Immunoglobulins in Health and Disease

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Immunoglobulins in Health and Disease

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Preface

Rapid advances continue to be made in all areas of immunology, not least the biology of the immunoglobulins. This knowledge has resulted in a better understanding of antibody responses and helped to clarify pathogenic mechanisms in many diseases, particularly autoimmune and allergic diseases, as well as expand our comprehension of antibody deficiency diseases and mechanisms in therapeutic immunization. In addition, the recognition that diverse disease states may result in abnormalities of the amount of immunoglobulins in body fluids has resulted in the use of immunoglobulin assays for disease diagnosis and management.

The aim of this book has been to condense both the established and recent aspects of this knowledge, particularly that pertaining to clinical immunology. The contributions of different authors hopefully provide a comprehensive review of their particular field of interest as well as a discussion of how this information can be applied to clinical medicine. Immunological terms and concepts have been explained where appropriate so that the book can be read by those with only a basic knowledge of immunology. In producing a book on this one area of immunology some duplication of information has been accepted so that topics can be considered in different contexts.

I hope the book will be of value to those in training or already pursuing a career in clinical or laboratory medicine by providing a basic and short text on immunoglobulins.

M.A.H.F.

Series Editor's Note

The modern clinician is expected to be the fount of all wisdom concerning conventional diagnosis and management relevant to his sphere of practice. In addition, he or she has the daunting task of comprehending and keeping pace with advances in basic science relevant to the pathogenesis of disease and ways in which these processes can be regulated or prevented. Immunology has grown from the era of anti-toxins and serum sickness to a state where the study of many diverse cells and molecules has become integrated into a coherent scientific discipline with major implications for many common and crippling diseases prevalent throughout the world.

Many of today's practitioners received little or no specific training in immunology and what was taught is very likely to have been overtaken by subsequent developments. This series of titles on IMMUNOLOGY AND MEDICINE is designed to rectify this deficiency in the form of distilled packages of information which the busy clinician, pathologist or other health care professional will be able to open and enjoy.

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Structure and Function of Immunoglobulins

D. R. BURTON AND L. GREGORY

BASIC ANTIBODY STRUCTURE AND FUNCTON

All antibodies are based on a monomer consisting of three structural units (Figure 1.1). Two of the units are identical and involved in binding to the foreign material or antigen - the Fab, Fragment antigen binding arms of the molecule. The third unit - Fc (Fragment crystalline) - is involved in binding molecules generally related to antigen elimination, e.g. complement, receptors on cells such as macrophages, neutrophils and mast cells. These molecules are often termed effector molecules since their binding to antibody triggers host defence systems known as effector functions. *The structure of antibodies thus reflects their dual role in recognizing foreign material and triggering its elimination.*

There are five classes of antibodies or immunoglobulins termed immunoglobulin G (IgG), IgM, IgA, IgD and IgE. Structurally the antibody classes tend to differ most in their Fc regions, and on binding to antigen they tend to trigger different effector functions, e.g. IgM recognition of antigen might lead to activation of complement whereas IgE recognition might lead to mast cell degranulation and anaphylaxis. The antibody classes also differ in the state of polymerization of the monomer unit of Figure 1.1. Thus IgG and IgE are generally monomeric, whereas IgM occurs as a pentamer. IgA occurs predominantly as a monomer in serum and a dimer in seromucous secretions.

The major antibody in the serum is IgG, and as this is the best-understood antibody in terms of structure and function we shall consider it shortly. The other antibody classes will then be considered in relation to IgG. First, however, a very brief overview of the structure and function of the different immunoglobulins will be presented.

IgG is the major antibody class in normal human serum forming about 70% of the total immunoglobulin, and is evenly distributed between intra- and

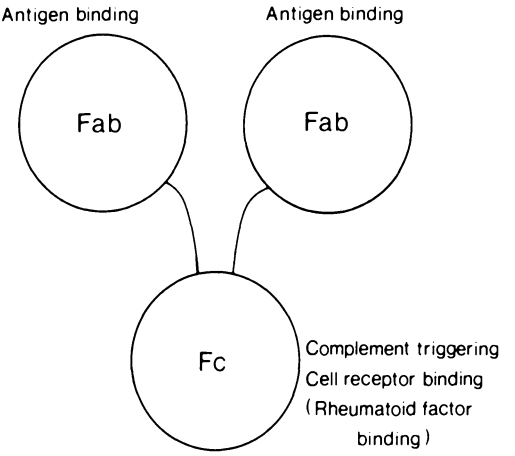


Figure 1.1 A schematic representation of antibody structure emphasizing the relationship between structure and function. The antibody molecule can be thought of in terms of three structural units. Two Fab arms bind antigen and are, therefore, crucial for antigen recognition. The third unit (Fc arm) binds effector molecules which trigger antigen elimination. The antibody molecule thus links antigen recognition and antigen elimination

extravascular pools. IgG is a monomeric protein which can be divided into four subclasses. It is the major antibody of secondary immune responses and the exclusive antitoxin class.

IgM represents about 10% of total serum immunoglobulin and is largely confined to the intravascular pool. It forms a pentameric structure and is the predominant antibody produced early in an immune response, serving as the first line of defence against bacteraemia. As a membrane-bound molecule on the surface of B-lymphocytes it is important as an antigen receptor in modulating the response of these cells to antigenic stimulation.

IgA forms about 15–20% of total serum immunoglobulin where it occurs largely as a monomer. In a dimeric complex known as secretory IgA (sIgA) it is the major antibody in seromucous secretions such as saliva, tracheobronchial secretions, colostrum, milk and genito-urinary secretions.

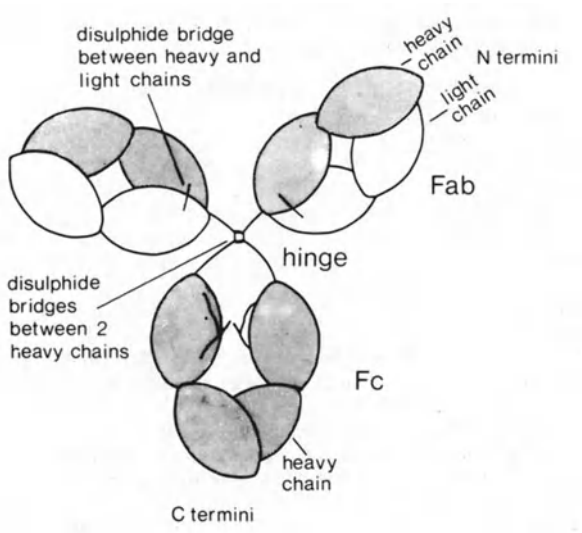
IgD represents less than 1% of serum immunoglobulin, but is widely found on the cell surface of B-lymphocytes where it probably acts as an antigen receptor similarly to IgM.

IgE, though a trace immunoglobulin in serum, is found on the cell surface of mast cells and basophils in all individuals. It is involved in protection against helminthic parasites but is most commonly associated with atopic allergies.

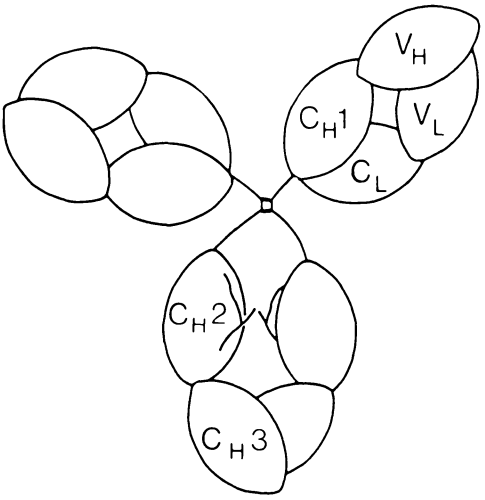
STRUCTURE AND FUNCTION OF IgG

IgG is a 4-chain structure composed of domains

As shown in Figure 1.2a IgG has a 4-chain structure consisting of two identical heavy (H) chains of molecular weight ~50000 and two identical light



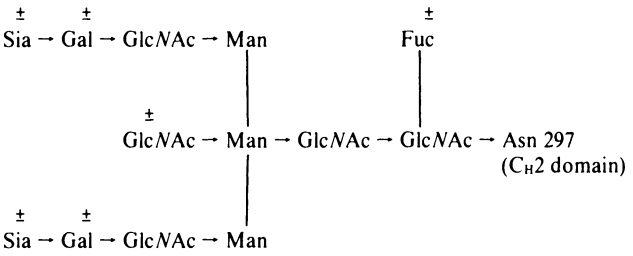
(a)



(b)

Figure 1.2 The 4-chain domain structure of IgG. (a) *The arrangement of heavy and light chains in relation to Fab and Fc*. Each heavy chain (shaded) is folded into two domains in the Fab arms, forms a region of extended polypeptide chain in the hinge and is then folded into two domains in the Fc region. The light chain forms two domains associated only with an Fab arm. Domain pairing leads to close interaction of heavy and light chains in the Fab arms supplemented by a disulphide bridge. The two heavy chains are disulphide bridged in the hinge (the number of bridges depending on IgG subclass) and are in close domain-paired interaction at their C-termini. (b) *Domain nomenclature*. The heavy chain is composed of V_H (variable-heavy), C_H1 (constant-heavy 1), C_H2 and C_H3 domains. The light chain is composed of V_L (variable-light) and C_L (constant-light) domains. All the domains are paired except for the C_H2 domains which have two branched N-linked carbohydrate chains interposed between them. The carbohydrate is not a

single oligosaccharide moiety but consists of a set of about 20 structures based on a mannosyl chitobiose core, i.e. there is considerable heterogeneity:



As shown, four types of mannosyl-chitobiose cores are found (± 'bisecting' *N*-acetylglucosamine [Glc*N*Ac] ± fucose [Fuc]) and outer chain variants include the presence or absence of galactose (Gal) and sialic acid (Sia).

Each domain has a MW of approx 12000 leading to a MW of ~50000 for Fc and Fab and 148000 for the whole IgG molecule. Antigen recognition involves residues from the V_H and V_L domains, complement triggering the C_H2 and Fc receptor the C_H2 and possibly the C_H3 domain (see text).

Proteolytic cleavage can yield various fragments. Papain cleaves the heavy chains to the N-terminal side of the disulphide bridges in the hinge to yield two Fab and a Fc fragment. Pepsin cleaves on the C-terminal side to the hinge disulphides and within the C_H2 domain to yield an $(Fab')_2$ fragment and a pFc' fragment (essentially a C_H3 domain dimer)

chains of molecular weight ~25000. The molecular weight of IgG is thus typically approximately 150000. The light chains are solely associated with the Fab arms of the molecule whereas the heavy chains span Fab and Fc parts. A single disulphide bond connects heavy and light chains as shown in Figure 1.2a. The two heavy chains are connected to one another via disulphide bridges in a region joining the Fab arms of the molecule to the Fc part and known as the hinge.

The light chains exist in two forms known as kappa (κ) and lambda (λ). The heavy chains exist in four forms known as $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ which correspond to the four subclasses of human IgG, i.e. IgG1, IgG2, IgG3 and IgG4. The principal difference between the subclasses is in the hinge region of the molecule as will be discussed later. In a single molecule the two heavy chains are identical, as are the two light chains – hybrid molecules are not found.

The Fc and Fab parts of IgG are organized into a total of 12 domains as highlighted in Figures 1.2a,b. Amino acid sequence comparisons of a number of monoclonal IgG proteins reacting with different antigens reveals some very interesting features of these domains. The N-terminal domains of both chains are found to show considerable sequence variation between different IgGs, and are consequently termed V_L (variable-light) and V_H (variable-heavy) domains (Figure 1.2b). The other domains show highly conserved sequences and are termed constant domains; C_L (constant-light) and C_H1, C_H2 and C_H3 (constant-heavy) domains. (As the H chain of IgG is a γ chain, the C_H domains are sometimes denoted $C\gamma1$, $C\gamma2$ and $C\gamma3$.)

