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Recent Developments in Clinical Immunology

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W.G. Reeves

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Editor

W.G. Reeves

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Preface

Major advances and technological achievements in basic immunology have already had a significant impact on clinical medicine. The need for expertise in clinical immunology has progressively increased during the last decade and many countries have seen the development of departments seeking to apply basic advances in immunology to human disease (Austen, 1982; IUIS Report, 1976; Pruzanski, 1983; Reeves, 1983; W.H.O., 1972). The intricacy and sheer volume of the literature generated by basic immunologists highlight the need for specific contributions designed to bridge the gap between basic science and immunologically-mediated disease. *Recent Developments in Clinical Immunology* contains eight contributions selected for their relevance to the investigation and management of immunological disorders.

Clinical immunologists often know the questions they wish to ask but all too often have not had the means with which to answer them. Testing out the immune response *de novo* has been an area fraught with difficulty in the past but chapter 1 describes the advent of well-documented test antigens of use in clinical investigation. Investigative applications of monoclonal antibodies derived by hybridoma technology are discussed in chapter 7. Methods and instrumentation available for the assessment of fluid-phase immunoprecipitation are described in chapter 2 and a review of the multipurpose role of the small computer in the clinical immunology laboratory is presented in chapter 6. The major histocompatibility complex has recently been the subject of intense study and some of these new approaches are discussed in chapter 3. Ways of studying the human phagocyte are critically assessed in chapter 4. The initial flurry of activity devoted to measuring amounts of immune complexes present in

the circulation has now been largely superseded by the immunochemical characterisation of such material and its biological activity and this is described in chapter 5. Lastly, chapter 8 reviews current experience of bone marrow transplantation with special reference to prospects for the correction of immunological as well as haematological disorders.

Nottingham, U.K. W.G. Reeves

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Contents

Preface v

List of contributors vii

Contents ix

Chapter 1. **The immune response to primary immunogens in man, by C.G.M. Kallenberg, R. Torensma and T.H. The** 1

Chapter 2. **Turbidimetric and nephelometric techniques, by I. Deverill** 27

Chapter 3. **Analysis and function of the major histocompatibility complex, by P. Mackintosh and L. Wells** 51

Chapter 4. **Evaluation of phagocyte function, by R.G.Q. Leslie** 77

Chapter 5. **Characterisation of circulating immune complexes and their solubilisation by complement, by S-E. Svehag, G. Baatrup and I. Petersen** 111

Chapter 6. **The small computer in the clinical laboratory, by I. Deverill** 135

Chapter 7. **Clinical applications of monoclonal antibodies, by A.A. Epenetos** 161

Chapter 8. **Bone marrow transplantation, by A.J. Barrett** 179

Subject index 207

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CHAPTER 1

The immune response to primary immunogens in man

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The function of the immune system is to maintain the integrity of the organism against invasion from outside, especially against microorganisms. In higher animals, an elaborate system has evolved which enables the host to react with a high degree of specificity to exogenous antigens. When this system is defective qualitatively or quantitatively, infections by opportunistic microorganisms take place more easily. Defective functioning of the immune system is due either to congenital or acquired defects of essential parts of the system — primary immunodeficiencies — or is secondary to other disease processes including the use of immunosuppressive agents — secondary immunodeficiencies (WHO Report on Immunodeficiency, 1979). Variations in immune responsiveness are not restricted to patient groups but also occur in normal subjects. The immune response to exogenous antigens is genetically determined in mice (Benacerraf and Germain, 1978). Evidence is accumulating in man that the quality and intensity of the immune response has a genetic basis (De Vries et al., 1977; Sasazuki et al., 1978; Whittingham et al., 1980). The assessment of immune responsiveness in man is important in clinical immunology; not only for the analysis and diagnosis of primary and secondary immunodeficiencies but also for the evaluation of the pattern of immune responsiveness in healthy and diseased people in relation to genetic factors.

