardization held in London in 1961 (Standfast, Evans & Weitz 1962).

TABLE 49.1

Examples of some international standards*

Substance	Established	Wt. of 1 international unit (mg)
Diphtheria toxoid plain	1st Standard 1951	0.5
Schick test toxin	1st Standard 1954	0.0042
Pertussis vaccine	1st Standard 1957	1.5
Tetanus antitoxin	1st Standard 1928	0.3094
Diphtheria antitoxin	1st Standard 1922	0.0628
Gas gangrene antitoxin (*Cl. welchii* Type A)	5th Standard 1963	0.3346
Staphylococcus antitoxin	2nd Standard 1938	0.2376
Anti-streptolysin O	1st Standard 1959	0.0213
Penicillin	2nd Standard 1952	0.0005988
Insulin	4th Standard 1958	0.04167
Hyaluronidase	1st Standard 1955	0.1

* Taken from *WHO Tech. Rept. Series*, No. 361, 1967.

DISTRIBUTION OF INTERNATIONAL STANDARDS

The responsibility for holding and supplying the international standards rests principally with three centres, the State Serum Institute, Copenhagen, for the immunological substances (antigens and antibodies), the National Institute for Medical Research, London, for all other substances, mainly antibiotics and hormones, and the Central Veterinary Laboratory, Weybridge, for those substances used exclusively in veterinary medicine such as anti-*Brucella abortus* serum and Newcastle disease vaccine. Small quantities of the standards are dis-

tributed free of charge on request throughout the world to the various National Control Laboratories which are in close touch with the three principal centres and are responsible for making standards available to manufacturers and research workers in their own countries.

Biological standards, unlike the physical standards for weight and length, are unfortunately expendable materials and although large quantities are held by the three centres, the problem of their replacement arises from time to time when supplies become low. The National Laboratories are, therefore, encouraged not to use the international standards for routine assay work, but to prepare their own national standards assayed in terms of the international ones, so that the supply of the international standards is maintained for a much longer period. It is interesting to note that the first international standard for diphtheria antitoxin established in 1922 and for tetanus antitoxin established in 1928 are still in existence and this is due in no small measure to the preparation of various national standards. In Britain, the National Institute for Medical Research, in addition to being responsible for international standards for antibiotics and hormones, also acts as a National Laboratory for the immunological standards held in Copenhagen and has prepared its own national immunological standards for distribution throughout Britain and the Commonwealth. Furthermore, the manufacturing laboratories who are often the most frequent users of biological standards are also encouraged to prepare their own working standards assayed in terms of the national ones and this procedure in turn considerably extends the life of the national standards.

Establishment of International Standards

Apart from holding and distributing the standards, the three main centres in Copenhagen, London and Weybridge are also entrusted by the World Health Organization with the task of establishing new standards and replacements for existing ones for which the supply is almost exhausted. The procedure is an exacting and lengthy one. In the first place the choice is made of a suitable and homogeneous sample which is usually supplied by manufacturing laboratories. The material is distributed into a large number of glass ampoules, extreme care being taken to ensure that during and after distribution each ampoule is subjected to the same procedure. The whole batch is then dried under sterile conditions over phosphorus pentoxide if the substance is a solid, or by freeze-drying and then over phosphorus pentoxide if the substance is a liquid. The ampoules are filled with pure dry nitrogen, sealed by fusion of the glass and then stored in the dark at $-10°C$; in this way the substance is protected from high temperature, oxidation, moisture and light. These precautions directed towards maintaining stability are most essential, for at all costs stability must be ensured otherwise the material will fail in its primary function of serving as a standard of reference. In

addition, checks are made of stability by carrying out accelerated degradation tests, in which the final preparation is assayed after subjecting it to heat for various periods.

The next stage in the procedure is to send out, to about twelve laboratories in different countries, a number of ampoules of the proposed standard. These laboratories, which are experienced in the biological assay of the particular substance under investigation, examine the preparation for its suitability as a standard for the assay of other preparations. On the other hand, if the proposed standard is a replacement, the laboratories assay it in comparison with the existing standard. As a result of this international collaborative study the proposed standard is assigned a potency and the unit defined as the activity contained in a given weight of substance. The proposed standard is thus elevated to the status of an international standard and is ready for distribution throughout the world to all laboratories who require it.

In this way the work of biological standardization continues year by year. Despite the major advances in physicochemical methods which it is hoped will ultimately bring about the extinction of biological standardization, new substances are regularly added to the quite imposing list of international standards, and each year the two centres distribute more than 4000 ampoules of standards. There can be no doubt that this fundamental and important work, which provides the only basis for agreement and unification in the study of biological substances whose chemical constitution is yet unmasked, brings enormous advantages to scientific research and to the practice of medicine throughout the world.