Figure 1.2b illustrates that all of the domains except for C_H2 are in close lateral associaton with another domain; a phenomenon described as domain

pairing. The C_H2 domains have two N-linked branched carbohydrate chains interposed between them. The domains also exhibit weaker (*cis*) interactions with neighbouring domains on the same polypeptide chain.

The hinge is a region of extended polypeptide chain which is generally believed to permit movement of the Fab arms of the IgG molecule relative to the Fc. This is an attractive idea in that it would allow an IgG molecule to attach to antigen molecules on a foreign cell surface via both its Fab arms without the need for the antigen molecules to be precisely spaced (the antibody has a 'variable reach'). Such divalent antibody binding greatly enhances affinity as compared to monovalent binding. It also allows cross-linking of particulate antigens to form soluble or insoluble immune complexes and agglutination of cells.

Domain structure: a closer look

Since domains are at the heart of antibody structure and function it is worthwhile considering their structure in a little more detail. Crystallographic studies have revealed that each domain has a common pattern of polypeptide chain folding as depicted in Figure 1.3. This pattern, the 'immunoglobulin

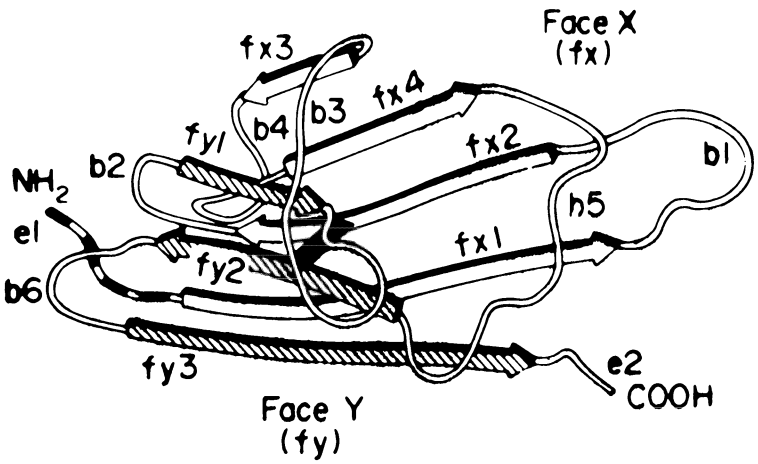


Figure 1.3 Peptide chain folding of a constant domain. The segments fx1-4 (unshaded) and fy1-3 (shaded) form two roughly parallel faces of antiparallel β-pleated sheet linked by an intrachain disulphide bridge. Between the β-pleated segments are other segments (b1-6) forming helices, bends and other structures. Segments fx3, fx4, fy1 and b4 are foreshortened in this three-dimensional representation. A variable domain differs from a constant domain in that the β sheets are more distorted and the variable domain possesses an extra loop.

Variable domains are paired by contact between two y faces and constant domains by contact between two x faces. In both cases the interacting faces are predominantly hydrophobic and the driving force for pairing is thus the removal of the amino acid residues of these faces from the aqueous environment. The disulphide bridge stabilizes the paired arrangement. Further contact is made between V_H and V_L domains by loops from each domain - hypervariable loops or complementarity determining regions - which come together in space to form the antigen binding site

fold', consists of two twisted, stacked β sheets surrounding an internal volume of tightly packed hydrophobic residues. The arrangement is stabilized by an internal disulphide bond linking the two sheets in a central position. One sheet has four and the other three antiparallel β strands. These strands are joined by bends or loops.

Antigen recognition involves loops from the variable domains

Comparison of amino acid sequences of the variable domains of antibodies binding different antigens reveals relatively short segments showing extreme variation in sequence. These hypervariable regions correspond to loops from both the V_L and V_H domains which come together in space to form the antigen recognition site (Figures 1.3 and 1.4).

Antibodies show enormous diversity in antigen recognition. A human being can produce of the order of a million different antibodies of differing antigen specificity. Structurally this feat is achieved in a very economical fashion by keeping the basic immunoglobulin fold structure of the variable domains relatively constant and varying the length and nature of the hypervariable loops to produce the enormous variety of antigen recognition sites.

Variable and constant domains: diversity and commonality

IgG molecules of a given subclass can recognize an enormous range of different antigen molecules and yet all will activate the same effector functions. This is because antigen is recognized by the hypervariable loops of the variable domains, whereas effector molecules are recognized by the constant domains of the Fc part of the molecule. *The structure of IgG thus consists of variable and constant elements perfectly suited to the dual role of recognizing diverse foreign material and triggering its elimination by a small number of common pathways.*

Before we move on to consider effector function recognition of IgG we need to consider the IgG molecule in a little more detail.

The three-dimensional structure of IgG

Crystal structures have been solved for two mutant IgG1 proteins in which the hinge region is deleted. These proteins adopt a T-shaped conformation. They are abnormal in the sense that they do not bind effector molecules such as complement or monocyte receptors as do complete IgG1 molecules. This may be because hinge-deletion brings the Fab arms closer in to critical binding sites on Fc so that they obstruct such sites. Unfortunately crystal structures have not been solved for complete IgG molecules because the presence of the hinge allows the Fc to move relative to the Fab arms even in the crystal, making its position indeterminate. (However, crystal structures have been solved for isolated Fab and Fc fragments of IgG.)

Therefore, for complete IgG molecules one must turn to less exact methods than crystallography, such as electron microscopy, hydrodynamic and

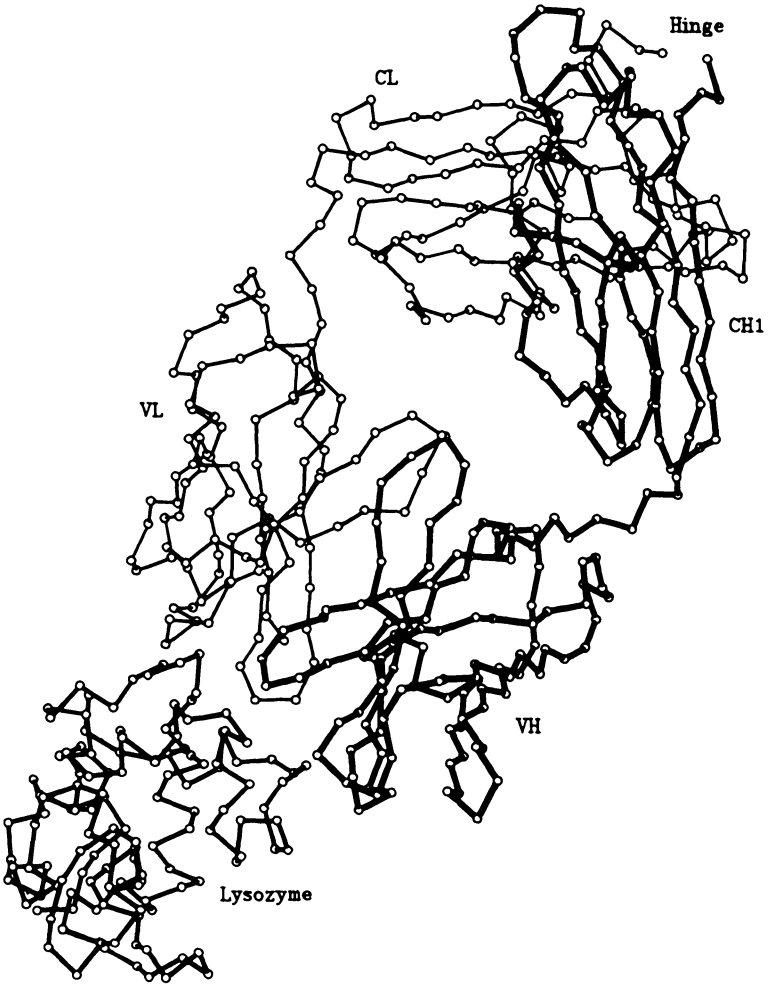


Figure 1.4 Structure of a complex of the Fab fragment of an antibody molecule with antigen. The complex was formed between hen egg-white lysosyme and the Fab fragment of a mouse monoclonal anti-lysosyme antibody. The diagram shows the crystal structure at 0.28nm resolution. Alpha carbon atoms only are shown; thick lines being used for lysosyme and the heavy chain of Fab and a thin line for the light chain.

The area of interaction between the two proteins is large, approximately 2 × 3 nm. The region of Fab in contact with the antigen includes hypervariable loops from both heavy and light chains with more interactions involving the former. However, the combining site is not a simple cleft enclosed by the hypervariable loops but extends beyond them. The antigenic site recognized on lysosyme is not a linear amino acid sequence - rather it is an arrangement of amino acids in three dimensions provided by different parts of the linear sequence. There is no evidence for significant conformational changes occurring in either antigen or Fab on complex formation.

This diagram was very kindly provided by Dr S. E. V. Phillips

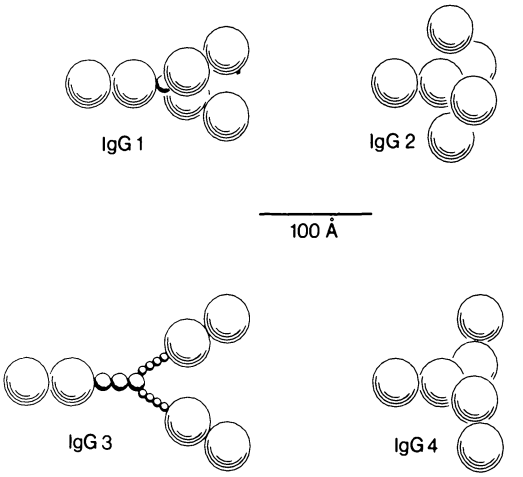


Figure 1.5 Proposed models for the structures of the subclasses of IgG. The models illustrate average solution conformations consistent with hydrodynamic and small angle X-ray scattering data (Gregory, Boyd, Jefferis, Nave and Burton, unpublished). The model for IgG3 is consistent with electron micrographs obtained by Dr R. S. H. Pumphrey and co-workers.

IgG1 is proposed as a Y-shaped molecule with the Fab arms subtending an angle of 40–60° to one another. The Fab arms are not co-planar and not in the same plane as Fc. In IgG2 the Fab arms are folded back leading to close approach of Fab and Fc. The opposite is the case for IgG3 which has an extended hinge as shown. IgG4 is proposed as an approximately T-shaped molecule with relatively close approach of Fab and Fc

scattering methods. We give in Figure 1.5 what we believe to be the best available models for the four subclasses of IgG at the current time. IgG1 is shown as a Y-shaped molecule: this is an average conformation. Hinge flexibility probably allows conformations between a T- and a nearly closed Y-shape. IgG3 has a very extended hinge region in which the Fab arms are distant from Fc. This contrasts with IgG2 and IgG4 where there is close approach of the Fab arms and Fc.

The triggering of effector functions by IgG

Effector molecules interact with the Fc part of IgG to trigger effector functions. Activity varies greatly between the subclasses. A rough rule of thumb is that IgG1 and IgG3 are generally much more effective than IgG2 and IgG4 in triggering effector functions. The antibody–effector molecule interactions are much less clearly understood than that between antibody and antigen. They will shortly be considered in turn, although we begin with the interaction between staphylococcal protein A and IgG which is an Fc interaction well understood in structural terms.