Contact with exogenous antigens commences early in life resulting in primary humoral and cellular immune responses and the development of immunological memory. This is maintained by recirculating T lymphocytes with specificity for the various antigens. Renewed contact with a previously encountered anamnestic or secondary antigen results in an immune reaction that peaks higher and more

rapidly than the primary response. This secondary immune response is not always a real reflection of the state of the immune system at that moment but is also determined by the prior reaction of the immune system to the antigen. For instance, the response to tuberculin in normal individuals depends upon the duration and the degree of prior antigenic exposure (Dahlstrom, 1940). However, for keyhole limpet haemocyanin (KLH) — a primary test antigen (see later) — the secondary response in skin tests appears to be independent of the dose of antigen used for primary immunization (Curtis et al., 1970). The interval between primary and secondary contact may influence the secondary immune response (Whittingham et al., 1978). These authors used flagellin derived from *Salmonella adelaide* as a test antigen. They found a strong correlation between the peak of the primary response and the response to secondary challenge. Such a correlation was not found by Peacock et al. (1973) using bacteriophage ØX-174. From these data, it will be evident that several variables, unrelated to the state of immune responsiveness at the time of investigation, influence the secondary immune response. To assess the capacity of the immune system reliably, one should evaluate the primary immune response to an antigen which is presented to the host in a standardized dose and physicochemical state and by a standard route of administration.

In this chapter, we will discuss technical aspects of measuring the antigen-specific response and the requirements for primary test immunogens. We will give a short survey of the several test antigens that are available and focus on the use of haemocyanin from the Roman snail (*Helix pomatia*). Technical and clinical aspects of the humoral and cellular immune response against this antigen will be dealt with as well as in vitro techniques for priming and restimulating human lymphocytes.

Primary test immunogens

Requirements for a primary test immunogen

An antigen suitable for use as a primary test immunogen should satisfy several conditions.

Its administration should not be harmful to the recipient at the time of sensitization or at planned or accidental renewed contact. The preparation should have been tested for sterility and for pyrogenicity. Nevertheless, several problems can arise when administering test antigens to a recipient.

Firstly, living organisms used as test antigens may cause generalized infections in patients with severe immunodeficiencies. Thus, live vaccines (BCG, vaccines for smallpox, poliomyelitis, measles, rubella and mumps) should never be given when a disorder is suspected (WHO Report on Immunodeficiency, 1979). Secondly, allergic reactions may occur at the time of intended primary immunization because of the unknown prior sensitization to the antigen. This potential danger underscores the importance of using a true primary test antigen. Moreover, it implies that one should not use antigens to which the recipients may be exposed later on. Thirdly, serum-

sickness-like disorders may develop depending on the dose of the antigen, the route of administration and the immune response of the recipient.

To exclude prior contact (sensitization) with a supposedly primary test antigen, antigen-specific antibody levels and, ideally, in vitro lymphocyte stimulation tests should be determined in every subject before immunization. Two problems arise. Firstly, negative results do not exclude prior immunological contact. Secondly, 'natural' antibodies have been described against many 'primary' immunogens (Boyden, 1966; Rowley, 1970) and antigen-specific memory cells detected in the peripheral blood of non-immunized subjects (Hersh and Dyre, 1974). This question will be discussed later on in this chapter.

The antigen should be stable and insensitive to minor temperature changes to allow its storage and distribution. It should be administered in a standard dose and physicochemical state. Parenteral administration is preferable unless local handling of antigen in the respiratory tract or the gut is to be studied.

The antigen should have good immunogenic properties. It should be effective in a single dose without adjuvant. This requires presentation of the antigen by macrophages to the antigen-recognizing cells. Interactions between macrophages, T lymphocytes and B lymphocytes follow and result in the development of immunological memory. Finally, the effector phase of the immune response consists of humoral and cellular components. Immunological memory as assessed by in vitro lymphocyte transformation, humoral immunity in all classes of immunoglobulins, and in vivo cellular immunity (delayed type hypersensitivity) should all be measurable with specificity and sensitivity.

Primary test immunogens — a short survey

Throughout the last three decades innumerable substances have been used as immunizing agents for assessing the immune response. However, at the present time only two antigens are widely used as primary immunogens, namely, bacteriophage ØX-174 and the haemocyanins. Before discussing these latter antigens, we will briefly mention other antigens which have been formerly used as test immunogens.