THE USE OF BIOLOGICAL STANDARDS

The principal use of biological standards is in controlling the potency of therapeutic and prophylactic substances which are used in human and verterinary medicine. In carrying out this control most countries throughout the world have now established, or adopted, regulations for potency based on the international standards. In Britain, for example, control is exercised through the Therapeutic Substances Act and the British Pharmacopoeia which lay down, wherever possible, specific potency requirements based on the international standards for vaccines, antisera, antibiotics, enzymes and hormones. For example, the Therapeutic Substances Regulations for tetanus antitoxin read as follows: 'Tetanus antitoxin in the form of a solution of the antitoxin globulins, or derivatives thereof, which has a potency of less than 1000 units per ml, or a dried preparation of such substance which has a potency of less than 7500 units/g, shall not be issued for prophylactic use.'

Similar requirements are given in the British Pharmacopoeia which in addi-

tion outlines suggested methods of assaying potency for all the biological substances which it covers and any reader who wishes to embark on potency testing will be well advised to turn to the British Pharmacopoeia for guidance.

Although the principle of measuring the potency of a biological substance in terms of a standard may be expressed by Fig. 49.2 in which the dose-response curves are parallel, it is evident in many biological assays, even though the biological system may be ideal and uniform for both the standard and the preparation under test, that parallelism does not always exist. Parallelism breaks down if the standard or the preparation under test is heterogeneous in the sense that the effect produced is not due to one compound but to a number of compounds having similar activity, as in the case of the penicillins, the D vitamins and tetanus antitoxin. Parallelism also breaks down when the standard or the test preparation contains substances which may either inhibit or enhance the activity of the biological substance. Furthermore, in the case of antitoxins, standard or test preparations may behave differently on account of differences in their power of combining with toxin, i.e. in their avidity. But although impurities and heterogeneous substances, present in either the standard or the test preparations, may make it impossible to perform the comparison under the ideal conditions of parallelism, this is no reason for delaying the establishment of a standard preparation until purification of the active principle has been achieved. In fact, it is due to the establishment of standards in the early stages when they are impure, that impurities and heterogeneous substances have been detected as a result of the variations obtained when assays in terms of the standard have been made using different biological systems. Thus the heterogeneity of tetanus antitoxin was shown to exist from the variation obtained when a number of antitoxins were assayed in terms of the standard using different toxin preparations and different test animals, namely mice and guinea-pigs.

There is a further reason why there should be no delay in establishing a standard for a preparation which is to be of prophylactic, therapeutic or diagnostic importance. Unless a standard is available and a unitage defined, it is highly likely that different workers will produce results which bear little or no relationship to one another. This can be well seen, for example, in the practice employed by some workers when measuring poliomyelitis antibody concentration, of determining the highest dilution of serum required to neutralize a quantity of virus suspension, and expressing antibody concentrations in terms of serum dilutions such as 1/4, 1/256, 1/10,000 and so on. In most cases it is completely impossible to make a meaningful comparison of the results of different workers because there is no point of reference. Moreover, the lack of uniformity in the way the test is performed makes even rough comparisons difficult; variations exist in the ratio of serum to virus suspension, in the time and temperature allowed for neutralization, in the test dose of virus, in the species of animal used for producing the cell cultures and so on, which all affect the

degree of sensitivity of the test and hence the resulting titre. This was borne out in 1959 when the World Health Organization conducted a collaborative study in which they distributed to different laboratories throughout the world a number of poliomyelitis antisera. The titres obtained by the laboratories varied widely, in some cases as much as a hundred-fold; in fact the study showed quite clearly the variability of the biological system and the inability of different laboratories to obtain comparable results by direct measurement. Those who employ these methods of expressing potency in terms of serum dilutions without reference to a standard are committing the same sin as those who in the past used 'cat units', 'mouse units' and 'rat units' and of whom Professor J.H.Burn (1930) wrote, '. . . all those who use these expressions, and who do not understand the fallacies underlying them, are still ignorant of certain principles which during the past few years have been shown to be capable of transforming this whole subject from the plane of an insidious means of self-deception to that of a well-ordered and progressive science.'

Although standards for poliomyelitis antisera have recently been established, many workers who have been using the titre notation for many years are reluctant to adopt them and to change over to expressing antibody potency in terms of *units*. In order to avoid situations such as this, it is essential that a standard should be prepared as soon as possible when a biological substance is shown to be important in prophylaxis, therapy or diagnosis. Although a standard made in the early stages of an investigation may not be entirely satisfactory and 'respectable' from an international point of view, it would nevertheless establish the principle of using a standard in determining the biological activity of the substance and of expressing potency in terms of units. Moreover, the first standard would serve as a stepping-stone towards the final establishment of an international standard, just as Ehrlich's standard for diphtheria antitoxin was the forerunner of the international standard which exists today.

RELEVANT LITERATURE

Most of the reports of the work done in connection with the establishment of international biological standards may be found in the *Bulletin of the Health Organization of the League of Nations* and in the *Bulletin of the World Health Organization*. A complete list of the international standards is published in the *World Health Organization Technical Report Series* which also show the progress made each year by the Expert Committee on Biological Standardization in establishing new standards. There are, in addition, a number of reviews which cover different aspects of biological standardization such as those by Burn (1930), Burn, Finney & Goodwin (1950), Dale (1939), Gautier (1935, 1945–46), Hartley (1945, 1945–46), Lightbown (1961), Maaløe & Jerne (1952) and Miles (1948, 1951).

