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ROBERT CRUICKSHANK

M.D., F.R.C.P., F.R.C.P.(Edin.), F.R.S.E.

Professor of Bacteriology, University of Edinburgh

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PREFACE

The past two decades have seen a great renaissance in immunology. The basic principles of the formation of antibodies in response to the stimulus of natural or artificial antigens, which led to the therapeutic and prophylactic uses of antisera and vaccines, began to attract detailed attention from chemists and physicists who have helped to define more precisely the nature and properties of antigens and antibodies. New tools and techniques such as immuno-fluorescence and radio-active isotopes for the detection of these substances have given fresh impetus to the study of the pathological manifestations of immune responses and reactions in cells and tissues, while at the same time new methods for the separation and identification of antigens and antibodies, for example, gel diffusion, electrophoresis, density gradient centrifugation and ion exchange celluloses, have helped to unravel their complexities. These technical tools, although they have contributed greatly to knowledge about immunological phenomena, could not in themselves expand the immunological horizon and it was the genius of men like Burnet and Medawar that carried the subject into new fields. Burnet's concept of ' self-markers ' and ' self-recognition ' as applicable to cellular antigens developing during foetal life without eliciting antibody responses was taken up experimentally by Medawar and his colleagues and so transplantation immunology was born. A natural extension of this new branch of the subject was auto-immunity which is concerned with phenomena and diseases where there may be some derangement of self-recognition. In this *Modern Trends* volume, these new developments in immunology, together with new knowledge about antigens and antibodies and their interactions, are reviewed in speculative fashion by workers who are themselves actively engaged in blazing new trails. But one must not forget that infection is the principal contributor to morbidity and mortality in the developing countries and still accounts for a sizeable proportion of the total sickness in economically advanced communities. There have been many advances in knowledge in this field of infection and immunity and, again, the growing points—and unsolved problems—in natural and acquired immunity, in artificial immunization and in tissue hypersensitivities are discussed by experts in these subjects. We hope this series of essays will help to attract an ever-widening circle of students of curative and preventive medicine in lands both new and old to the rejuvenated science of immunology.

Edinburgh, R. C.
July, 1963

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CHAPTER 1

ANTIGENS

D. A. L. Davies

INTRODUCTION

Antigenicity is a property of most biological macromolecules, but there is no example of a natural antigen for which the complete requirements for antibody induction, or all of the features involved in specificity, have been elucidated. There are trends in antigen studies in a great number of directions and only a small number of examples can be referred to here, where the emphasis will be on the chemical nature of antigens; their behaviour and effects are described in other chapters.

Antigens were originally defined in terms of antibody response, but some extension in concept is necessary for those substances which do not induce a detectable circulating antibody but can initiate some kind of ' cellular immunity ' or hypersensitivity; the term allergen is better used in this context. Where no primary immune response can be obtained by injection of a molecule which will react with a specific antibody, then it is a hapten. This term can extend to cover cases such as degraded bacterial polysaccharides which will precipitate with an antibody induced by immunization with the native antigen from which the hapten was derived, and also, for example, chemical groups artificially attached to some larger molecule to give an antigenic specificity, or a new or modified specificity. The terms ' epitope ' (specific antigenic determinant site, group or area), ' epitype ' (family of related epitopes), ' paratope ' (complementary site on antibody), etc. (Jerne, 1960), would be clarifying if brought into general use in this field.

From the point of view of definitions, there are, as always, borderline cases: for example materials of relatively low molecular weight which are rapidly excreted, some of inherently low antigenicity where a response can only be revealed by the use of adjuvants. Materials which are good antigens when tested in one animal species may give a poor response when tested in another, for example pneumococcus polysaccharides; this may occur when the animal being immunized carries a closely related antigen and is inherently tolerant for most of the specificities of the injected material.

The general sequence of stages in antigen studies are these: (1) detection of a specificity belonging to the desired product; (2) devising a means for measuring the activity of the antigen, allergen or hapten in some *in vivo* or *in vitro* system; (3) isolation, purification and characterization of the substance to allow unequivocal results in (4) studies of its properties in the absence of contaminating substances. For some kinds of molecules it is possible to proceed to (5) the identification of the epitopes and finally (6) to study the biosynthetic pathways.