Bacterial antigens

BCG is highly immunogenic (Medical Research Council, 1959). DTH is measured by injecting tuberculin intradermally after primary sensitization. A major drawback of using BCG is the danger of injecting living organisms in immunodeficient patients. The primary character of BCG as a test antigen is questioned since atypical mycobacteria are ubiquitous and cross react with one another as well as *Mycobacterium tuberculosis* (Smith, 1967). The lack of a humoral immune response to BCG makes this antigen also unsuitable as a primary test immunogen. Vaccines of *Pasteurella tularensis* (Levin et al., 1964) and other microorganisms have been described as tools for assessing the primary immune response. Besides the potential

danger associated with the use of vaccines, the problem of cross-reactivity is inherent to the use of any microbial product and prior exposure can never be excluded. This applies also to the use of supposedly harmless microbial products such as the Vi-antigen and flagellin from *S. adelaide* (Rowley, 1970). The Vi-antigen, which is a polysaccharide consisting of repeating units of *N*-galactosaminuronic acid is isolated from the Vi strain of *E. coli* 5396/38. Vi-antibody levels are estimated by haemagglutination of sheep or human erythrocytes coated with the purified antigen. Very low Vi-antibody levels were present in 20 out of 400 normal subjects before immunization (Landy, 1954). IgM class antibodies reactive with flagellin have been detected two weeks after birth. Those 'natural' antibodies were present in titres up to 1:2560 in 90% of healthy subjects prior to the immunization. The presence of these antibodies was not associated with a secondary type of antibody response after immunization (Rowley et al., 1972). This suggests that these antibodies result from previous exposure to antigens with similar determinants (Boyden, 1966).

Synthetic hapten-carrier conjugates

Conjugates of picryl or dinitrophenol with human serum albumin (Kantor and Bullock, 1966) and synthetic polymers of α-amino acids (Maurer et al., 1962) have been used as test antigens. These products are reasonably immunogenic. Whether or not their restricted antigenicity is an advantage is doubtful. More complicated antigens like haemocyanins are probably more representative of the average antigen to which the immune system is exposed. Coupling of the former antigens to human serum proteins might be a drawback, since alterations of these proteins by exogenous material can induce immune responses against these proteins (Lerner et al., 1967).

Bacteriophage ØX-174

The use of bacteriophage ØX-174 for testing the primary immune response in humans was first described by Uhr et al. (1962). Since that time several other reports have been published on this test immunogen (Ching et al., 1966; Ochs et al., 1971; Peacock et al., 1973). The antigen is highly immunogenic when given intramuscularly or intravenously. Pre-immunization antibodies have been detected in low titre in 4% of the subjects (Peacock et al., 1973). Both the clearance of ØX-174 particles from the circulation and specific antibody titres (determined as the 50% bacteriophage neutralization titres) can be measured after intravenous administration. In vitro lymphocyte stimulation tests or skin testing with the antigen have not been reported. No adverse effects have been described in the literature. Since the bacteriophage is capable of multiplication only within *E. coli C*, which carry the highly specific receptor with which it interacts, proliferation in human beings is excluded.

Haemocyanins

Haemocyanins from several species have been used extensively for assessing the primary immune response. Haemocyanins from the keyhole limpet (KLH) *Megathura*

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crenulata (Curtis et al., 1970; Curtis and Hersch, 1972; Herscowitz et al., 1972; Salvaggio et al., 1969; Swanson and Schwartz, 1967), from the horseshoe crab (LPH) *Limulus polyphemus* (Bandilla et al., 1969) and from the Roman snail (HPH) *Helix pomatia* (De Gast et al., 1973) have been described as test antigens.

In some instances, IgM class antibodies have been detected prior to immunization against the haemocyanins (Burke et al., 1977; De Gast et al., 1973; Moroz et al., 1973; Salvaggio et al., 1969). These antibodies can be removed by prior absorption of the sera with antigen-coated red cells or free antigen. 2 ME-resistant antibodies have also been found in some non-immunized subjects (Salvaggio et al., 1969). However, no relationship was found between pre-immunization IgM or IgG antibody levels and the subsequent immune response (Burke et al., 1977). Since haemocyanins are large molecules with many epitopes inducing many specific immunoglobulins, the antibodies present before immunization are most probably directed against carbohydrate structures which are part of many natural antigens. This is illustrated, e.g. by the cross-reactivity between KLH and various mammalian red cell stroma (Frick and Shimbor, 1970). Skin tests and in vitro lymphocyte stimulation tests can also be performed in non-immunized subjects. Whereas Salvaggio et al. (1969) found positive skin tests in some non-immunized individuals, Burke et al. (1977) found skin tests and in vitro lymphocyte stimulation tests with KLH to be negative in subjects with pre-immunization antibodies. In vitro studies also demonstrated the presence of lymphocytes with binding sites specific for KLH in the peripheral blood of normal controls before immunization, although in a very low percentage (0.13%) (Hersh and Dyre, 1974). Thus, IgM class antibodies are detectable against most test antigens when sensitive techniques are used. They probably result from prior exposure to cross-reacting determinants and do not influence the primary immune response. However, the virginity of the immune system with respect to these antigens is still a matter of debate.