REFERENCES

BURN J.H. (1930) The errors of biological assay. *Physiol. Rev.* **10,** 146.

BURN J.H., FINNEY D.J. & GOODWIN L.G. (1950) *Biological Standardization*. London: Oxford University Press.

DALE H.H. (1939) Biological standardization. *Analyst* **64,** 554.

GAUTIER R. (1935) The Health Organization and biological standardization. *Bull. Hlth Org. L.o.N.* **4,** 497.

GAUTIER R. (1945–46) The Health Organization and biological standardization. *Bull. Hlth Org. L.o.N.* **12,** 1.

HARTLEY P. (1945) International biological standards: prospect and retrospect. *Proc. roy. Soc. Med.* **39,** 45.

HARTLEY P. (1945–46) Notes on the international standards for antitoxins and antisera. *Bull. Hlth Org. L.o.N.* **12,** 76.

HARTLEY P. (1945–46) Notes on the international standards for drugs, hormones and vitamins. *Bull. Hlth Org. L.o.N.* **12,** 98.

LIGHTBOWN J.W. (1961) Biological standardization and the analyst. *Analyst* **86,** 216.

MAALØE O. & JERNE N.K. (1952) The standardization of immunological substances. *Ann. Rev. Microbiol.* **6,** 349.

MILES A.A. (1948) Some observations on biological standards. *Analyst* **73,** 530.

MILES A.A. (1951) Biological standards and the measurement of therapeutic activity. *Brit. med. Bull.* **7,** 283.

STANDFAST A.F.B., EVANS D.G. & WEITZ B. (1962) *Proc. 7th int. Congr. Biol. Stand.* Edinburgh: E. & S. Livingstone.

APPENDICES

APPENDIX A

ALLERGY

From the Imperial and Royal Paediatric Clinic of the University of Vienna

C. v. PIRQUET*

In the course of the last few years a number of facts have been collected which belong to the domain of Immunology but fit poorly into its framework. They are the findings of Supersensitivity in the immunized organism.†

These two terms clash with each other. Do we not regard an organism as immune if it is protected against the disease, is not attacked by it a second time? How can this organism at the same time be supersensitive to the same disease?

Already v. Behring had sensed this contradiction when he described as a 'paradoxical reaction' the death of animals hyperimmunized against tetanus when they were subsequently injected with a small dose of the same toxin.

However, the term 'paradox' can only be accepted in an exceptional case. But the deeper we penetrate into this field, the more closely is it found to obey a definite law. Already a large number of diseases is known in which evidence of supersensitivity has been obtained. Among them are the following:

Tetanus (v. Behring, Kretz), Tuberculosis (Courmont, Strauss and Gamaleia, Babes and Proca, Detre-Deutsch, B. Schick, Löwenstein and Rappaport, Möller, Löwenstein and Ostrowsky), Syphilis (Finger and Landsteiner), Diphtheria (Rigt), Serum sickness (Arthus, v. Pirquet and Schick, Lehndorff, Otto, Rosenau and Anderson), bacteria in general, organ extracts, various proteins, hay fever (A. Wolff-Eisner).

But are Immunity and Supersensitivity really connected with each other, or should one distinguish the processes in which pre-treatment causes immunity, from those in which it leads to supersensitivity?

A. Wolff-Eisner‡ would insist on such a separation: those processes in which toxins are implicated would lead to the production of antibodies and to immunity; those in which the active agent is an endotoxin to supersensitivity.

Yet from the experience with tetanus it is already evident that in pure antitoxic

* *Münch. med. Wochenschr.* **30,** 1457 (1906). Translated from the German original by Carl Prausnitz.

† See v. PIRQUET und SCHICK Üeberempfindlichkeit und beschleunigte Reaktion. *Münch. med. Wochenschr.* 1906, 2

‡ *Zentrabl. f. Bakteriolog.* **37** (1904); *Münch. med. Wochenschr.* 1906, No. 5; *Das Neufieber.* Müchen: Lehmann (1906)

processes supersensitivity can occur. Wolff-Eisner's objection that it occurs only as an exception, does not seem to me to meet the principle of the problem.

Richet* was the first to recognize the significance of supersensitivity, which he called Anaphylaxis. He discovered that his actinia poison produced, at the same time, both immunity and anaphylaxis. If the poison was re-injected after a suitable interval, the animals mostly died acutely; but if they survived the first shock, they overcame the disease more rapidly than the control animals receiving their first shock injection.

v. Pirquet and Schick† have come to similar conclusions from their study of Serum sickness: following the re-injection of serum the symptoms run a more stormy but a shorter course.

Recently Rosenau and Anderson‡ have shown that in spite of the very high level of supersensitivity acquired by the guinea-pig after injection of a minute quantity of horse serum, this is nevertheless accompanied by certain processes of immunity. For if instead of giving a single first injection the horse serum is injected for 10 days running, the animal injected ten times will not succumb to the subsequent re-injection of horse serum, whilst the animal pre-injected only once will die.