There is now an increasing awareness of the necessity for purifying antigens for study and providing adequate evidence for physical, chemical and immunological homogeneity. The use of impure products in the past has resulted in many dubious or uninterpretable results. The general level reached in antigen studies in different fields largely reflects the difficulties peculiar to those fields though with an element derived from what has happened to become fashionable at various times. Many bacterial antigens are well advanced into the stage of study and identification of epitopes, because as starting material for isolation, micro-organisms have the advantage of ease of availability in large quantities where all the cells may be of the same kind, subject to adequate control of variation. In fact, batch-type growths of bacteria may include cells reflecting the history of the culture, which grew possibly in an environment slowly changing with respect to pH or some nutrient factor, but stable continuous cultures can give batches of identical cells. For viruses and bacteriophage, the desired quantities of starting material are difficult to obtain free from host products, though improvements in methods are constantly allowing smaller amounts of material to be adequate for study. In this area, therefore, studies are largely at the stage of detection though many means of measurement are available and some work on isolation is also proceeding.

For antigens of higher animals quantity of material is frequently a problem because many interesting compounds are subject to polymorphism or allotypy within a species; for such iso-antigens material from different individuals cannot be pooled at random and for small animals only species where inbred lines are available make some kinds of study practicable. The existence of a polymorphism, as, for example, in the blood groups of man, itself draws attention to the antigens involved and as the study of the readily available serum components increases, more cases of allotypy appear. This further decreases the justification for pooling from different individuals, even for substances not known to vary in this kind of way.

For antigens of higher animals only the investigation of blood group substances has advanced to the study of epitypes at the chemical level; this reflects the simpler situation in carbohydrates as compared with proteins and in this instance the finding that body secretions contained blood group specific substances enabled an early start to be made. Only recently have these materials been isolated from cells. Because of their ready availability, serum components have been the subject of a vast amount of research whereas tissue-cell antigen studies are only beginning; for the latter a large amount of serological information is already available, but much of this is ill-defined and chemical information is very limited. This has now become a fashionable area and an important one, and the present trend is likely to be very profitable. A sound knowledge of the situation with respect to antigens of normal tissue cells seems essential to provide information with which to compare the antigens of abnormal cells, particularly, for example, tumour cells. Some of the difficulties here are due to inadequacies in the test systems available for measurements of activity and to the association of activities with insoluble material. As there is no reason to suppose that cell antigens are necessarily obtainable in a soluble form convenient for experimental study, improvements in methods for handling insoluble products would be very helpful.

For animal tumours there is much information of variable quality but it now seems clear enough that tumour cells do possess specificities not detectable in cells of the autochthonous host and that host cells have antigens not detectable in tumours derived from them.

METHODS OF IDENTIFICATION

The purification of antigens can only proceed when adequate tests are available for measurement of their specific properties. Many tests are laborious but all new tests stimulate bursts of activity in new directions and those of wide applicability are inclined to change the whole face of the subject. Gel diffusion methods, for example, have provided for the immunologist an analytical method analogous to chromatography for the chemist, putting immunochemistry in a most favourable situation for development. Just as recording many spots on chromatograms became a popular sport a decade ago, before the true impact of the methods was felt, so we have had a phase where animals have been immunized with complex antigen mixtures and large numbers of precipitin lines recorded using the resulting antisera. It is now more generally

appreciated that finding lines is not an end in itself but a key to unravelling a situation, and to lead towards isolation of the antigens responsible. If, for an antigen requiring further study, no better method for measuring activity is available in the early stages, a gel diffusion dilution method can be used up to a stage when other properties of the substance are revealed. Some appropriate absorption of complex antisera is often desirable for such tests but unabsorbed antisera reveal what impurities are present.