In the following section we will discuss the structure and physicochemical characteristics of HPH in particular, its use in the assessment of the primary immune response and some of its clinical applications.

Structure of *Helix pomatia* haemocyanin

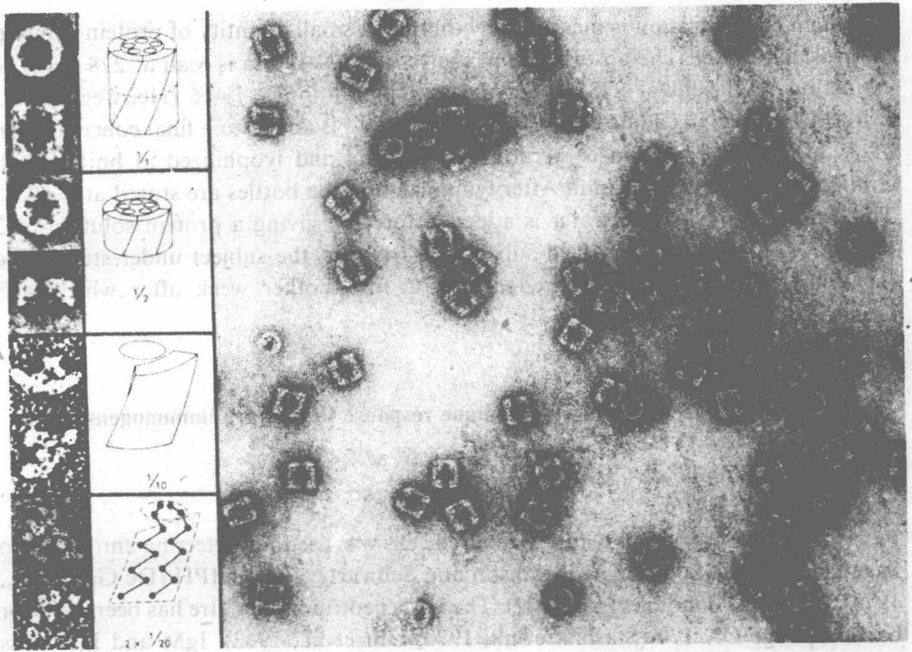
Haemocyanins occur freely dissolved in the haemolymph of several arthropods and molluscs. They are large proteins and contain copper atoms. They serve as oxygen carriers: one oxygen molecule is bound by two copper atoms. The molecular weight of molluscan haemocyanins ranges from 4.5×10^6 (*Colus gracilus*) to 2.7×10^7 (*Megathura crenulata* or keyhole limpet). Molluscan haemocyanins have been used to assess immunological responses in both animals and man (Curtis et al., 1970; De Gast et al., 1973; Dixon et al., 1966). *H. pomatia* haemocyanin (HPH) is a typical molluscan haemocyanin and has a cylindrical appearance at both ends partly closed