The relations between immunity and supersensitivity seem to me most clearly exemplified by what is seen in vaccination.§ A recently vaccinated individual appears supersensitive as compared with a person receiving his first vaccination, for he reacts far more quickly to the infection; yet at the same time his is protected, for in him the disease process extends only over a small localized area, and he is spared all generalized symptoms. Very similar observations have been made quite recently by Finger and Landsteiner¶ in the case of syphilis. At any stage of the disease re-inoculation has a pronounced effect: this occurs more rapidly than after the primary inoculation (shortened period of incubation). In tertiary syphilis a local erythema may even develop immediately after the re-inoculation; this is equivalent to the 'immediate reaction' seen after repeated serum injection.

'Immunity' and Supersensitivity can therefore be most closely interrelated.

And yet these two terms contradict each other, their union is a forced one. In fact, the concept of immunity has been carried on since a time when supersensitivity was unknown.

* *Archivio di Fisiologia*, 1904, p. 129. *Soc. de biologie.* 21.1.1905.

† *Wien. klin. Wochenschr.* 1903, Nos. 26, 45; 1905, No. 17. *'Die Serumkrankheit'*, Wien, Deutike, 1905

‡ 'A study on the cause of sudden death following the injection of horse serum.' *Hyg. Lab. U.S. Pub. Health and Mar. Hosp. Serv., Washington*, 1906, Bull. No. 29

§ v. PIRQUET: *Verhandlungen der Gesellschaft deutscher Naturforscher und Aerzte*, Kassel, 1903; *Wien. klin. Wochenschr.* 1906, No. 28. *Klinische Studien über Vakzination und vakzinale Allergie.* Wien, Deutike, 1906 (to be published shortly)

¶ Sitzungsbericht der Kais. Akad. d. Wiss. in *Wien. M.-N. Klasse*, April 1906

As F. Hamburger‖ has pointed out, the specific change which an animal undergoes after an experimental disease is almost as often an increase in susceptibility as a raised power of resistance.

What we need is a new generalized term, which prejudices nothing but expresses the change in condition which an animal experiences after contact with any organic poison, be it animate or inanimate.

The vaccinated person behaves towards vaccine lymph, the syphilitic towards the virus of syphilis, the tuberculous patient towards tuberculin, the person injected with serum towards this serum, in a different manner from him who has not previously been in contact with such an agent. Yet he is not insensitive to it. We can only say of him that his power to react has undergone a change.

For this general concept of a *changed reactivity* I propose the term *Allergy*. 'Allos' implies deviation from the original state, from the behaviour of the normal individual, as it is used in the words Allorhythmia, Allotropism.

The vaccinated, the tuberculous, the individual injected with serum becomes *allergic* towards the corresponding foreign substance. A foreign substance which by one or more applications stimulates the organism to a change in reaction is an *Allergen*. This term—not quite in accordance with philological usage—traces its origin to the word Antigen (Detre-Deutsch) which implies a substance capable of giving rise to the production of antibody. The term Allergen is more far reaching. The allergens comprise, besides the antigens proper, the many protein substances which lead to no production of antibodies but to supersensitivity. All the agents of infectious diseases which are followed by immunity are allergens. Among the allergens should be included the poisons of mosquitoes and bees in so far as their stings are followed by hypo- or hypersensitivity. For this reason we may also enrol under this term the pollen causing hay fever (Wolff-Eisner), the urticaria-producing substances of strawberries and crabs, and probably too a number of organic substances leading to idiosyncrasy.

The term Immunity must be restricted to those processes in which the introduction of the foreign substance into the organism causes no clinically evident reaction, where, therefore, complete insensitivity exists; may this be due to alexins (natural immunity), antitoxins (active and passive immunity in diphtheria and tetanus), or even to some kind of adaptation to a poison (Wassermann and Citron).

The new names do not conflict with the nomenclature in use up to now. The well-defined concepts of antitoxins, cytolysins, haemolysins, precipitins, agglutinins, coagulins are not affected thereby. Supersensitivity is a new field of research where only in the last few years new concepts have been evolved under laborious adaptation to the old names. From the need to clarify these conceptions I propose the new terms; I hope that by simplifying the outer form I will have made it easier for new research workers to study the interesting phenomena in this field.

‖ 'Eine energetische Vererbungstheorie'. *22. Kongr. f. innere Medizin*, Wiesbaden, 1905

APPENDIX B

STUDIES ON SUPERSENSITIVITY*

From the Institute of Hygiene, University of Breslas
Director: Geh. Med. Rat Prof. Dr. R. Pfeiffer

CARL PRAUSNITZ & HEINZ KÜSTNER

Among the idiosyncrasies to foodstuffs the supersensitivity to fish has so far received little attention. Since one of us (K.) suffers from this affection, we took this opportunity of investigating the mechanism of the reaction and the serological conditions concerned. In the course of the work a series of controlled tests were carried out on the other one (P.), who is a hay-fever patient, and on several non-sensitive colleagues who kindly placed themselves at our disposal.