Protein A

Protein A is a major cell wall component of most strains of *Staphylococcus aureus* which binds to the Fc regions of a number of antibodies but

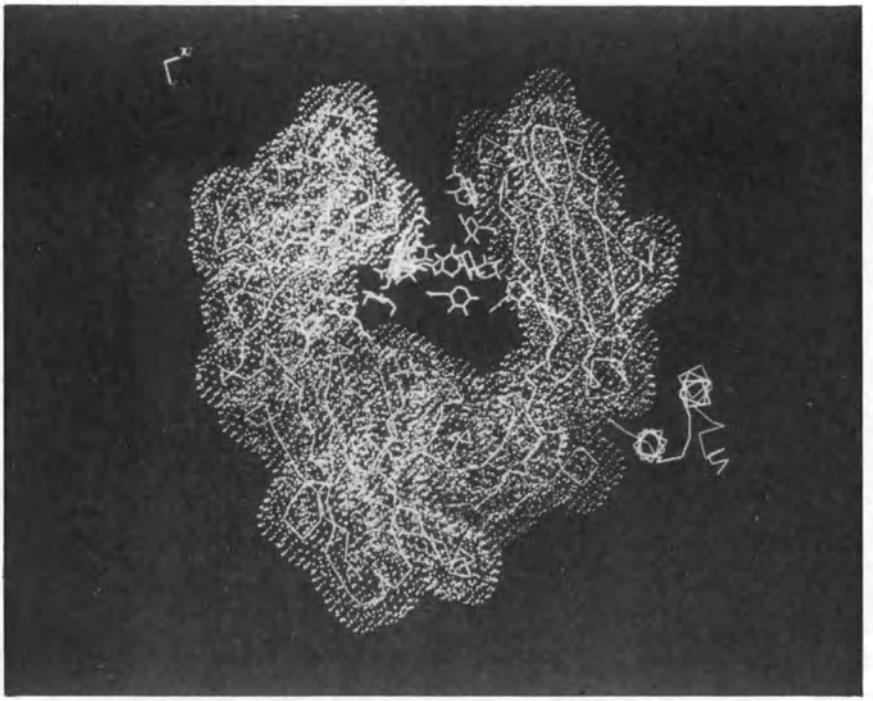


Figure 1.6 A view of the interaction of fragment B of protein A with human Fc. This diagram is obtained from the co-ordinates to 0.28 nm of the crystal structure of complexed human Fc and fragment B of protein A, solved by Dr J. Deisenhofer and available from the Brookhaven Data Bank. The diagram was obtained using the SERC Northern Computer Graphics Display with the kind assistance of Drs G. C. Ford and J. White.

The diagram shows an α-C trace of the two heavy chains of Fc with associated van der Waals surface (dotted), the two C_H2 carbohydrate chains and an α-C trace of fragment B of protein A (right). Note that the two C_H3 domains are in a close pairing interaction whereas the two C_H2 domains have the two branched carbohydrate chains interposed between them. The interheavy disulphide bridges and early residues in the C_H2 domain are in a region of flexible polypeptide chain and do not give defined electron density for crystallographic structural solution. Fragment B binds at a site between C_H2 and C_H3 domains - only one interaction site is shown, although there is of course a similar site on the other side of the molecule. Note that the Fc molecule contains an approximate two-fold axis of symmetry (not a mirror plane - so that a site close to the observer on, e.g. one heavy chain will be distant on the other chain)

particularly those of the IgG class. It is useful for isolating IgG and detecting IgG, e.g. radiolabelled or fluorescent labelled protein A can be used in IgG estimation. Protein A binds to Fc at the interface between C_H2 and C_H3 domains as shown in Figure 1.6. This binding is found for IgG1, IgG2, IgG4 and those IgG3 antibodies bearing allotypic markers characteristic of Mongoloid populations. IgG3 antibodies from Caucasian populations do not bind to protein A. This is explained in terms of a difference in a single amino acid residue in the protein A binding site. Protein A possesses four IgG

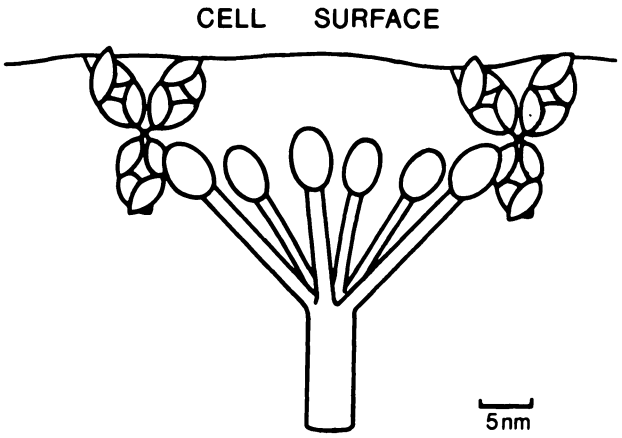


Figure 1.7 A schematic view of the binding of complement C1q to two IgG molecules on a cell surface. C1q is a hexavalent molecule of MW approximately 460000. It adopts a structure likened to a bunch of tulips in which six collageneous stalk regions are connected to six globular head regions which contain the IgG binding site. The dimensions for C1q used here have longer 'arm' regions than those originally proposed from electron microscopic studies. There is evidence from physical measurements that the arms of C1q possess some flexibility although this appears less in C1 than isolated C1q.

Any flexibility of C1q may complement that of Fc in reducing steric requirements in the C1q-IgG interaction

binding sites and IgG has two sites for binding protein A (Figure 1.6). Hence if added to one another in the correct proportions they will form extended complexes and precipitate.

Complement

The classical pathway of complement is a cascade system generating a variety of potent biological molecules including anaphylatoxins and chemo-attractants and leading ultimately to lysis of antibody-coated cells. In health foreign cells will be the primary target. In disease host tissue may be attacked on a large scale, e.g. in autoimmune disorders. The pathway is triggered by the interaction of the first complement component, C1, with IgG in an associated state, i.e. coating a target cell or aggregated by antigen in an immune complex. The pathway is clearly not triggered by monomeric IgG which is at high concentration in the serum.

C1 is a complex of the complement components C1q, C1r and C1s. It is the subcomponent C1q which interacts with the C_H2 domain of IgG to initiate the enzymatic process of the pathway as shown in Figure 1.7. C1q is a molecule having the appearance of a 'bunch of tulips', and it is multivalent in its binding to IgG (Figure 1.7). This multivalency is probably the key to why complement is only triggered by IgG in an associated form. Binding of C1q to monomeric IgG is only weak, whereas binding to associated IgG and the consequent use of two or more of the tulip heads makes binding much tighter

and allows the activation process to proceed. Theories of activation which involve binding of antigen to the Fab arms of IgG and the induction of conformational changes which are passed down the molecule to Fc, thus affecting the interaction with C1q, are now rejected by most workers. It is particularly hard, for instance, to visualize a common conformational change being transmitted through the extended hinge of IgG3 (Figure 1.5) by the binding of a wide variety of different antigens at the extremities of the Fab arms.

The flexibility of Fc is complemented by some flexibility in the arms of the C1q molecule which may be important in complement triggering. Thus flexibility reduces the stringency of steric requirements when C1q binds to an array of IgG molecules. The precise location of the C1q binding site on IgG is a matter of controversy although there is general agreement about the importance of ionic interactions in the binding process.

In considering the triggering of complement it is important to distinguish C1q binding, C1 binding, C1 activation and whole complement activation. The most used assay is the measurement of the end-product of the whole complement cascade, i.e. cell lysis. The inability of an IgG to promote efficient lysis does not necessarily indicate an inability to bind C1q. A later stage may be implicated. For example, it appears that C1q binding is not always directly related to C1 activation and furthermore later components of complement, e.g. C4b, C3b, also interact with IgG. A further complication is that a small change in C1q binding affinity may, through the amplification nature of the complement cascade process, produce a large change in whole complement activation measured as cell lysis.

Comparison of the IgG subclasses provides the following view. All the subclasses in a monomeric state bind C1q with measurable affinity with the order of binding constants IgG3>IgG1>IgG2>IgG4. IgG3 and IgG1 activate C1 and whole complement efficiently. IgG2 is less efficient in complement activation. IgG4 does not appear to bind C1 and does not activate complement. Interestingly the Fc fragment of IgG4 (Fc4) does bind C1 and does activate complement implying that the C1 binding site is probably sterically obstructed in the parent IgG4 molecule by the Fab arms. This is consistent with the model for IgG4 proposed in Figure 1.5 where the Fab arms make a close approach to the C_H2 domains.

Cell Fc receptors

Receptors for the Fc region of IgG are found on a number of cell types and are associated with a variety of functions including phagocytosis (monocytes, macrophages, neutrophils), antibody-dependent cellular cytotoxicity (monocytes, macrophages), transport (trophoblasts) and possibly immunomodulation (lymphocytes). 'Fc receptor' is an operational term and does not imply the same molecular species is found on the different cell types. Indeed there is good evidence that at least two and probably more molecular species are involved. In terms of binding affinity of monomer IgG there are three broad categories: the tight binding monocyte receptor, the intermediate affinity trophoblast receptor and the weaker binding receptor found on neutrophils,

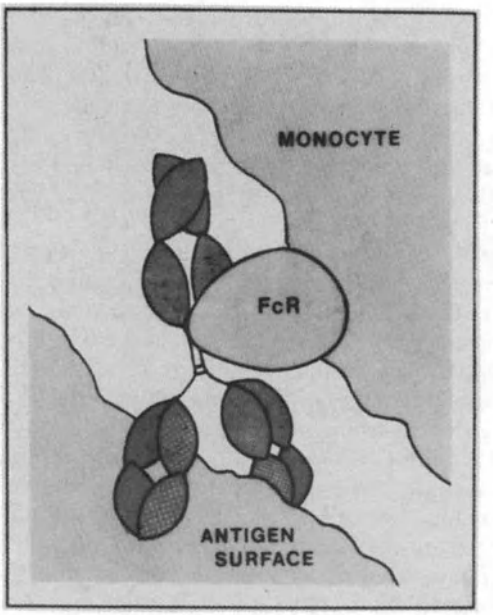


Figure 1.8 A model proposed for the interaction of IgG and human monocyte Fc receptor. The model is proposed primarily on the basis of a comparison of sequences of IgG of different species and subclass showing differing affinity for monocyte Fc receptor (Woof J. M. *et al.* [1986]. *Mol. Immunol.*, In press). The region of interaction on Fc is suggested to be that between the interheavy disulphides and the folded C_H2 domain (hinge-link) and possibly part of the N-terminal end of the C_H2 domain. The monocyte Fc receptor has a MW of approximately 70000 and is represented as a globular protein of appropriate size. An antigenic surface is included in the diagram to show the close approach of foreign cell and monocyte required by the interaction, although most studies have been carried out using unliganded monomeric IgG

platelets and a proportion of lymphocytes and macrophages. All these cell types exhibit a similar subclass specificity pattern in binding affinity, i.e. IgG1 ~ IgG3 > IgG4 >> IgG2. For most purposes IgG4 can be viewed as somewhat less effective than IgG1 and IgG3 with IgG2 seen as not interacting with Fc receptors.

Opinion has tended to favour the C_H3 domain of IgG as interacting with Fc receptors, although we have recently argued that in many instances the C_H2 domain may be a better candidate. For the monocyte we have proposed a site of interaction close to the hinge as depicted in Figure 1.8.

Other functions of IgG

The molecular mechanisms by which IgG is removed from the circulation are referred to as *clearance*. The number, nature and relative importance of the molecular species interacting with IgG in this process are unclear as is the nature of 'damaged' or altered IgG subject to clearance. However, it is apparent that the C_H2 domain is critical in control of clearance.

Fc-Fc interactions

These are believed important in the formation of immune precipitates, and a small peptide corresponding to the region connecting C_H2 and C_H3 domains has immunoregulatory properties.