As a method in taxonomy, agar diffusion provides exceptional opportunities if wisely used. A set of precipitin lines generally reflects the structure of a number of constituent proteins, direct products of the genetic inheritance of a species. This gives a much more basic characteristic of, for example, a bacterium than the host of variable metabolic features normally used in intrageneric classification. Bacterial inter-relationships are very evident for species adequately studied and this method extends, for example, to species relationships in different genera of Agarics and no doubt has almost unlimited possibilities among higher animals and plants. The association of imperfect stages of fungi with identifiable species is an obvious application of economic importance.

Because of the variability of individual animals in the degree of their immune reaction to the injection of the same amount of the same antigen, and the unfavourable dose-response relationship, a direct response is necessarily a poor measure of the activity of the substance, unless very large numbers of test animals are available. Thus, whereas the direct response is an essential property to be recorded for a purified product, purification is better followed in some other way than measurement of induction of precipitins, agglutinins and the like if an alternative exists. Inhibitions of serological reactions frequently provide convenient methods although it should be borne in mind that for 1 : 2 dilution series no further information is provided after a product has reached 50 per cent purity. If some characteristic constituent of a desired product can be identified at an early stage, a chemical estimation of its increase in amount, in parallel with a serological method, is very convenient.

The adsorption of specific substances on to red cells or some inert particles provides useful test systems for inhibition methods particularly for specificities not already present on cell surfaces. Enhancement of agglutinability or adsorbability is sometimes necessary, for example by tanning, or pyruvic aldehyde treatment. Treatment of the substance to be adsorbed may be necessary (for example heat or alkali treatment for lipopolysaccharides). Lipid

components of complex antigens generally inhibit their adsorption on to red cells and hydrophobic particles have to be used. For testing an antigen in a mixture by this kind of method, a monospecific antiserum is necessary. For iso-antigens a careful choice of individuals is required; thus the products of an inbred mouse strain may be used to immunize some suitable hybrid or coisogenic line for monospecific histocompatibility antisera. If not directly available, a monospecific antiserum may be obtainable by absorption, for example an antiserum against a pathogenic microorganism may be absorbed with a closely related non-pathogenic strain.

For localization of antigens on cell surfaces there are various methods based on agglutination and mixed agglutination, and for localization generally, fluorescent techniques are providing valuable information.

For fractionation and purification of antigens from complex starting materials, new methods of ever-increasing discrimination are becoming available. Ion exchange substituted celluloses, preparative electrophoretic techniques and density gradient centrifugation have been particularly valuable, but the many other methods cannot be detailed here. Few methods of general application have emerged because antigens are of such varied chemical nature but many practical instructions have been provided by Kabat and Meyer (1961).

THE STATUS OF DIFFERENT CLASSES OF COMPOUNDS

Carbohydrates

For progress through the stages outlined, carbohydrates have proved to be easier to isolate, purify and handle than other kinds of macromolecules. In the first place a particular source usually contains a small number of different specific carbohydrates; for example bacterial cells frequently possess an internal glucan, a heteropolysaccharide outside the cell wall, and an acidic carbohydrate polymer on the surface. Few species have a greater variety than this and many have less. Information for animal cells is insufficient to generalize but there is no indication of a wide range of carbohydrates. The materials are relatively stable to heat and a reasonable pH range though very alkali-labile O-acetyl residues sometimes occur which may (Pneumococcus I polysaccharide) or may not (*Klebsiella aerogenes* polysaccharide) constitute an epitope. Acid-labile sugars (for example 3,6-dideoxyhexoses) may occur,

but a range of specificities on bacterial cell surfaces which have not been identified chemically may be due to residues which have been lost by too harsh treatment during extraction. A vast background of chemical data on sugars predated the study of specific polysaccharides though a variety of novel sugars have since been found in the natural specific substances. These include representatives of new classes of sugars, for example 3,6-dideoxyhexoses, aldoheptoses, aminodeoxyhexuronic acid, and di- and tri-amino sugars. In addition there are many unusual members of known classes of sugars (Davies, 1960) and also muramic acid and sialic acids. The epitypes provided by these residues cover a wide range but rarely do more than five different sugars occur in one polysaccharide and in such instances the more exotic sugars generally occupy non-reducing end group positions and provide most of the specificity. Reducing end group residues have never been shown to provide an epitope though it should be borne in mind that only a single reducing end normally occurs. Due to the small number of different sugars which most frequently occur, cross reactions are found between products of quite unrelated organisms, from bacteria to man.