The patient, an otherwise healthy man of 24, is known to have been fish-super-sensitive since the age of 6. After eating the merest trace of marine or freshwater fish the following symptoms appear:

> After half an hour itching of the scalp, neck, lower abdomen, dry sensation in the throat; soon afterwards swelling and congestion of the conjunctivae, severe congestion and secretion of the respiratory mucous membranes, intense fits of sneezing, irritating cough, hoarseness merging into aphonia, and marked inspiratory dyspnoea. The skin of the entire body, especially the face, becomes highly hyperaemic, and all over the skin of the body there appear numerous very itching wheals, 1-2 cm large, which show a marked tendency to confluence. Increased perspiration has not been noted. After about 2 hours heavy salivation starts and is followed by vomiting, after which the symptoms very gradually fade away. Temperature, cardiac and renal function have always been normal. After 10 or 12 hours all the symptoms have disappeared; only a feeling of debility persists for a day or so. After each attack there is a period of oliguria and constipation; this may be due to dehydration and vomiting, but perhaps it is better explained by retention of water similar to what occurs in serum sickness.

What may perhaps be regarded as an abortive form of the illness is the patient's observation that after careless working with fish-glue he has occasionally had urticaria of the lips. The symptoms and signs of fish supersensitivity can have no

* *Centralbl. f. Bakteriol. 1. Abt. Orig.* **86,** 160–9 (1921). Translated from the German original by Carl Prausnitz.

psychological basis. They have frequently occurred when he had no idea of having eaten any fish e.g. once after eating tunny fish which he had taken for meat; another time after eating parsley which had been chopped up on a board previously used for cutting up anchovies. But the patient has found that he can eat caviar with impunity. The active substance appears to occur practically only in the muscle flesh of bony fish. It occupies a peculiar position among the agents of supersensitivity, since for our patient fish is completely harmless in the *raw* state and *only becomes poisonous when heated* (cooked, baked or fried).

I

Starting from these observations it was first tried to produce the signs of supersensitivity by *parenteral* administration of the fish antigen. A standard solution was prepared by mincing fresh marine fish (usually haddock), boiling it in ten times its weight of distilled water, filtering through paper and sterilizing the filtrate for half an hour in the steam sterilizer. The clear, non-opalescent fluid thus obtained proved inactive on the patient's conjunctiva, but highly active on intradermal injection. After *intradermal* injection of 0.1 c.c. of the standard solution (the utmost care being taken to avoid subcutaneous injection) there developed at the site of injection within 10 minutes a very itching wheal which rapidly, under our eyes, grew to about 4 cm in diameter. The fully developed wheal was raised high above the surrounding skin, white, with an indented margin; it was surrounded by a deep red flare about 10 cm wide. After 20 minutes there developed the syndrome of severe generalized intoxication previously described (urticaria of the entire body, intense congestion of the conjunctivae and upper air passages, irritating cough, dyspnoea). The generalized signs, which we were able to produce repeatedly in the same form, gradually fade away after several hours. Subcutaneous injection of 1 mg atropine sulphate quickly and completly relieves the respiratory symptoms, 0.1 mg 'suprarenin' the urticaria. But even after a day, sometimes even 2 days, oedematous infiltration of the injection site persists. In our experience this oedema is very characteristic of positive supersensitivity reactions.

A distinct local reaction could be obtained by intradermal injection of as little as 0.1 c.c. of a thousandfold dilution of the standard solution, but not by 0.1 c.c. of a ten-thousandfold dilution. The limiting active dose therefore corresponds to the boiled extract of about 0.01 mg of fish muscle.

However, a 50 per cent watery solution obtained by leaching fish muscle *in the cold* was completely inactive in the patient. If this solution was boiled, a precipitate formed which was inactive, whilst the filtrate was weakly active (about 1000 times less than the original standard solution described previously). After heating to 50°C the solution remains inactive. At 55°C—i.e. the lower limit of protein coagulation—a toxic effect begins to appear; above 60°C its toxicity is found not

to differ from that of the boiled extract. In agreement with this finding is the fact which greatly astonished our patient, that *he can eat raw fish (5 g!) without the slightest discomfort.* It appears, therefore, that the active agent is only formed in fish muscle when it has been heated. Evidently in the fish extract prepared in the cold and filtered, small particles of finely dispersed fish protein had remained in suspension and from them antigen was formed on boiling.

The intradermal tests have proved that even after technically unobjectionable injection of sufficient amounts of antigen* so great an absorption of the poison can take place so rapidly that the picture of a severe general intoxication will develop. This observation is not unique.

With *hay fever* similar severe reactions have been described by Dunbar, Prausnitz and others after *subcutaneous* injection of the active pollen protein. But according to our investigations the same severe syndrome can be produced even by the *intradermal* injection of pollen protein. The other one of us, a hay-fever patient, was given 0.1 c.c. of a 1 per cent solution of rye pollen protein† intradermally: that is 2000 times the minimum amount required to produce a conjunctival reaction in this patient.