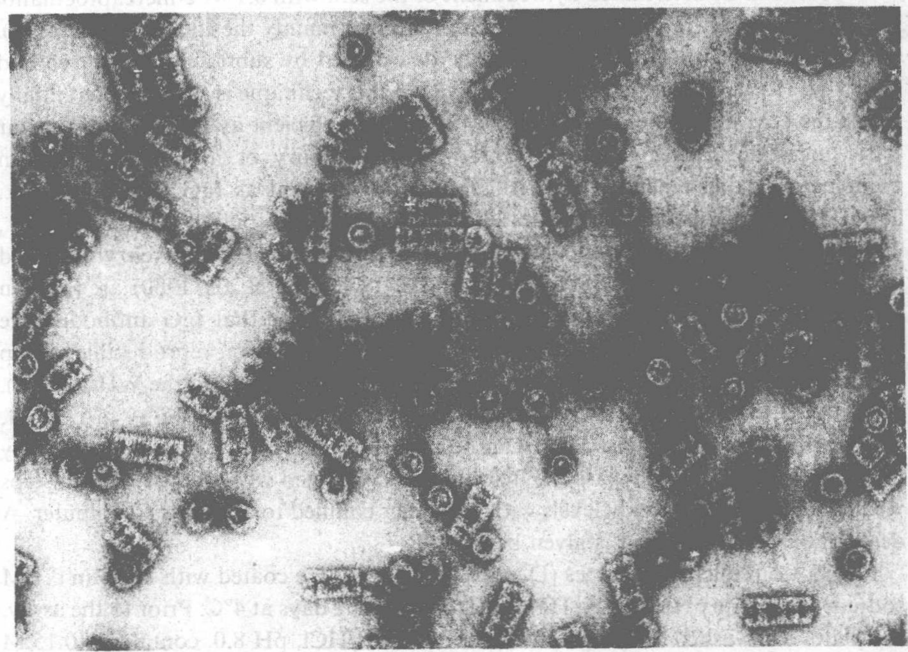
by a collar, as observed on electron micrographs (Fig. 1.1). The cylindrical molecule has a diameter of 35 nm and a height of 38 nm. Raising the pH and removing divalent cations (i.e. Ca^{2+} and Mg^{2+}) causes the protein to dissociate into smaller, but distinct components. Dissociation into half molecules occurs perpendicular to the axis of the cylinder. Each half molecule dissociates into 5 sector-shaped pieces containing part of the wall and part of the collar. These one-tenth molecules can be split further into one-twentieth molecules that are revealed in electron micrographs as a beaded chain (Fig. 1.1). They cannot be dissociated further without disrupting polypeptide bonds. Biochemical analysis indicates the presence of eight globular domains per one-twentieth molecule connected by more extended parts of the polypeptide chains (Gielens et al., 1977). Each domain bears an oxygen-binding site and has specific functional and structural properties (Torensma et al., 1981a, b). Evidence is accumulating that they also have different immunological properties (Préaux et al., 1981) since antibodies specific for a given domain can be raised. The dissociation into one-twentieth molecule is fully reversible. In most cases, the resulting association product is indistinguishable from the native form. However, for some species like *Neptunea antiqua*, *M. crenulata* and *Busycon canaliculatum* association to larger structures than the native molecule can occur (Van der Laan et al., 1981; Senozan et al., 1981; Roxby, 1981). Since the physicochemical state of the antigen is one of the features that determine its immunogenicity, the influence of the isolation procedure on the physicochemical state of the antigens has to be known when it is used as a primary test immunogen.

Extraction and purification of HPH

Helix pomatia snails can be obtained commercially. A hole is made in their shell making the heart visible. Blood is obtained by heart puncture. Care has to be taken not to puncture the stomach because it contains cellulase which will destroy dialysis tubing during subsequent isolation steps. Blood is collected and centrifuged at low speed to remove cells and debris. The supernatant is centrifuged at 100 000 × *g* for 4 h. The blue pellet is dissolved in 0.1 M NaAc pH 5.7. The resulting protein solution is dialyzed against 0.01 M NaAc pH 5.3 for several days. After 2 days, a precipitate develops in the solution. The dialysis is continued until no more precipitate is formed. This precipitate is obtained by centrifuging at low speed. The pellet contains haemocyanin that is immunologically and functionally different from the haemocyanin in the supernatant. The supernatant is collected and dialyzed against 0.1 M NaAc pH 5.7.

Fig. 1.1. A. Electron micrograph of *Helix pomatia* haemocyanin. A homogeneous distribution is seen. The rectangular appearances represent side views of the cylindrical molecule. Magnification × 260 000. The line drawings are a schematic representation of the electron micrographs on the far left showing the dissociation products. B. Electron micrograph of *Busycon canaliculatum* haemocyanin. A heterogeneous size distribution is evident. Magnification × 260 000.