Rheumatoid factors

Rheumatoid factors are autoantibodies directed against epitopes (antigenic determinants) expressed in the Fc region of IgG. Although most patients with rheumatoid arthritis show a heterogenous population of rheumatoid factors a common specificity has been detected in most rheumatoid sera. This antibody is of the IgM class and reacts with the C_H2–C_H3 interface region showing many features in common with protein A binding.

Membrane or surface IgG

A small percentage (about 1%) of peripheral blood B-lymphocytes express detectable levels of membrane-bound IgG on their cell surfaces. This IgG has extra heavy chain residues attached to the C_H3 domains which anchor the molecule to the membrane and indeed traverse it. The role of this surface IgG, as for surface Ig generally, is thought to be in binding antigen and thereby triggering the proliferation and differentiation of the cells on which it is expressed. Since one B-cell makes one specific immunoglobulin this is of course a key stage in a specific antibody response to a given antigen.

Proteolysis of IgG

The hinge region of IgG is generally sensitive to attack by proteases to yield large fragments. Papain gives two monovalent Fab and an Fc fragment, whereas pepsin gives a single divalent $(Fab')_2$ and a smaller pFc′ fragment (Figure 1.2). These patterns were crucial in allowing Rodney Porter in 1962 to propose the now well-accepted structure of IgG.

Proteolytic cleavage can be very useful in separating antigen and effector function binding, when this is required. For example, consider the case of a mouse monoclonal antibody raised against an antigen prevalent on a particular tumour cell where it was desired to use the antibody, perhaps labelled with a cytotoxic agent, in anti-tumour therapy. Some mouse IgG subclasses will bind to certain non-tumour human cells via Fc receptors and this is clearly inappropriate. It can, however, be circumvented by preparing the corresponding $(Fab')_2$ fragment. Similar considerations apply to the use of antibodies in histopathology.

STRUCTURE AND FUNCTION OF IgM

Membrane or surface IgM

IgM is a principle class of immunoglobulin on the surface of B-lymphocytes where it acts as an antigen receptor modulating the response of these

Light (L) chain
λ or κ chain
$L = V_L + C_L$ domains

Heavy (H) chain

$\gamma = V_H + C_H1 + \text{hinge} + C_H2 + C_H3$	**(IgG)**
$\mu = V_H + C_H1 + C_H2 + C_H3 + C_H4 + \text{tail piece}$	**(IgM)**
$\alpha = V_H + C_H1 + \text{hinge} + C_H2 + C_H3 + \text{tail piece}$	**(IgA)**
$\delta = V_H + C_H1 + \text{hinge} + C_H2 + C_H3 + \text{tail piece}$	**(IgD)**
$\epsilon = V_H + C_H1 + C_H2 + C_H3 + C_H4$	**(IgE)**

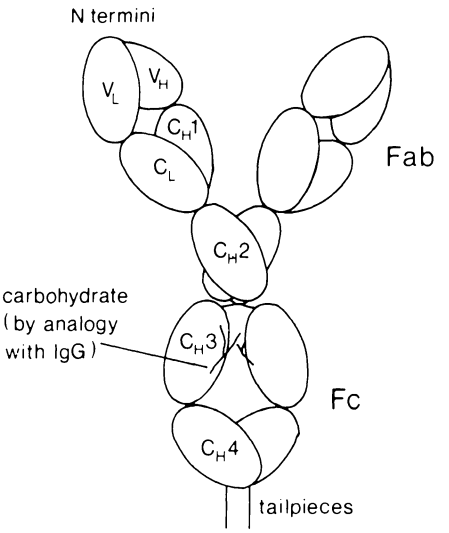
Figure 1.9 Domain composition of immunoglobulin chains

antibody-producing cells to antigenic stimulation. Membrane IgM has an extra C terminal heavy chain sequence to traverse the membrane and anchor the molecule as for IgG.

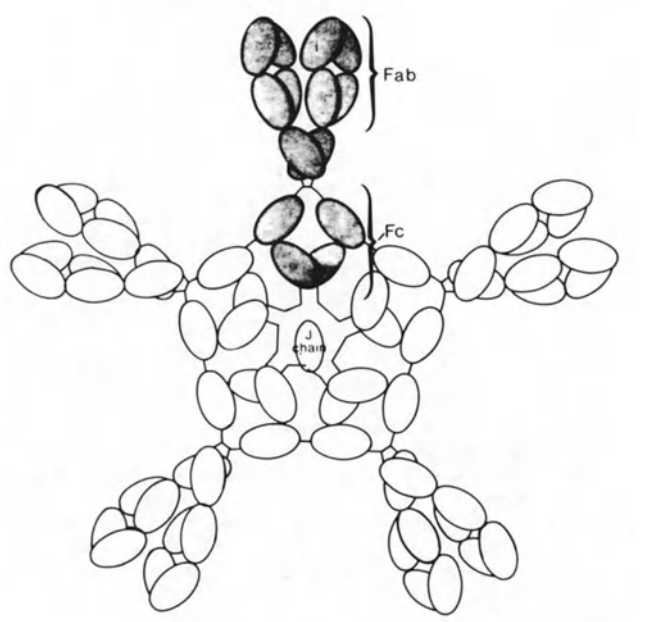
Structure of IgM

As for all antibodies, the structure of IgM is based on domains. In fact the monomeric unit of IgM is proposed to be similar to IgG except that the hinge region is replaced by two paired domains (C_H2) and there is a short tailpiece at the C terminal ends of the heavy chains (Figures 1.9, 1.10a). The C_H2 domains have been suggested to adopt a very loose structure, and they may function as a hinge in allowing molecular flexibility as in IgG.

IgM is found in the body not as a monomer but as a pentamer, and has been suggested as adopting a star shape as shown in Figure 1.10b. Polymerization of IgM is believed to occur through disulphide bridges linking the C_H3 domains of neighbouring monomer units, the tailpieces of neighbouring units and through the involvement of a separate molecule



(a)



(b)

Figure 1.10 The structure of IgM. (a) *The monomeric unit.* This schematic representation relies greatly on comparison of the amino acid sequence of IgM (μ chain) and IgG (γ chain) and extrapolation from known features of IgG structure. The Fab arms are as for IgG, the paired C_H2 domains replace the hinge and are thought to possess a loose structure, the C_H3 domains are suggested to resemble the C_H2 domains in IgG being unpaired with interposed carbohydrate and the C_H4 domains to resemble the paired C_H3 domains of IgG. A disulphide bridge connects the heavy chains between C_H2 and C_H3 domains. An additional feature is a tailpiece of 18 residues at the C termini of the heavy chains. The MW of the monomer is ~190000. Further carbohydrate chains found in the C_H1, C_H2, C_H3 and C_H4 domains with unclear structural/functional roles are not shown in this representation. (b) *The pentameric structure.* Monomer units are joined by disulphide bridges between C_H3 domains and between tailpieces. One pair of monomers are joined by a J chain. One monomer unit is shown shaded. The MW of the pentamer is 970000

known as the J chain. This molecule probably adopts a structure resembling an immunoglobulin domain and links together the tailpieces of two heavy chains (Figure 1.10b). The J chain is necessary for the correct assembly of the IgM pentamer, for the secretion of IgM from plasma cells and for the transcellular transport of IgM into the external secretions. It is also found associated with secretory IgA as discussed below.

Functions of IgM

Antigen binding

In theory IgM is decavalent for antigen, whilst in practice close approach of Fab arms on the same monomer unit mean it is maximally pentavalent. The binding of IgM to a particulate antigen appears to involve a conformational

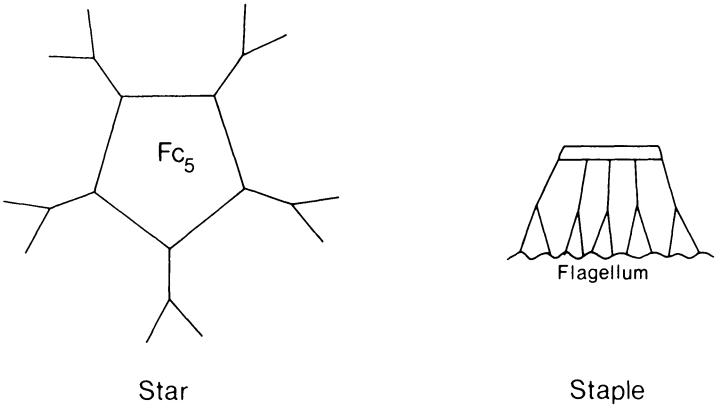


Figure 1.11 Structural forms of IgM. These representations are based on electron micrographs obtained by Dr A. Feinstein and co-workers of an uncomplexed IgM paraprotein ('star') and a specific sheep IgM bound to *Salmonella paratyphi* flagellum as antigen ('staple'). The star form corresponds to the IgM pentameric structure shown in Figure 1.10b with the Fc_5disc and $5F(ab')_2$ units in an approximately planar configuration. When bound to the flagellar antigen the $F(ab')_2$ arms are dislocated out of the plane of the Fc_5 disc to give a staple or 'crab-like' configuration. Complement C1 is activated on binding to complexed IgM (staple) but interacts only very weakly, yielding no significant activation, with uncomplexed IgM (star) implying an important role for the dislocation process in complement triggering

change from a star shape to a 'staple' form (Figure 1.11). The Fab arms are dislocated out of the plane of the molecule to leave a central Fc_5 disc.

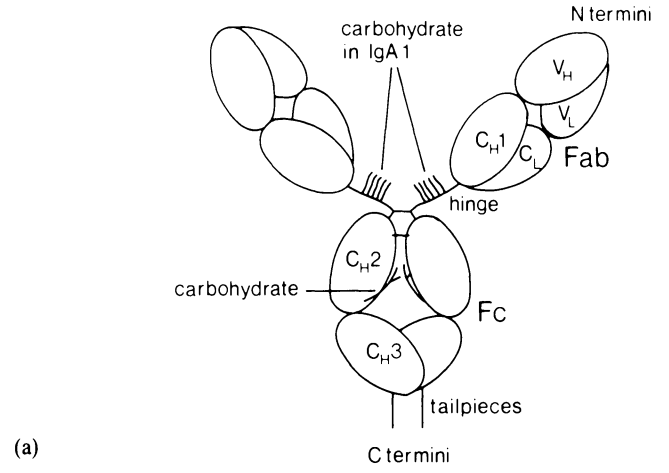
Complement activation

IgM is the antibody most efficient in activating the classical pathway of complement. IgG, normally monomeric, only activates complement when in an aggregated form, e.g. on an antigenic cell surface when the multivalent C1q can bind through multiple 'tulip' heads (Figure 1.7). IgM, normally pentameric, may therefore be expected to bind C1q even in the absence of antigen. It appears that this does not occur in the star form of IgM but that binding to antigen converts the molecule to a staple form which does bind C1q and activate complement.

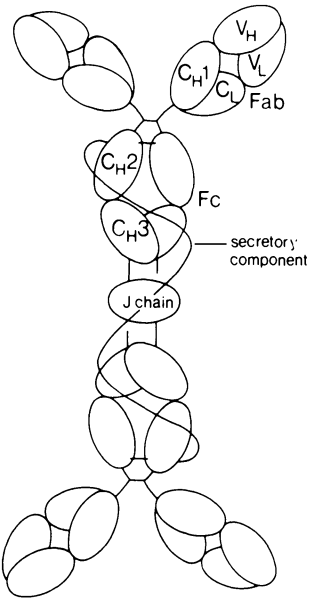
The two antibody molecules, therefore, solve the problem of activating complement when in contact with antigen but not when free in serum, by two different routes. Monomeric IgG relies on the aggregating ability of antigen. Polymeric IgM is already aggregated but in an inactive form and relies on antigen to dislocate the molecule to allow complement activation.