> After 10 minutes there developed a white, raised, very itching wheal with indented border, 22 × 17 mm in size, with a deep red flare 60 × 45 mm. Ten minutes later intense conjunctival congestion with severe lachrymation, a burning, itching sensation and chemosis. After a further 10 minutes there was severe inspiratory dyspnoea, panting respiration, stridor, distressing cough. The wheal was now 45 × 23 mm, the flare 200 × 90 mm in size. The eyelids became very oedematous, the palpebral fissure narrowed to a slit, the face so swollen as to be almost unrecognizable. This disfigurement only gradually disappeared after several days. For several days the site of injection presented a doughy swelling and remained painful.

II

In order to *determine the active principle* the original standard fish solution was first mixed with an eightfold volume of absolute alcohol, and after standing for 24 hours was filtered through paper. The filtrate was evaporated to dryness on the water-bath; from 10 c.c of the standard solution 0.027 g of a greenish-yellow amorphous material was obtained. It was suspended in 10 c.c of normal saline solution; it proved quite inactive on intradermal injection. The aclohol precipitate (0.035 g from 10 c.c of standard solution) was also suspended in 10 c.c normal saline: this solution, just like the original standard solution, was active in one thousandfold dilution. Therefore the antigen had passed quantitatively into

* 1000 times the quantity required to produce a local reaction

† We are indebted to Professor Dunbar for kindly supplying this valuable preparation

the alcohol precipitate. The minimal active dose of the alcohol precipitated antigen is 35×10^{-5} mg.

No active substance can be extracted from fish muscle by alcohol or ether in the cold or by heat.

The substance was then tested for dialysability. Ten c.c of the standard solution were dialysed in a Schleicher and Schüll dialysing sheath for 24 hours against frequent changes of distilled water. The dialysate (4 litres) was inspissated on the waterbath to a volume of 20 c.c; it proved inactive by intradermal test. The fluid within the sheath had retained its original activity. It gave a distinct biuret and Million reaction.

Therefore the antigen is neither fat nor lipid. It is allied to the proteins but does occupy a peculiar distinct position: coctostable antigens, it is true, have long been known, e.g. pollen protein. But what is new is the fact that *the active substance is only formed when the solution is heated beyond the temperature of denaturation of protein.* We would assume that it still is a fairly high molecular decomposition product of fish protein, since it is precipitated by alcohol, is non-dialysable and gives the above-mentioned protein reactions.

If the standard solution is treated in the cold with 10 per cent acetic or 10 per cent nitric acid, a precipitate forms; this, like the filtrate after neutralization, is intradermally inactive. If acetic acid is added to the standard solution to a concentration of 0.1 per cent in the cold, its activity is reduced to about one-tenth; when boiled this solution becomes quite inactive. After treatment with 3 per cent potassium hydrate the standard solution remains clear and active.

It was not possible to detoxicate the standard solution with midly acidified pepsin of proved activity, nor with alkaline trypsin.

The fact that the patient can eat caviar without suffering any trouble suggested testing the various organs of fish for activity. Only the cross-striated muscle of bony fishes was found to contain the antigen in considerable quantity. Decoctions of stomach and liver were very weakly active (about 1000 times less so than the standard solution described above). Inactive were decoctions of spleen and roe, as well as pure, undiluted fish serum, both raw and boiled. Of cartilaginous fishes the ray proved to be about 100 times weaker than the bony fishes investigated. Naturally whale flesh was inactive.

III SEROLOGICAL INVESTIGATIONS

In view of the strictly specific character of the reaction described the serum of the supersensitive individual was tested for the presence of antibodies. With the most varied testing arrangements it was impossible to demonstrate *in vitro* precipitins or complement-binding substances in his serum or in that of normal persons.

Following the observation of Bauer (*Münch. med. Wochenschr.* 1911, p. 71)

that in serum sickness the patient's serum agglutinates the corresponding erythrocytes, we tested our patient's serum for activity against fish red blood corpuscles, but with no result. Nor could we discover in a test on the human being any neutralizing substances in the serum: mixtures of equal parts of the standard fish solution and freshly drawn serum of the patient, as well as of a normal person, were kept for 2 hours at 37° C and overnight in the icebox; their toxicity by intradermal injection to our patient remained uninfluenced.

These results show a close agreement with the similar behaviour of pollen protein in the hay-fever patient. This substance is also a *coctostable* protein-like substance which, it is true, behaves differently towards acids and alkalis but is also very resistant to digestive enzymes. Both substances are strictly specific for the corresponding supersensitive persons, although they show a considerable quantitative difference in their active dose. Their chief difference is that the poisonous pollen protein is preformed, whilst the poisonous substance from fish does not develop until the protein has been denatured. Pollen protein, injected subcutaneously or intradermally to the hayfever patient, produces exactly the same symptoms as fish extract in the fish-sensitive individual. A further difference, though hardly so important a one, is the fact that pollen protein is inactive after oral, fish extract after conjunctival administration.