(B)

Protein concentration is measured by diluting a small quantity of protein solution in saturated borax. The absorbance of the resulting solution is read at 278 nm. The extinction coefficient at 278 nm for a 1 mg/ml solution is 1.416 (Heirwegh et al., 1961). The protein is adjusted to 10 mg/ml. Sucrose is added to a final concentration of 25 mg/ml. The protein is sterilized by filtering and lyophilized in bottles each containing 6 mg haemocyanin. After lyophilization the bottles are stored at −20°C. Three millilitres of sterile water is added before use giving a protein solution of 2 mg/ml; 0.5 ml (1 mg) is injected subcutaneously into the subject under study. The remaining solution can be stored at +4°C for another week after which it is discarded.

Technical aspects of measuring the immune response to primary immunogens

Antigen-specific antibody assays

Passive haemagglutination using tanned red cells was used for detecting antibodies to flagellin (Wistar, 1968), KLH (Swanson and Schwartz, 1967), HPH (De Gast et al., 1973) and LPH (Bandilla et al., 1969). The virus neutralization titre has been used for bacteriophage ØX-174 (Stashak et al., 1970; Uhr et al., 1962). IgM and IgG class antibodies can be determined by treatment of the sera with 0.1 M 2-mercaptoethanol (2-ME), the 2-ME resistant antibody titre reflecting mainly the IgG antibodies (7S). The 2-ME-sensitive IgM antibody can be determined by subtraction (Deutsch and Morrow, 1957). One of the major drawbacks of this technique is that it preferentially measures IgM antibodies since IgM is 750 times as efficient as IgG on a molecular basis in agglutinating human erythrocytes (Greenbury et al., 1963). Also, in complement-fixing reactions IgM is 120 times as efficient as IgG (Robbins et al., 1965). If antigen binding techniques like radioimmunoprecipitation (Bandilla et al., 1969), quantitative precipitation techniques (Dixon et al., 1966) or enzyme-linked immunosorbent assays (ELISA) (Weits et al., 1978; The et al., 1980) are used in conjunction with haemagglutination techniques, it is clear that IgG antibodies are underestimated by the latter. The ELISA-technique is sensitive, reproducible, cheap and relatively simple compared to many other methods (Bulletin of the WHO, 1976). With the ELISA technique class- and subclass-specific (IgG_1–IgG_4, IgA_1, IgA_2, IgM, IgE) antibody levels against HPH can be measured in a sensitive and specific way (Weits et al., 1978). To bypass the inaccuracies connected with the use of titre steps we now determine antibody levels with a scanner coupled to a desk-top computer. A description of our method is given below.

Flat-bottom microtitre plates (Dynatech M 129 A) are coated with HPH in 0.1 M sodium carbonate pH 9.6 (5 μg HPH/ml) for at least 2 days at 4°C. Prior to the assay, the plates are washed thoroughly with 10 mM Tris/HCl, pH 8.0, containing 0.15 M NaCl and 0.05% Tween 20. Subsequently, the plates are incubated with 100 μl

diluted serum (1:100 to 1:3200) in 0.01 M Tris/HCl pH 8.0 containing 0.05% Tween 20, 0.3 M NaCl and 4% BSA for 45 min at 37°C. The plates are washed to remove unbound material and are incubated with 1000 times diluted commercially available heavy-chain specific antisera conjugated to horse radish peroxidase in 10 mM Tris/HCl pH 8.0 containing 0.3 M NaCl, 0.05% Tween 20 and 2% BSA for another 45 min, and washed again. Bound enzyme activity is measured by incubation with 100 μl 50 mM phosphate buffer, pH 5.6, containing 0.2 mg/ml ortho-phenylene-diamine (OPD) and 0.0045% H_2O_2 for 10–30 min at room temperature. The reaction is stopped by the addition of 100 μl 1 N H_2SO_4. The optical density at 492 nm is read in a Titertek Multiskan apparatus and the values are stored in a desk-top computer for subsequent calculation. Each plate contains blank incubations and a reference sample assayed in duplicate at six concentrations. This reference sample consists of pooled sera from 20 high responders three weeks after the primary HPH immunization. Unknown anti-HPH concentrations are computed from the linearized titration curve obtained after log–logit transformation of the concentrations of the reference serum and the corresponding optical densities. Antibody concentrations in unknown sera are expressed as a percentage of the reference sample. Antibody concentrations have to be covered by the titration curve. Therefore, six serum dilutions are measured.