Interaction with cell receptors

There have been reports that monocytes possess Fc receptors for IgM but there is no general agreement about this phenomenon.



(a)



(b)

Figure 1.12 The structure of IgA. (a) *Serum IgA1*. The structure proposed closely resembles that of IgG on which it is based with the differences being an extended hinge region containing ten O-linked carbohydrate chains, a disulphide bridge between the C_H2 domains and C terminal tailpieces. Further carbohydrate chains are found on the C_H3 domains. In IgA2 the hinge is much shorter and the L chains are disulphide linked not to the H chain but to one another. The MW of IgA1 is 160000. (b) *Secretory dimeric IgA*. Electron micrographs indicate a double Y-shape. The J chain (MW ~15000) resembling an Ig domain is thought to link tailpieces of monomer IgA via disulphide bridges. The secretory component (MW ~70000) appears to link the two IgA molecules again via disulphide bridges - the representation shown is purely hypothetical as the structure of the secretory component is unknown

STRUCTURE AND FUNCTION OF IgA

Structure of serum IgA

More than 80% of serum IgA occurs as a monomer with the rest occurring as relatively small polymers (dimers, trimers, etc.). There are two subclasses of IgA, IgA1 and IgA2, with IgA1 being the predominant (80–90%) subclass in serum. The structure of both subclasses is thought to be very similar to that for IgG with the addition of a tailpiece (*cf* IgM) in the case of IgA as shown in Figure 1.12a. A principal difference between the subclasses is that IgA1 has a more extensive hinge region carrying five O-linked carbohydrate chains on each heavy chain. Also in most IgA2 molecules (allotype $A_{2m}(1)$) the L chains are not covalently linked to H chains but are disulphide bridged to one another thereby covalently linking the two Fab arms together.

Structure of secretory IgA

IgA is the predominant immunoglobulin in seromucous secretions such as saliva, tracheobronchial secretions, genito-urinary secretions, milk and colostrum where it is found in a dimeric form. Dimerization involves the J chain linked to two tailpieces as in IgM and another molecule known as the secretory component (SC). Secretory immunoglobulins are discussed in detail in Chapter 5. The structure of the dimer is not understood in detail, although electron microscopy studies indicate a double-Y shape as shown in Figure 1.12b.

In contrast to serum IgA, secretory IgA shows roughly equal proportions of the two subclasses.

Functions of IgA

Both subclasses of serum IgA in aggregated form activate the alternate pathway of complement but the structural basis for this function is not understood at the present time. Receptors for IgA have been reported on both peripheral blood neutrophils and monocytes.

STRUCTURE AND FUNCTION OF IgD

Several antibody responses include the production of specific IgD antibody, but the concentration of IgD in serum is very low. Since the antibody does not appear to activate any effector system a possible protective role for IgD in serum has not been identified. However, IgD, together with IgM, is a principle class of immunoglobulin found on the surface of B-cells where it too is thought to act as an antigen receptor modulating the response of these cells to antigenic stimulation.

The structure of IgD resembles that of IgG with an extended heavily glycosylated hinge region as shown in Figure 1.13. Membrane IgD possesses an extra heavy chain C terminal sequence which anchors the molecule to the B-cell surface in a manner very similar to that for other immunoglobulins.

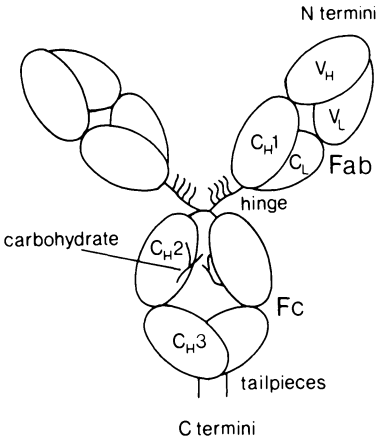


Figure 1.13 Structure of IgD. The structure of IgD proposed is similar to that of IgA1 with again a heavily glycosylated extended hinge region. Further carbohydrate chains are located in the C_H3 domains. The hinge region of free IgD is extremely susceptible to proteolytic attack. However, IgD is normally found anchored to the B-lymphocyte cell surface via extra C terminal heavy chain sequences where it is much more stable. The MW of IgD is ~184000

Serum IgD is extremely susceptible to enzyme cleavage in the hinge region whereas membrane IgD is much more stable.

STRUCTURE AND FUNCTION OF IgE

Structure of IgE

The structure of IgE is very similar to that of monomeric IgM with an extra domain, C_H2, replacing the hinge region as shown in Figure 1.14. As for IgM the C_H2 domains are suggested as adopting a relatively loose structure and may function effectively as a hinge. Unlike IgM, IgE shows no tendency to polymerize.

Function of IgE

The major effector function activities of IgE are related to its binding to cell Fc receptors specific for the Fc region of IgE. These are basically of two types: a tight binding receptor found on mast cells and basophils and a weaker binding receptor found on lymphocytes, monocytes and macrophages.

When monomeric IgE bound to Fc receptors on the surface of a mast cell is cross-linked by contact with antigen, degranulation of the mast cell occurs releasing chemical mediators. The function of Fc receptors for IgE on lymphocytes is probably related to regulation of IgE synthesis and those on monocytes and macrophages to IgE-mediated phagocytic reactions.

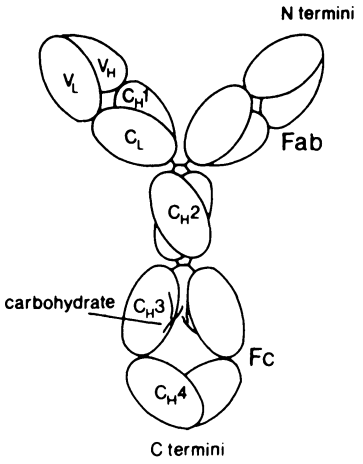


Figure 1.14 The structure of IgE. The proposed structure of IgE closely resembles that of the IgM monomer with an extra constant domain (C_H2) replacing the hinge region. Again the C_H2 domain has been suggested as adopting a loose structure allowing flexibility of the molecule. Additional carbohydrate chains are found on the C_H1, C_H2, and C_H3 domains. Lacking tailpieces the IgE molecule shows no tendency to polymerize. The MW of IgE is ~188000

SUMMARY

For purposes of ready reference Table 1.1 summarizes the physicochemical properties of the human immunoglobulins.

Table 1.1 Physicochemical properties of human immunoglobulins

	IgG					*IgA*				
Heavy chain	*IgG1* γ1	*IgG2* γ2	*IgG3* γ3	*IgG4* γ4	*IgM* μ	*IgA1* α1	*IgA2* α2	*sIgA* α1/2	*IgD* δ	*IgE* ε
Mean serum concentration ($mg ml^{-1}$)	9	3	1	0.5	1.5	3.0	0.5	0.05	0.03	5×10^{-5}
Basic structural form	mon	mon	mon	mon	pent	mon	mon	dim	mon	mon
Valency for antigen	2	2	2	2	5(10)	2	2	4	2	2
Molecular weight ($\times 10^{-3}$)	148	148	158	148	970	160	160	385	184	188
Number of heavy chain domains	4	4	4	4	5	4	4	4	4	5
Carbohydrate (%)	3	3	3	3	12	7 11	7 11	7 11	9 14	12

Acknowledgement

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2
Immunoglobulin Genetics

J. P. PANDEY

Immunoglobulin (Ig) molecules are exceptions to the 'one gene–one polypeptide chain' concept of genetics in that a single polypeptide chain is coded by more than one gene. Three gene families located on human chromosomes 14, 2, and 22 code for the Ig heavy (H) and κ and λ light (L) chains, respectively.

GENE REARRANGEMENTS

The multiple genetic elements encoding the H and L polypeptide chains of Ig molecules are physically separated from one another in the embryonic (germ line) DNA[1–4]. Prior to their expression, these scattered genetic elements must be correctly juxtaposed to form a single transcription unit. The variable (V) region of an Ig molecule which determines the specificity of the antibody is encoded by two gene segments for the κ and λ light chains and three segments for the H chains. The two gene segments required for the L chains are designated V and J [joining segment, because it joins the variable (V) and constant (C) region genes]; for H chains, an additional segment – D (for diversity, corresponding to the most diverse region of the H chains) – is required. The Ig gene, like most eukaryotic genes, is a mosaic structure: the gene is an alternating sequence of coding regions (exons) and intervening non-coding regions (introns)[5].

To form a functional L chain gene, the V gene to be expressed is translocated to the J gene and then the VJ segment is brought in proximity with the C_κ or C_λ gene. The DNA segment coding for VJC, along with the introns, is transcribed into heterogeneous nuclear RNA. The introns are subsequently removed by an RNA splicing mechanism, leaving mature messenger RNA to be translated into a L chain protein. For the H chain expression, an additional rearrangement is necessary since a third region – D – is incorporated in the product. A D segment is brought in direct contact with a J segment and this is followed by a second translocation event, yielding

a contiguous VDJ segment. Each translocation results in the deletion of some intervening DNA sequences. The VDJ complex is then translocated by successive recombination events to one of the C gene complexes - $C\mu$, $C\delta$, $C\gamma_3$, $C\gamma_1$, $C\alpha_1$, $C\gamma_2$, $C\gamma_4$, $C\epsilon$ or $C\alpha_2$. After transcription of this VDJC-DNA complex, the intervening introns are removed by an enzyme-splicing mechanism. IgM is the first Ig molecule produced from this RNA transcript.

The gene rearrangements discussed thus far occur before any antigenic stimulation. After antigenic stimulation, the H gene may undergo further rearrangements resulting in Ig class switching. This occurs by deletion of the DNA lying between J and the C gene to be expressed. The VDJ combination is not altered during class switching and therefore the antigen-binding specificity of the antibody molecule is not affected. Thus the same V gene can be associated with more than one C region gene. As a result, one can find Ig molecules of different classes (say IgM and IgG2) with identical V region amino acid sequences.

ANTIBODY DIVERSITY

The immune system is capable of synthesizing at least 10^8 antibody molecules with different specificities. How this vast diversity is generated has been one of the most interesting and intriguing problems in immunology. It is now clear that several mechanisms contribute to this phenomenon[1-4]

Multiplicity of V, D, and J genes

There is a substantial repertoire of germline genes. Thus, in humans, there are approximately 50 V and 5 J genes for the κ type L chains; for the λ type L chains, the numbers of V and J genes are approximately 25 and 6, respectively. The human H chain family has about 100 V, 10 D, and 6 J genes.

Combinatorial freedom of various genes

The VJ and VDJ genes can combine quite freely to form a functional L and H chain gene. Thus 100 V, 10 D, and 6 J genes can produce 6000 different H chain genes. Likewise, numerous L chain genes can be made from different V and J genes.

Junctional diversity

There is some flexibility in the joining ends of various gene segments, and this imprecision in joining can augment diversity of the amino acid sequences in L and H chains.

Insertion and deletion of nucleotides

During V D and D J joining, certain enzymes can add or delete a few nucleotides and thus contribute to diversity.

Combinatorial association of L and H chains

It appears that any L chain can combine with any H chain and thus further amplify antibody diversity. For instance, random association of 1000 L and 1000 H chains can produce 10^6 antibody molecules with unique specificities.

Somatic mutations

Mutation as a source of antibody diversity has been a controversial subject for many years. It is now clear that mutations occur in both L and H chain genes. Mutations appear to be restricted to the V gene segments and not to the C genes. Very little is known about the precise mechanism and time of occurrence of these mutations.

ANTIGENIC DETERMINANTS OF IMMUNOGLOBULIN MOLECULES

Isotypes

These determinants distinguish C regions of various classes and subclasses of H chains and the two types of L chains. Isotypes are not polymorphic, i.e. every individual of a given species normally possesses all isotypes unique to that species.