The reactions observed show a remarkable resemblance to those of genuine anaphylaxis. True, there is *one* difference: in fish- and pollen-supersensitive persons after intradermal injection the reaction sets in almost without any incubation period—within a few minutes—and fades relatively soon; in the person sensitive to serum or tuberculin the intradermal reaction only begins after several hours and remains at its height for days. Still, these differences might only be of a quantitative nature. Whether the forms of supersensitivity discussed here come under the heading of true anaphylaxis would most readily be decided if they could be passively transferred to non-susceptible beings. In guinea-pigs it was impossible to produce passive anaphylaxis by intraperitoneal injection (1 or 2 c.c) of the fish-supersensitive patient's serum followed 24 hours later by intravenous injection of 0.5 c.c standard fish solution. By a similar technique, using the serum of a hay-fever patient and pollen protein, again no result was obtained.* The attempt was therefore made to transfer the supersensitive state passively to non-susceptible *human beings*. The technique used in guinea-pigs was out of the question, since corresponding to the weight of the individual to be tested far too much serum would have to be taken from the donor, and above all because of the danger of producing a severe-anaphylactic shock. We therefore tried to localize the anaphylactic reaction *within the skin* by

* Note during the printing of this article. In promising experiments with the somewhat similar syndrome of acquired supersensitivity of hide-dyers to 'Ursol' (quinone di-imino polymers) H. Curschmann has recently succeeded in rendering guinea-pigs passively anaphylactic to this substance with the patient's serum (*Münch. med. Wochenschr.* 1921, No. 5)

injecting both reactants (serum and antigen) *intradermally into the same spot of skin.*

1. Mixtures of standard fish solution with the fish-sensitive patient's serum in varying proportions, injected intradermally to normal persons in 0.1 c.c amounts, were inactive.

2. Even after being kept for 2 hours at 37° C and for 24 hours in the icebox, they failed to become active.

3. *A positive result was only achieved* when, conforming exactly to the passive anaphylactic experiment in the guinea-pig, *the serum was first injected intradermally and the standard fish solution was injected the following day into the same spot of skin.*

On July 19th, 1920, a person, not sensitive to fish solution, received into the abdominal skin intradermal injections of 0.1 c.c of the following substances:

(1) Serum of the fish-sensitive patient, undiluted;
(2) Serum of the fish-sensitive patient, diluted 1 in 10;
(3) Serum of the fish-sensitive patient, diluted 1 in 100;
(4) Serum of a healthy person, free from any idiosyncrasy;
(5) Normal saline solution.

On July 20th, 1920, 0.1 c.c of standard fish solution was injected into each of these spots and (6) into an untreated spot of skin. The arrangement of the wheals was as follows:

	4		1	
right	3	navel	5	left
	6		2	

After 15 minutes a marked subjective and objective reaction was present *only* in the spots 1, 2 and 3 pretreated with the *specific* serum. It was strongest where the undiluted serum had been administered. The control wheals 4, 5 and 6 showed only a trifling traumatic reaction (see Table 1).

After 1 hour the wheal and flare in the skin spots 1, 2 and 3, pretreated with *specific* serum, were still about the same size as after 15 minutes, but already the border between wheal and flare had become indistinct and the flare was starting to fade. On the next day there was still in these spots the distinct oedema which was always seen in the fish-sensitive patient after intradermal injection of the fish antigen. But the spots 4, 5 and 6 remained free from any trace of oedema.

The experiment was repeated in this person, who incidentally is supersensitive to pollen, and in two others, one male and one female, who are free from any idiosyncrasy: the result was the same. In a further person, supersensitive to peptone, there were also differences in favour of the spots pretreated with the specific serum, but they were less marked.

It has thus been proved by this test that, according to the technique which we have elaborated, the state of supersensitivity can be transferred passively to normal persons by the serum of the supersensitive individual.

It had now become obvious to try out the technique in other forms of supersensitivity. For this purpose we chose hay-fever and sensitivity to tuberculin and to horse serum. In all of them the result of numerous tests was negative. One might still consider the possibility that, e.g. in hay-fever not the serum but certain cells were the carriers of supersensitivity; as such the cells of the subcutis and cutis were the most likely ones since the skin shows a specially high degree of pollen sensitivity. To decide this question a piece of the hay-fever patient's skin, 15 sq. cm in size was excised; the subcutis and deeper layer of the cutis were carefully

TABLE 1

Result of the Test

Skin spot No.	*After 15 minutes*		*After one hour*
	Wheal	*Flare*	
1	21×15 mm Tense, pale, raised to a plateau with irregular protrusions, very irritating	120×70 mm Bright red	Flare still bright red
2	12×11 mm Same appearance	80×50 mm Bright red	Flare still bright red
3	12×8 mm Same appearance	55×28 mm Red	Flare red
4	7×5 mm Slightly raised, circular, quite insensitive.	60×25 mm Pale	Pale area
5	7×6 mm Slightly raised, circular, quite insensitive	40×25 mm Pale pink	Pale area
6	8×7 mm Slightly raised, circular, quite insensitive	55×30 mm Pale	Pale area

scraped off and ground with glass powder in an agate mortar to a fine pulp; 6 c.c of normal salline was slowly added and stirred to make a suspension which was shaken for 15 minutes and spun for 1 minute in the hand centrifuge to sediment the coarser particles. The supernatant, a slightly opalescent, blood-stained fluid, was microscopically free from cells. It was tested in three persons according to the technique used previously for testing passive transmission of pollen sensitivity—but without any result.