Assessing immunological memory in vitro

In vitro lymphocyte stimulation is performed to assess the capacity of the immune system to recognize a previously encountered antigen. Peripheral blood mononuclear cells are isolated using Isopaque–Ficoll and incubated in microtitre plates with several concentrations of the test antigen (De Gast et al., 1975; Kallenberg et al., 1981). Depending on the state of the immune system in vitro contact between antigen and antigen-specific B and T lymphocytes results in proliferation of these lymphocytes. This is measured by the amount of incorporation of ^{3}H-labelled thymidine, which is added during the last 16 h of the culture. It is necessary to perform an antigen dose–response curve in order to establish the optimal concentration and it is important to set up control lymphocyte cultures with non-specific stimulants (mitogens) and anamnestic antigens. In this way, specificity with respect to the antigen in question can be evaluated. Another method to study antigen-recognizing cells in the peripheral blood is described by Hersh and Dyre (1974). Cells able to bind KLH were detected among human peripheral blood leucocytes and KLH-stimulated cultured lymphocytes by their formation of rosettes with KLH-coated human O red blood cells. The low percentage of cells binding the antigen in the blood (0.13% to 0.80%) and in the cultures (0.1% to 8.5%) renders the method inaccurate, unless a very high number of cells is counted. Antibody-forming cells, both direct (19S) and indirect (7S), may be detected by a haemolytic plaque assay, which is much more sensitive (Herscowitz et al., 1974). Lymphocyte transformation tests are not

necessarily equivalent to delayed type hypersensitivity. A correlation between in vitro blastogenesis and in vivo cellular immunity was observed in one (McFarland, 1966) but not in another study (Benezra et al., 1969).

Assessing delayed hypersensitivity

Cellular immunity — delayed type hypersensitivity (DTH) — can be measured by intradermal injection of a low dose of the antigen after primary sensitization. Positive DTH reactions can be elicited 5 days after subcutaneous or intradermal immunization with KLH (Curtis et al., 1970). Positive skin tests were present on challenging with 0.1 mg HPH intradermally, 28 days after primary sensitization with 1 mg HPH subcutaneously (De Gast et al., 1973). Bacteriophage ØX-174 does not seem to be suitable for measuring DTH. In both studies with haemocyanin, skin infiltration was read at 24 and 48 h. With HPH induration was more extensive at 24 than at 48 h. This poses a problem in the interpretation of skin reactions. Immediate (weal and flare) reactions sometimes occur supposedly based on IgE-mediated reactions (Ishizaka and Ishizaka, 1967). Arthus reactions produced by local contact of antigen with free circulating antibody give inflammatory reactions approximately 8 h after challenge (Cochrane and Janoff, 1974). Shortly after sensitization skin testing may result in the 'Jones–Mote phenomenon', an erythematous, usually non-indurated lesion with the same rate of onset and duration as the DTH reaction. It is mediated by antigen-specific lymphocytes which attract basophils without the intervention of antibodies. The phenomenon frequently disappears when the subject makes prominent humoral responses to the same antigen (Colvin et al., 1973). For this reason, skin testing with a primary test antigen should not be performed before antibody formation has started. The typical DTH reaction takes place from 24–72 h after local application of the antigen. Thus, one should read skin reactions immediately (15 min), and at 8, 24, 48 and 72 h after challenge in order to discriminate between the several types of reactions.

The test most extensively used for assessing the primary cellular immune response is that using dinitrochlorobenzene (DNCB) (Brown et al., 1967). Sensitization occurs by application of DNCB epidermally. The mechanism of contact hypersensitivity is reviewed by Claman et al. (1980). We use a modification of the DNCB test in which the degree of skin reactivity is determined semi-quantitatively (Bleumink et al., 1974). Details are given below.

DNCB sensitization is performed by applying 2000 μg twice recrystallized DNCB in acetone topically within an area of a polythene ring (diameter, 2 cm) on the volar aspect of the right arm. Challenges are done by patch testing on the back of the subjects with 30, 10 and 3 μg of DNCB in acetone 2 wk later. The reactions are read after 48 h and are graded 1+ to 4+: 1+ = erythema, 2+ = erythema plus induration, 3+ = erythema, induration and vesiculation, 4+ = 3+ with ulceration. The

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