Allotypes

These are hereditary antigenic determinants on Ig polypeptide chains; they may differ between individuals of the same species, i.e. they are polymorphic, hence they can be used as genetic markers. Allotypic determinants follow Mendelian laws and are inherited as autosomal codominant genes. With one exception (discussed later), allotypes discovered thus far in humans are localized on the C region of $\gamma 1$, $\gamma 2$, $\gamma 3$, $\alpha 2$, ϵ H chains, and κ type L chains.

Idiotypes

These markers characterize the V region of Ig molecules. They are specific for a given antibody. Idiotypes are usually located in or near the antibody-combining site. Ig molecules having the same allotypes may have different idiotypic determinants.

HUMAN IMMUNOGLOBULIN ALLOTYPES

Allotypes associated with IgG1 are designated with the prefix Glm, and those on IgG2 and IgG3 are called G2m and G3m, respectively[4,6–8]. Currently testable Gm specificities for each subclass are given in Table 2.1. All Gm determinants are present on the Fc portion of IgG, with the exception of Glm(3) and Glm(17) which are on the Fd portion. Although the amino acid substitutions for the Fd markers are on the H chain, the presence of L chain is

Table 2.1 Allotypes of human IgG

Heavy chain subclass	*Numeric*	*Alphanumeric*
γ1	G1m (1)	(a)
	(2)	(x)
	(3)	(f)
	(17)	(z)
γ2	G2m (23)	(n)
γ3	G3m (5)	(b1)
	(6)	(c3)
	(10)	(b5)
	(11)	(b0)
	(13)	(b3)
	(14)	(b4)
	(15)	(s)
	(16)	(t)
	(21)	(g1)
	(24)	(c5)
	(26)	(u)
	(27)	(v)
	(28)	(g5)

required for their optimal expression. More than one determinant may be associated with a single polypeptide; Glm(1) and Glm(17) are usually present on the Fc and Fd portion of the same H chains.

The Gm antigens are transmitted as a group (haplotype) via codominant alleles. Each major race has a distinct array of haplotypes (Table 2.2).

Table 2.2 Gm haplotypes commonly present in major races of the world*

Race	*Haplotype*
Caucasoid	(1, 17; 21), (1, 2, 17; 21), (3; 5, 13, 14)
Negroid	(1, 17; 5, 13, 14), (1, 17; 5, 14), (1, 17; 5, 6) (1, 17; 5, 6, 14)
Mongoloid	(1, 17; 21), (1, 2, 17; 21), (1, 17; 13) (1, 3; 5, 13, 14)

*When tested for Gm 1, 2, 3, 17; 5, 6, 13, 14 and 21

Two allotypic determinants, which behave as alleles of one another, have been localized on the Fc portion of IgA2 molecules. They are designated A2m(1) and A2m(2). Some IgA deficient individuals possess anti-IgA antibodies directed against isotypic or allotypic determinants; these antibodies have been shown to cause severe anaphylactic reaction following transfusion of IgA incompatible whole blood or gammaglobulin.

Recently, the first genetic marker of IgE – Em(1) – was discovered by use of a monoclonal anti-IgE antibody.

The inheritance of the three allotypic determinants of κ type L chains – Km(1), Km(2), and Km(3) – can be explained by postulating three alleles: Km^1, $Km^{1,2}$, and Km^3; Km^1 allele is very rare. Although the amino acid substitutions for these markers are on the κ chains, association with H chains is required for their optimal expression.

A V region allotypic marker of Ig H chains has been described. Designated Hv(1), it is located in the framework of the V regions of μ, γ, α, and possibly also on δ and ε chains.

Haemagglutination-inhibition, using antisera derived from fortuitously immunized human donors, is the most widely used method for detecting human Ig allotypes. Radioimmunoassays for some markers have been developed and monoclonal antibodies for a few determinants have been produced.

ALLELIC AND ISOTYPIC EXCLUSION

An Ig-producing B-cell synthesizes only one H and one L chain. Only one of the H chain loci is expressed in a given plasma cell; furthermore, only one of the two alleles of that H chain locus, either paternal or maternal, but never both, is productive. This phenomenon has been termed allelic exclusion. Isotypic exclusion refers to the production of either κ or λ type L chain by the B-cell. The regulatory mechanisms for allelic and isotypic exclusion are poorly understood. Using the Gm system as an example, a given B-cell from an individual heterozygous for Glm^3/Glm^{17} will produce IgG molecules carrying either Glm(3) or Glm(17). Since this gene activation process is random, the serum from such an individual will of course have both allotypes secreted by different Ig producing cells.

IMMUNOGLOBULIN GENES AND BURKITT'S LYMPHOMA

Chromosome 8 of neoplastic cells from patients with Burkitt's lymphoma undergoes reciprocal translocation with either chromosome 14, 2, or 22. As mentioned above, the latter chromosomes carry the genes for H, κ, and λ chains, respectively. It appears that a proto-oncogene (c-myc) normally present on chromosome 8 is activated when juxtaposed to an Ig-locus[9]. The molecular mechanisms responsible for this malignant transformation are not very well understood.

ROLE OF IMMUNOGLOBULIN ALLOTYPES IN IMMUNE RESPONSE AND DISEASES

The polymorphic nature of Gm and Km loci, non-random combinations of various Gm allotypes, and a marked difference in their frequency in different ethnic groups point toward the existence of some kind of natural selective mechanism. One possible mechanism could be association of these allotypes

Table 2.3 Association of Gm and Km allotypes with immune responsiveness to various antigens

Antigen	*Study population*	*Gm and/or Km*
Flagellin	White	Gm 1, 17; 21
Insulin	Japanese	Gm 1, 2; 13, 15, 16, 21
Haemophilus influenzae type b	White	G2m(23)
Haemophilus influenzae type b	White and Black	Km(1)
Epstein–Barr virus in Burkitt's lymphoma	Black	Complex Gm–Km interaction
Osteosarcoma-associated antigen	White	Gm 1, 3, 17; 5, 13, 14, 21
Meningococcal group B polysaccharide	White	Km(1)
Type III group B streptococcal antigen	White	G2m(23) and Km(1) interaction
Native human type II collagen	White	Gm 1, 3; 5, 10, 11, 13, 21

Table 2.4 Association of Gm allotypes with various diseases

Disease	*Study population*	*Gm*
Susceptibility to systemic lupus erythematosus (SLE)	White	Gm 1, 3, 17; 5, 13, 21
Resistance to SLE	White	Gm 3; 5, 13
Susceptibility to SLE	Black	Gm 1, 17; 5, 6, 13
Susceptibility to alopecia areata	White	Gm 1, 2, 3, 17; 5, 13, 21
Resistance to alopecia areata	White	Gm 1, 3, 17; 5, 13, 21
Susceptibility to multiple sclerosis	White	Gm 1, 17; 21
Susceptibility to myasthenia gravis	Japanese	Gm 1, 2; 21
Susceptibility to Graves' disease	Japanese	Gm 1, 2; 21
Susceptibility to Hashimoto's disease	Japanese	Gm 1, 2; 21
Susceptibility to neuroblastoma	White	Gm 1, 3; 5
Susceptibility to lung cancer	Japanese	Gm 1, 2; 13, 15, 16, 21
Susceptibility to multiple myeloma	Black	G3m(28)
Susceptibility to familial polyposis coli	White	Gm 3; 5, 13
Susceptibility to malignant melanoma	White	G2m(2)

with resistance or susceptibility to certain diseases or immune response to certain pathogenic organisms. Several studies have presented evidence for these associations[6-8]; some of these are summarized in Tables 2.3 and 2.4.

It appears that in certain diseases, susceptibility or resistance is caused by the simultaneous involvement of Gm (on chromosome 14) and HLA (on chromosome 6) loci[10]. Examples in this category include autoimmune chronic active hepatitis, Graves' disease, systemic lupus erythematosus, juvenile rheumatoid arthritis, *Haemophilus influenzae* meningitis and epiglottitis. The mechanism for the interactive effect of these unlinked genetic systems is not understood.

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3
Immunoglobulin Isotype Diversity and its Functional Significance

L. HAMMARSTRÖM AND C. I. E. SMITH

PHYLOGENY OF IMMUNOGLOBULIN CLASSES

Cellular immune mechanisms have been observed in a number of primitive species but antibody molecules are found only in vertebrates. The most common class of immunoglobulin is IgM, which appears first in cyclostomes and persists through the evolutionary tree all the way to mammals. The IgM molecules exist in various forms (monomeric in sharks, tetrameric in bony fishes and pentameric in most other vertebrates) and may be found both in serum and to some extent in secretions. In some of the bony fishes a second immunoglobulin class appears (IgG), and subsequent evolution has led to the appearance of yet another class, IgA, in mammals and birds. IgD and IgE have thus far only been demonstrated in mammals. Further evolutionary diversity of IgG and IgA in higher mammals has resulted in there being four IgG subclasses and two IgA subclasses in man and, therefore, a total of nine different immunoglobulin classes or subclasses (otherwise known as isotypes). The different immunoglobulin isotypes have probably arisen due to environmental pressure and different specialized effector mechanisms have been assigned to each. Thus, the isotype diversity of antigen specific antibodies may profoundly influence their biological function.

Immunoglobulin light chain types (κ and λ) are differently organized in various species, and there is also a high degree of variability in the amounts of the respective types actually found in serum. In general, phylogenetically closely related species tend to have similar κ:γ ratios. In lower vertebrates, the majority of antibodies appear to contain λ light chains. In mammals such as mice, rats and rabbits, κ light chains predominate whereas in humans and baboons, the ratio is in the order of 2:1. In cats, dogs and sheep, λ light chains are predominantly found, and in mink and horse only λ light chains are normally expressed (for review see ref. 1).

GENERATION OF THE ANTIBODY REPERTOIRE

Individual immunoglobulin domains, which together form the antibody molecule, appear to have a common ancestral origin. Little is as yet known about the evolution of the antigen binding domain and no differences in affinity of antibodies from the various vertebrates have been found. It, therefore, seems that the genes coding for the variable portions of the antibody molecule, and thus the repertoire of available antibodies, developed early in immunoglobulin evolution.

Immunoglobulin gene rearrangement and subsequent expression can be divided into multiple separate stages. First, D_H–J_H joining occurs and in the second step, a V_H gene is physically linked to the D_H–J_H segment, thereby deleting intervening sequences. If the latter process is non-productive, a rearrangement of the germ line genes in the remaining heavy chain locus will follow. Yet another non-functional rearrangement is thought to result in the death of the B-cell. A productive V_H–D_H–J_H gene sequence on the other hand appears to inhibit further variable gene rearrangements.

The V_H–D_H–J_H segment encodes half of the antigen binding site of the molecule and the number of possible combinations (reviewed in Chapter 2) allows the formation of antibodies against any given antigen. Although the potential repertoire of antibodies is thereby virtually unlimited, the actual repertoire of antibodies at any given time, may, depending on the limited number of B-cells in the system, be decidedly smaller. The subsequent differentiation step involves the rearrangement of the V_κ and J_κ light chain gene segments to form a complete antigen binding site, a step which is suggested to require productive heavy chain gene rearrangement although certain exceptions have been described. The successful rearrangement of either of the V_κ–J_κ genes and the ensuing expression of a κ light chain protein and formation of cell surface immunoglobulin, terminates further light chain rearrangements. If, however, the κ chain rearrangements are non-productive, λ light chain genes will in turn be rearranged. Again, if only non-functional genes are formed, further differentiation is prevented and the cell dies. Due to the presence of the specific enhancer and promotor elements within the immunoglobulin gene segments, antibodies will only be produced in lymphoid cells.