Many excellent chemical methods of degradation and analysis are available, for example methylation, periodate oxidation, borohydride reduction, to reveal structural features which can be correlated with epitopes. Using the homopolymer dextran, the extent of the epitope could be revealed by inhibition studies with isomaltose oligosaccharides; it was found that most specificity was contributed by the non-reducing end group residue and its glucosidic link, and progressively less by subsequent residues and their links as far as the sixth or seventh residue. This gives some information about the nature of the paratope (Kabat, 1960). The situation in heteropolysaccharides is more complex; in the blood group mucopolysaccharides, which have rather similar overall compositions and each contain the same four sugars, different arrangements give quite different specificities which do not overlap. Even here, inhibition studies with hetero-oligosaccharides have revealed details beyond the end group residues and their glycosidic links.

Simple polysaccharides generally make rather poor antigens, but association with protein or lipoprotein renders them powerfully antigenic, as they are when attached at the surface of microorganisms, bacteria, microfungi and protozoa. Antigenicity of plant polysaccharides has not been so extensively studied. In the metazoa, sponges have glycopeptides on cell surfaces. Materials related to blood group specific mucopolysaccharides in their general composition are widely distributed. The sulphated and acidic poly-

saccharides of connective tissues are also very widely distributed; these have not been shown to have specific immunological reactivity but whether this is an inherent property, or a reflection of tolerance following their presence in all higher animals, is not clear.

Proteins

The situation with respect to proteins is very different; most sources contain a great variety of different molecular species which have sometimes proved very difficult to separate. Proteins are generally labile, easily lose their native specificity and on slight maltreatment reveal new cryptotopes. Structural studies are extremely laborious, of recent date and limited applicability. The variety of amino acids in proteins is almost always much greater than sugars in the most complex carbohydrates, and protein antigen studies have not reached the stage of identification of epitopes, except in one limited field. Cross-reactions do not generally occur except between proteins of closely related organisms, for example in bacteria usually within one genus, or for truly analogous products such as between some enzymes and between serum proteins in mammals. The more specialized the protein functionally the wider the cross-reaction with analogous proteins, where one presumes that for very specialized functions a limited number of alternative structures will provide the desired properties.

Approaches to closer studies of protein specificities are being made by both degradative and synthetic methods. Denaturation of native globular proteins gives loss of tertiary structure and loss of native specificity with the appearance of cryptotopes. The small number of proteins whose amino acid sequences are known or at least largely resolved are, however, providing an opportunity to find information about the native epitopes. Large peptides inhibit interaction of the parent antigen with its antibody in some instances and certain amino acid residues or structures (such as disulphide bonds) have been shown to be necessary for specificity. Peptides from fibrous proteins are generally more active than those from globular proteins, but when, for example, ribonuclease is oxidized, then a peptide derived from it has inhibitory activity when antibody against the oxidized (extended) form is used (Brown, 1962). An extended but enzymically active form of ribonuclease prepared by treatment with subtilisin becomes unreactive with homologous antibody on removal of a peptide component but regains specificity on recombination (Singer and Richards, 1959). This kind of approach generally suggests that amino acid residues are unlikely

to be epitopes but that areas of the molecules are more likely to be involved. Thus denaturation will generally destroy the site by distributing the residues contributing to that area to different positions on the extended helices, and a resolution of the primary (amino acid sequence) and secondary (helix form) structures of globular proteins may not necessarily provide information sufficient to describe the epitopes. However, where total structures are being interpreted, for example haemoglobin, myoglobin, there are exceptional prospects for studies of protein specificity.