Possibly there is a difference of principle between fish supersensitivity and the other idiosyncrasies. But we consider it to be more probable—particularly in view of the striking resemblance already stressed between fish and pollen supersensitivity—that such differences are merly *quantitative*. This assumption is favoured by the fact that the fish-sensitive individual presents an unusually high degree of sensitivity whereas the human donors used for the hay-fever and

horse-serum tests were only moderately supersensitive. Perhaps we might have succeeded in transferring pollen and horse-serum supersensitivity passively by our test, if more highly sensitive individuals had been available. However, the patient used in the tuberculin test reacts so strongly to tuberculin that in his case such an assumption can hardly be justified.

According to the experiments described above it is highly probable that fish supersensitivity should be regarded as a true anaphylactic phenomenon.

IV

The possibility of *active immunization* against hay-fever is known from the experiments of Noon, Freeman, Dunbar, Eskuchen and others. One of us (P.) studied the question in detail in the summer of 1919; with some highly active rye

TABLE 2

Test (July 6th, 1920)

	Left forearm, flexor aspect after repeated injections	*Right forearm, flexor aspect no previous injections*
Intradermal injection 0.1 *c.c.*	*Standard fish solution undiluted*	*Standard fish solution diluted* 1 *in* 10
After 15 minutes	Very *weak* local reaction Wheal 20×13 mm, Flare red, indistinct, no sharp margin	Very *strong* local, almost a general reaction Wheal 36×25 mm, Flare 135×70 mm, deep red On the extensor aspect a small, very irritating daughter wheal has developed

pollen solutions supplied by Professor Dunbar he obtained promising results in two out of four hay-fever patients. The mechanism of this protection is not yet sufficiently understood. It has not by any means been proved that genuine immunity is involved here. Opposed to such a view would seem to be the fact that so far substances neutralizing the hay-fever poison have not been discovered in the serum of rabbits, goats and horses immunized against pollen. Perhaps, as Bessau suggests, this is a case of anti-anaphylaxis or katanaphylaxis. Nor has finality been reached as to the technique of this immunization. The dose employed should not be too small, otherwise success is usually not achieved. But if it is increased too rapidly, acute symptoms readily develop in the skin and respiratory passages; they may occasionally become very severe and require treatment with atropin and suprarenin (Noon, Freeman, Bessau, personal experiences).

The *possibility*, however, of producing a transitory, perhaps even a permanent

control of supersensitivity can be deduced from observations on the fish-sensitive patient. After a considerable number (about forty) of intradermal injections of different fish extracts had been carried out during about 6 weeks, always on the flexor aspect of the left forearm, a very marked *local* reduction of his sensitivity was observed (see Table 2).

Subsequently, after a number of fish-extract injections had been performed on the right arm, a corresponding reduction of local sensitivity occurred here also. After stopping the tests for 8 weeks the sensitivity had almost returned to its original level. It follows, therefore, that a kind of local immunization is possible; however, so far as our present experience goes, it is only transitory. We can not yet say whether it will be possible to immunize the whole body against the result of oral administration of the antigen. Experiments in this direction are contemplated.

CONCLUSIONS

1. According to our investigations on a highly fish-sensitive patient, fish supersensitivity exists both towards oral and intradermal administration of the antigen. The active principle is present in the muscle, but not in the serum and most of the organs of bony fishes, and only in small amounts in the muscles of cartilaginous fishes. It is not demonstrable in raw fish muscle but only develops on heating to the temperature of protein coagulation.

2. The active principle is insoluble in cold or warm alcohol and ether; it is non-dialysable, is speedily inactivated by acids, but not by alkalis, pepsin or trypsin.

3. The reaction is strictly specific. Precipitins, complement-binding and neutralizing antibodies were not demonstrable in the serum of the sensitive individual.

4. It was not possible to sensitize guinea-pigs passively with the patient's serum. But *with his serum the specific transfer of fish supersensitivity to normal, not fish-sensitive human beings, was successfully accomplished. The technique consisted in the intradermal injection of the serum and re-injection of the same spot of skin, 24 hours later, with the antigen.*

5. It was not possible with this technique to transfer sensitivity to pollen protein, horse serum or tuberculin. Perhaps we might have succeeded with the sera of persons more highly sensitive than those available to us.

6. After repeated performance of intradermal injections of fish extract there resulted a local reduction of sensitivity lasting for several weeks.

INDEX