The antibody diversity arises mainly from apparently random combinatorial mechanisms affecting a limited number of germ line antibody genes within the V, D and J loci. However, the functional antibody repertoire appears to follow a characteristic development programme where antibodies against selected protein antigens appear during early fetal life. Responsiveness against most other antigens, develop gradually with advancing age. There is reason to believe that this may be due to the localization of the utilized V gene where 3′ genes are being preferentially rearranged during early life, whereas more distal V gene families are sequentially used as the immune system matures. The number of V genes (within the different V gene loci) may differ quite substantially between various species and the limited number of V_λ genes in the mouse may put a severe constraint on the possible antibody repertoire. Recent experimental data support the notion that a large proportion of the

initially used V genes actually encode the binding site of antibodies directed against self-antigens. As a consequence, antibodies directed against autologous antigens may be formed and the detection of low levels of autoantibodies in serum is therefore a normal finding. With time, environmental modulation of the genetically encoded repertoire in the form of tolerance or T-cell or antibody mediated idiotypic network effects may significantly alter the expression of certain specificities, and in experimental animals, a number of systems have been described where antigen-specific silenced B-cell clones have been shown to exist, thereby revealing the true repertoire. In addition, somatic mutations may lead to the appearance of antibodies not encoded by the germ line genes.

Utilization of J segments within the immunoglobulin chain loci has been suggested to be a random event. Recently, however, evidence has been put forward which support the non-randomness of rearrangements of κ chain joining segments. This has been definitely shown in the mouse, where two out of the four functional J_κ segments are preferentially expressed, both at the RNA and protein level. The mechanism underlying this phenomenon is at present not understood.

IMMUNOGLOBULIN CLASSES AND SUBCLASSES

The heavy chain constant region genes for the five immunoglobulin classes are encoded on chromosome 14. These classes differ from each other in size, charge, chemical composition and carbohydrate content. Electrophoretically, the immunoglobulins are markedly heterogeneous and extend from the γ- to the α-fraction of normal serum.

Table 3.1 Biological properties of immunoglobulins

Function	*IgM*	*IgD*	*IgG1*	*IgG2*	*IgG3*	*IgG4*	*IgA1*	*IgA2*	*IgE*
Complement fixation (classical pathway)	+++		++	+	+++		—	—	
Complement fixation (alternative pathway)						[a]	+	+	
Placental transfer			+	+	+	+	—		—
Mean serum concentration (g/l)	1.5	0.05	6.6	2.4	0.7	0.3	1.5	0.2	0.00005
Halflife (days)	10	3	21	21	7	21	5	5	2

[a]Aggregated molecules may possibly activate the alternative pathway

Antibodies may be effective in a number of biological systems (Table 3.1). However, the two main effector mechanisms are complement induced cell lysis and Fc receptor mediated phagocytosis. In addition, antibodies may serve to directly neutralize biologically active compounds such as bacterial toxins, and they may also prevent attachment of micro-organisms to cells by agglutination or by binding to adhesion molecules such as pili.

Only IgM and IgG will effectively fix complement via the classical pathway, whereas IgA on the other hand may activate complement via the alternate pathway. This is of considerable interest in the elucidation of the pathogenetic mechanisms underlying immune complex induced cell damage. The majority of immune complexes are normally of the IgG class (mostly IgG1 and IgG3) which may fix complement and subsequently lead to innocent bystander lysis which is thought to be one of the main mechanisms in immune complex induced kidney disease. IgA containing immune complexes may be seen in Henoch–Schönlein purpura. In both instances, the complement activation will allow the formation of a membrane attack complex which will lead to pore formation, leakage of the cytoplasmic contents and ultimately cell death.

Table 3.2 Interaction of immunoglobulins with Fc receptors

Cell type	*IgM*	*IgD*	*IgG1*	*IgG2*	*IgG3*	*IgG4*	*IgA1*	*IgA2*	*IgE*
Neutrophil granulocytes	–	–	+	–	++	+	+	+	–
Eosinophil granulocytes[a]	–	–	+	?[a]	+	?[a]	–	–	+
Basophil granulocytes	–	–	–	–	–	–	–	–	+++
Lymphocytes[b]	+	+	+	+	+	+	+	+	+
Mast cells	–	–	–	–	–	–	–	–	+++
Platelets	–	–	+	+	+	+	–	–	?[a]
Macrophages	–	–	+	–	+	–	–	–	+

[a]Insufficient data

[b]Binding is mostly restricted to subpopulations of these cells

The other main mechanism by which antibodies contribute to the immune defence is the binding of immune complexes to Fc receptor bearing cells such as granulocytes and monocytes/macrophages. The complexes are subsequently phagocytosed and the engulfed material is then degraded. Fc receptors have also been described on a number of additional effector cells including lymphocytes and mast cells (Table 3.2). In the former cells. Fc receptors for all major immunoglobulin classes have been described and these cells are capable of expressing multiple isotype-specific Fc receptors simultaneously. The presence of Fc receptors for different classes were previously suggested to delineate functionally distinct lymphocyte subpopulations. However, recent data do not support such a relationship and the biological relevance of the receptors is still unknown, although a possible participation in facilitating or enhancing differentiation has been suggested. A wide range of properties and molecular weights have been reported for Fc receptors from different sources, and there is evidence to suggest that these discrepancies result from the existence of fundamentally different receptors on separate cell populations. In addition to their established role on phagocytosing cells suggested above, Fc receptors are essential in the antibody dependent cell-mediated cytotoxicity exerted by K (killer) cells. Receptors for a number of complement factors have also been found on granulocytes, monocytes/macrophages and B lymphocytes. By trapping

antigens to which complement factors have bound, micro-organisms may be effectively removed even prior to the completion of the complement cascade.

Antibodies may also regulate an ongoing immune response by a feedback mechanism, possibly simply by removing the available antigen or by more intricate means such as interaction via Fc receptors, by which subsequent antibody production is prevented. The different immunoglobulin classes clearly differ with respect to their suppressive capacity and most often, IgG has been found to be the most potent antibody molecule in this respect. Antibodies may also interfere in the immune network by interacting with idiotypic determinants on other cell surface bound antibody molecules. It has previously been claimed, at least in experimental animal systems, that the class and subclass of the anti-idiotypic antibody is of major importance in determining whether binding will result in a stimulatory or inhibitory signal to the B-cell.

IgM is a pentameric molecule, chiefly produced in the bone-marrow, which accounts for roughly 10% of the total immunoglobulin pool. Due to its size, the molecule is largely confined to the intravascular pool. The IgM molecule has 10 antigen combining sites but often uses only five of these which has been attributed to the lack of flexibility of the Fab_2 arms (due to absence of the hinge region). The biological properties of the IgM molecule include the fixation of complement (Table 3.1) but its pentameric structure also makes it an ideal antibody for agglutination.

IgM is the main immunoglobulin class which is synthesized by the fetus. Usually, little immunoglobulin is synthesized *in utero*, but exposure to antigen in the form of intrauterine infection, may lead to synthesis of IgM antibodies. In such circumstances, presence of specific IgM antibodies in the serum of the newborn is indicative of infection during pregnancy and, therefore, has considerable diagnostic value.

IgG, which is a functionally divalent molecule, is the main immunoglobulin class in normal human serum, accounting for 70–75% of the total immunoglobulin pool. Plasma cells contained within the bone-marrow are normally the main source of IgG and the antibodies are distributed evenly between the intra- and extravascular pools. One important property of the IgG class is its ability to cross the placenta during pregnancy and although some IgG may be synthesized by the fetus, the vast majority of IgG molecules are of maternal origin at birth. IgG can be subdivided into four highly homologous subclasses where the corresponding heavy chain constant region genes are encoded by separate genes; the gene order being μ, δ, γ_3, γ_1, α_1, γ_2, γ_4, ϵ and α_2. Serum levels of the four IgG subclasses appear to be independently regulated during ontogeny and adult levels of IgG3 and IgG1 are reached at an early age, whereas adult levels of IgG2 and IgG4 are not reached until adolescence. This may suggest a sequential utilization of the immunoglobulin heavy chain constant region in genes where 3′ genes are expressed late in ontogeny. This hypothesis is also supported by the known association between the serum levels of closely linked genes such as IgG3 and IgG1 on the one hand and IgG2 and IgG4 on the other, a correlation which is found both in sera from normal donors and in patients with various forms of immunoglobulin subclass deficiencies. A similar pattern of the maturation of

the various IgG subclasses has also been described in patients reconstituted with allogeneic bone marrow cells due to immunodeficiency or leukemias. Total serum levels of the various IgG subclasses do not appear to be markedly influenced by sex or HLA although somewhat higher levels of IgG4 have been found in men. Gm allotypes may, however, have a profound influence on serum levels. IgG1 normally constitutes 66% of the total IgG. IgG2 on the other hand constitutes only 24% and IgG3 and IgG4 contribute 7 and 3% respectively to the total IgG. Biological properties differ markedly between the subclasses (Table 3.1) (for review see Ref. 2) and only IgG1 and IgG3 effectively fix complement. A number of additional important biological differences such as Fc receptor binding and biological half life have previously been noted. IgG4, which, in spite of its divalent structure is functionally univalent, has been suggested to be able to bind to mast cells and basophils and may thus serve as a sensitizing antibody in allergic responses. Furthermore, the mean affinity of the IgG4 antibodies, at least against an antigen such as tetanus toxoid has been suggested to be markedly lower than the corresponding IgG1 antibodies which may be of functional importance with regard to toxin neutralizing effects.

IgA is present not only in serum but also in a variety of body fluids such as saliva, intestinal and bronchial mucus, nasal secretions, sweat, breast milk and colostrum. Serum IgA constitutes roughly 10–20% of the total immunoglobulin pool. A majority of these antibodies are in monomeric form but some polymeric IgA may be seen where the units are held together by sulphide bridges and by the J chain. No functional biological role has as yet with certainty been ascribed to the serum IgA. Secretory IgA consists of two IgA monomers, one J chain, and one secretory component, a complex which is far more resistant to proteolysis than the native IgA molecule. The two monomers can either be stacked on top of each other or joined tail to tail. The secretory IgA is synthesized by plasma cells lining mucosal surfaces and the IgA is then passed through epithelial cells where they acquire the secretory component. The J chain is added to IgA already in the plasma cell. The complete molecule is secreted into the body fluids. In contrast to serum IgA, secretory IgA is of major importance in the immune defence, and antibodies are produced locally at mucosal surfaces against a variety of bacterial and viral antigens. If cells producing secretory IgA are taken into account, IgA may in fact be the major immunoglobulin class in humans. IgA molecules do not activate the classical complement pathway. However, aggregated IgA antibodies may activate the alternate complement pathway and the antibodies may also be active in promoting phagocytosis via Fc receptor binding.

IgA is subdivided into two subclasses, IgA1 and IgA2, encoded by separate constant region genes. The two subclasses differ mainly with regard to methods of heavy chain pairing and carbohydrate composition. In serum, IgA1 is highly predominant. In secretions, however, the two subclasses are roughly equally distributed with a proportional increase in IgA2 distally in the gastrointestinal tract. Apart from the sensitivity of IgA1 to bacterial proteases, no major differences in biological functions have been observed for the two subclasses.

IgD is normally present in serum only in low levels. High rate secreting cells

are rare and the majority of serum IgD antibodies appear to stem from cell surface shedding of the molecules. IgD is co-expressed with IgM on the surface of a majority of mature B-lymphocytes. No biological function has as yet been clearly demonstrated for serum IgD, although a role in lymphocyte differentiation and/or tolerization has previously been suggested for the membrane bound form of IgD.