The number of epitopes carried by some proteins may be very large; a large number frequently occur in carbohydrates but often many of these are of the same epitype. This is less likely to be the case with proteins. A protein isolated from mouse ascitic fluid cross-reacts (with 'spur' formation on gel diffusion tests) with the analogous product of many other rodents, where tests make use of rabbit antisera. The analogous products of other rodents overlap among themselves. A Coypu anti-mouse serum, however, reveals that the rabbit also carries some of the epitopes of the mouse product and for which it is therefore tolerant (Haughton, 1962).

It is possible that in fibrous proteins and polypeptides with little or no folded structure, the situation may be more akin to that in polysaccharides. Thus, the synthetic approach reveals that attachment of small peptides to the free amino groups of antigenic proteins alters their specificity. Further, using non-antigenic protein, peptidylation can produce immunologically specific products, for example the attachment of tyrosine to gelatin. The imposition of some rigidity upon the molecule appears to be an important factor. Wholly synthetic peptides have also been made antigenic; an immunologically inert polyalanine chain is antigenic when tyrosine and glutamic acid are added. End residues do not appear to be specially important but rather the accessibility of residues forming epitopes (Sela, 1962; Sela, Fuchs and Arnon, 1962). Whereas studies at this level possibly do not yet bear on native globular protein specificity, they are of obvious importance in relation to the large number of biologically active peptides involved in physiological processes.

Nucleic Acids

The status of nucleic acids as antigens is barely resolved. Although purified undegraded DNA has been claimed to be antigenic and to give good species specificity, most workers have had less success. There is agreement, however, that at least they are haptens which will react with antisera prepared by immunization with nucleo-

protein or nuclei. Such antisera react both with nucleohistone and purified DNA (Phillips, Braun and Plescia, 1958). Species cross-reactions have been reported (Miescher, Cooper and Benacerraf, 1960). Studies using thermally denatured bacteriophage DNA have been illuminating; material prepared from coliphages T2, T4 and T6 cross-reacted when measured by complement fixation using rabbit antisera, but no reactions were obtained with preparations from a variety of animals, plants and bacteria, where glucosylated hydroxymethylcytosine is not present. The cross-reactions were due to differences in the glucose substituents (α, β or multiple); thus for the T6 reaction gentiobiose (6-β-D-glucopyranosyl-D-glucose) was the best inhibitor of five diglucosides tested, whereas for T2 and T4 DNA reactions maltose (4-α-D-glucopyranosyl-D-glucose) was the most effective. Since glucosylated hydroxymethylcytosine was a much more powerful inhibitor than any disaccharides, the extent of the epitopes would appear to extend beyond the sugar substituents (Levine and colleagues, 1960; Levine, 1962). A natural source of anti-DNA is the serum of patients suffering from systemic lupus erythematosus, where auto-antibodies against other nuclear components and cell structural material also occur (Deicher, Holman and Kunkel, 1959). The specificities of antibodies in different lupus sera appear to differ from one another; in one instance pentathymidylic acid made a better inhibitor than other thymidine oligonucleotides or any cytosine derivatives tested. The sugar residue (deoxyribose) did not appear to be involved (Stollar and colleagues, 1962).

Less is known about the immunological specificity of RNA. Microbial ribosomes appear to induce three classes of antibody: (1) directed against proteins common to the ribosomes of closely related species, (2) against protein specific for homologous ribosomes and (3) against ribosomal RNA, which has a broad species specificity. The precipitation of ribosomes by heterologous antisera, where the protein interactions were minimal, was inhibited by RNA. Purified anti-RNA antibody was prepared by ribonuclease treatment of immune RNA- anti-RNA precipitate. This purified antibody precipitated RNA and the reaction was inhibited by polyadenylic acid (Barbu and Panijel, 1960; 1961).

In the light of this kind of progress, it seems possible that immunochemical methods may yet have a contribution to make to genetics.

Lipids

It has long been known that lipids are involved in immunological reactions, but in rather an ill-defined way. The main diffi-

culty in this field is in detection and measurement of activity on account of insolubility in aqueous media and anti-complementary effect. It has been found that the incorporation of auxiliary lipids such as lecithin and cholesterol in the medium allows detection and measurement. This situation reflects the limitations of our methods to the examination of compounds susceptible to convenient handling. The presence of specificity on the surface of a cell may not be difficult to demonstrate even if it is due to lipid, which may be antigenic *in situ;* but to prove that it is due to lipid is much more difficult.

The Wasserman hapten is well known; this is esterified glyceryl-phosphoryl-glyceryl-phosphoryl-glycerol (Macfarlane, 1958). The Forssman antigen also has a long history; it is in any case a generic term for a family of overlapping specificities whose structures have not been resolved. The material is extractable with organic solvents but reactions can be obtained with lipid free material; for example with the specific polysaccharide of *Shigella dysenteriae* which is composed of galactose, glucosamine and rhamnose (Meyer and Morgan, 1935; Morgan, 1937). Forssman antigen is evidently glycolipid of some kind. Specific glycolipids are better known as bacterial products, occurring especially in Mycobacteria (MacLennan, 1961; Lederer, 1961), where a variety of unusual sugars occur.

Recently evidence has accumulated to suggest that an important role may be played by lipid specificities in mammalian tissues; one such hapten, cytolipin H, isolated from human sources is sphingosine-fatty acid-galactose-glucose. The reaction of this material in complement fixation tests, in the presence of suitable non-specific lipids, can be inhibited by lactose (Rapport, 1961). The extent to which the aglycone is involved in specificity is not known nor is there much information for lipids which do not possess sugar residues. However, if complex lipids are involved in tissue-cell specificities, and there is suggestive evidence that they are, then epitopes formed by the labile secondary structure imposed by their mode of aggregation or attachment to protein may be particularly difficult to study.

ANTIGENS IN MICRO-ORGANISMS

Knowledge of bacterial antigens is very extensive particularly (*a*) for pathogenic species, where many biologically active components useful for protective immunization or identification have been purified and described, and (*b*) for those ' guinea-pig ' species

which are convenient to handle in the laboratory. Many important products are proteins, such as the classical exotoxins of the diphtheria bacillus and the pathogenic clostridia (actually endo- in the sense that they are present inside the cells and usually released into the medium by autolysis). The endotoxins which are present outside the bacterial cell walls are protein-lipid-polysaccharide complexes and will be discussed below.

Proteins are among the antigens which sometimes differentiate virulent from non-virulent forms of pathogens; gel diffusion methods are particularly useful in this sphere using absorbed antisera (Burrows, 1960). The possibility of important antigens being produced by pathogens when grown *in vivo*, which are not produced or occur in relatively small amount in the less convivial environment of culture vessels is being explored (Smith, 1960). In the case of pathogens recent evidence for common specificities between parasite antigens and components of their particular animal host suggests that tolerance may play some part in pathogenicity and host specificity. There are two examples, for *Salmonella typhimurium* and the mouse (Rowley and Jenkin, 1962) and for haemolytic streptococci of Group A and man—heart specific antigen (Kaplan and Meyeserian, 1962a). It might therefore be wise to check for antibodies produced in animal species unrelated to the particular hosts of pathogens. Tolerance can also obscure (to the immunologist) the presence of bacterial components which are poor antigens or non-antigenic because of general distribution; hyaluronic acid may fall into this category.

For material forming the structural parts of microbial cells and whose physiological functions are not well known, antigenic studies have proved most helpful. The cell wall and associated surface components play roles other than containing the cell contents and providing protection against variable osmotic environments, for example control of entry and egress of metabolites. Bacterial cell surface patterns have been elucidated largely from immunochemical data. The basic cell wall (muramic acid–peptide) is probably never exposed and antibodies specific for its components have not been prepared. The materials closely associated with cell wall, however, are important antigens. These may be protein, polysaccharide or one of the teichoic acid family (for example poly-ribitol-phosphate). In Gram-negative species a polysaccharide-lipid-protein ' endotoxin ' always occurs outside the cell wall but protein antigens may also be present on the surface. Frequently, if the polysaccharide is neutral, some acidic component is found also, such as hyaluronic acid, polyamino-hexuronic acid (Vi antigen),