IgE is an immunoglobulin class that is normally only present in trace amounts in serum. The main physiological function of IgE mediated responses is thought to be the defence against parasite infections, and high serum levels are seen during the course of some of these diseases. The killing mechanism is mediated via cells such as eosinophils, mast cells and basophils. The IgE antibodies are bound by Fc receptors on the cell surface of the effector cells and although the serum half life of the IgE is only 2–3 days, mast cell bound IgE may remain for weeks or even months. Binding of antigen (or in experimental systems by addition of anti-IgE antibodies) crosslinks the receptors and causes the release of chemical mediators which are stored in the granules. These mediators will induce a complex tissue response which is ultimately aimed at the killing of the parasite. Another mechanism of killing is induced by the binding of specific IgE antibody to the parasites to which Fc_ϵ receptor bearing eosinophils will attach and lyse the cells. The presence of IgE may not only be advantageous but may also give rise to type 1 hypersensitivity reactions such as conjunctivitis, rhinitis and asthma, which are characterized by allergic symptoms immediately following contact with the antigen (allergen).

The serum levels of the light chain types would theoretically develop according to the general pattern of DNA rearrangements which start with a functional rearrangement of the κ light chain genes. However, in about 10% of human cord blood sera a skewed balance, with an increment in the λ light chain type, has previously been noted. This particular imbalance becomes more marked in infants during the first months of life and between the 4th and 12th months, a further shift in favour of the λ light chain is the rule. With advancing age the pattern stabilizes toward the normal 2:1 κ/λ ratio which is normally observed in adults. The light chain distribution is similar in all immunoglobulin classes with the exception of an almost exclusive λ light chain type preference in IgD secreting tumours (in contrast to other malignant immunoglobulin producing cells).

THE ANTIBODY RESPONSE

The exposure of an individual to any given foreign antigen will normally evoke an immune response. The type of response is determined by multiple factors such as the chemical nature of the antigen, the amount of antigen that the individual is exposed to, the route of immunization and the immune status of the individual.

IgA producing cells normally line mucosal surfaces, and localized, superficial mucosal infections may thus induce an immune response restricted to secretory IgA. Specific antibody levels may in these cases not even be

noticeable in serum. More pronounced gastrointestinal or respiratory tract infections will result in a systemic immune response dominated by IgA. The same response pattern is seen after oral vaccination which induces a local immune response in the gastrointestinal tract where protective levels may again be reached in spite of undetectable serum antibody levels. Recent observations, however, suggest that protection against mucosal infections, particularly in the lower respiratory tract, could also be due to serum IgG antibodies.

Following a primary challenge with an antigen that is introduced into the body there is an initial lag phase where no antibody can be detected. This phase is followed by a rapid increase in circulating antibody levels which are mainly of the IgM class. These antibodies normally display a relatively low affinity. The initial phases are followed somewhat later by a switch to IgG antibodies retaining the original specificity. These antibodies may disappear or remain at low levels for long periods of time. The IgM antibodies produced in the primary response will effectively agglutinate micro-organisms and consequently induce a complement mediated lysis via the classical pathway. A secondary antigenic challenge normally results in a rapid increase in antibody levels mainly of the IgG class. Some IgA antibodies may also be produced. It is still not established with certainty whether a given cell will only switch from IgM to IgG or IgA or whether consecutive switches from IgG to IgA may occur. Most data would appear to support the concept of direct switches from IgM to IgG or IgA but switches from IgG to IgA have been observed in some malignant B-cell clones. The antibodies of the secondary response normally show a high affinity for the antigen. The magnitude of the secondary response is influenced by a number of factors including feedback suppression by antibodies which combine with the antigen and thus compete with the antigen receptors on the responding B-cells. Other regulatory mechanisms include the Fc mediated effects of immune complexes formed during the immune response and also idiotype–anti-idiotype interactions. Compartmentalization of cells producing the various immunoglobulin classes or subclasses has recently been suggested, based both on immunofluorescence staining of tissues and analysis of secreted immunloglobulin, but its full influence of antibody responses has yet to be determined.

Selected protein antigens (allergens) may also induce IgE production. Most allergens are derived from plants or animals whereas viruses and most bacteria apparently cannot induce allergy, the possible exception being the IgE antibodies against staphylococcal antigens found in the hyper-IgE syndrome. The majority of allergens have a relatively low molecular weight and even simple haptens such as dinitrophenyl are capable of inducing high IgE titres when administered on an appropriate carrier. Most antigens which tend to induce an antibody response of the three major immunoglobulin classes induce a poor or non-existent IgE response, and a combination of several properties is probably required to make a protein allergenic. In situations where the same molecule will induce both IgG and IgE, glutaraldehyde treatment may often abolish IgE formation while retaining its IgG stimulatory capacity. This principle may be used in immunotherapy (hyposensitization) where a predominant IgE response is converted to an IgG

response, thereby inducing putatively blocking antibodies. According to prevailing theories, this will prevent the antigen from reaching the IgE on mast cells and thus inhibit an allergic reaction. Apparently the dose of antigen is important in provoking an immune response, and IgE antibody formation is favoured by repeated exposure to low antigenic concentrations. This particular reactivity pattern is normally seen only in genetically predisposed individuals, and genes within the major histocompatibility complex may influence these responses.

B-lymphocytes respond to various thymus-dependent and thymus-independent antigens. In the former case, macrophage and T-helper cell collaboration are needed to induce the response. Protein antigens are mainly thymus-dependent antigens, whereas carbohydrate antigens may induce antibodies without the aid of collaborating T-cells. A secondary challenge of B-cells with a thymus-independent antigen will normally not induce the switch to IgG and/or IgA production characteristic of thymus-dependent antigens. It has previously been suggested that B-cells responding to thymus-dependent and thymus-independent antigens respectively, belong to two distinct lineages. T-helper cells secrete factors which influence the switch pattern of immunoglobulin producing cells and a number of growth factors and differentiation factors have been described. Some of the genes coding for these factors have already been identified and sequenced.

IMBALANCES OF IMMUNOGLOBULIN SUBCLASSES

The proportions of the various IgG and IgA subclasses in serum is usually quite stable, but imbalances may be found in immunodeficiency or during ongoing infections. Chronic infections may thus often lead to elevated levels of IgG1 and possibly also IgG3 and IgG4. In patients with various forms of atopic diseases, increased levels of IgG4 are often found. Patients with recurrent or chronic infections have often been shown to have reduced levels of IgG2 and/or IgG4. No selective increase in either of the two IgA subclasses has been observed to date, and although IgA deficient patients may suffer from bacterial infections, no major increase in disease susceptibility has been noted in patients lacking either of the IgA subclasses.

SUBCLASS RESTRICTION OF SPECIFIC ANTIBODIES

The IgG and IgA subclass distribution of specific antibodies profoundly influence the biological consequences of any given immune response due to the various effector functions evoked by the different antibodies. Antibodies against protein antigens will mainly be of the IgG1 subclass in man but low amounts of IgG3 and IgG4 may also be formed (for review see Ref. 3). However, repeated exposure to the antigens will lead to the formation of appreciable amounts of specific IgG3 or IgG4 antibodies and hyperimmunization may result in an IgG3 or IgG4 restricted response. Antibodies against carbohydrate antigens on the other hand are usually found only in the IgG2

subclass in adult individuals (for review see Ref. 3). Protein antigens also induce substantial amounts of IgA1 antibodies and there appears to be a sequential appearance of IgG1-IgA1 and IgG4 antibodies. IgA antibodies against carbohydrate antigens may in hyperimmune sera be of the IgA2 subclass but usually the majority of antibodies are of the IgA1 subclass. In infants antibodies against carbohydrate antigens are restricted to IgG1 with a gradual shift towards IgG2 as the immune system matures. Adult levels of IgG2 antibodies against carbohydrate antigens are not reached until adolescence, an increase which is parallelled by the rise in total serum IgG2.

RESPONSE TO MICROBIAL ANTIGENS

Antibodies against microbial products arise as a consequence of antigenic exposure and will in most cases be due to natural infection. However, vaccination against a number of important pathogens is widely used and has contributed substantially to eradicate or drastically reduce a number of infectious diseases.

Immunity to most infections is passed from mothers to their offspring and can be passively conferred by serum antibodies or purified IgG. This is clearly indicative of the importance of an intact humoral immune system in the defense against infections. Antibodies may also prevent re-infection and protective serum antibody levels have been determined in a number of systems.

Children respond to both protein and carbohydrate antigens by the production of specific IgG1 antibodies. There are also indications that young children respond to selected protein antigens (such as viral antigens) by the production of quite significant amounts of IgG3 antibodies. The appearance of the latter antibodies have been suggested to carry prognostic significance in cases of potentially lethal viral infections. This is somewhat surprising since IgG3 only constitutes a minority of the total serum IgG and may indicate that IgG3 antibodies in fact have a highly specialized biological function. The early appearance of responsiveness to protein antigens also has practical implications since vaccinations (using protein antigens) may be initiated early in infancy. Children do, however, respond poorly to carbohydrate antigens. The antibodies formed are mainly of the IgG1 subclass and are gradually replaced with advancing age by antigen specific IgG2 antibodies. It has been shown, based on animal experimental models, that the affinity for antigen differs within the various subclasses and thus, that different V gene families are expressed in the different subclasses (for review see Ref. 2). This would have a profound influence on the biological efficiency of the antibodies produced. Thus, it is possible that the IgG1 antibodies formed against carbohydrate antigens in children may be less efficient in removal of the pathogen. This suggestion is partially substantiated by previous findings on the presence of marked levels of IgG1 antibodies against bacterial polysaccharides even in infection prone children. To improve the low responsiveness to carbohydrate antigens used for vaccination (such as pneumococcal or *Haemophilus influenzae* polysaccharides) a strategy has

been adopted of conjugating the polysaccharide to a protein antigen to which the children respond. The IgG subclass distribution of these antibodies is, however, as yet not known but may, as suggested above, be of clinical importance. Usually there is a strong correlation between the amount of total serum IgG2 and the subsequent response to vaccination with the polysaccharides in children. The presence of the G2m23 allotype marker (normally associated with an increased total level of serum IgG2) has also been associated with an increased response to vaccination with pneumococcal and *Haemophilus influenzae* polysaccharides in children.

In adults, protein antigens mainly give rise to IgG1 antibodies, regardless of whether these antibodies are induced by natural infection or vaccination (for review see Ref. 4). In addition, repeated or prolonged exposure to a given antigen will often induce quite marked levels of IgG4. This has previously been taken as support for the hypothesis that hyperimmunization with a protein antigen will ultimately result in an IgG4 restricted response, and that the IgG subclass pattern of any given antibody will thus change with time. Presence of IgG4 antibodies is also normally seen against selected antigens such as tetanus toxin, diphtheria toxin, alpha toxin from *Staphylococcus aureus* and M-protein from *Staphylococcus pyogenes* (for review see Ref. 3). However, antibodies against protein antigens from latent viruses such as the herpes virus group may be restricted to IgG3 (in addition to the dominant subclass: IgG1). Furthermore, chronic infections with trypanosoma, malaria or spirochetes induce specific antibodies which, again, are restricted to the IgG3 subclass (in addition to IgG1). This may suggest that the antibody repertoire that can be expressed in a given subclass is limited and that certain V genes may only, or at least preferentially, combine with a limited number of immunoglobulin constant region genes. This proposal would appear to be in contrast to recent findings in the mouse where V genes in the beta chain locus of the T-cell antigen-specific receptor can combine with either of the two available constant region genes. However, although of similar evolutionary origin, B- and T-cells may have adopted different strategies for the generation of their receptor repertoires. The alternative approach is of course to suggest that the induction of the antibodies (via T-cell help) differ, and that particular sets of T-helper cells will produce factors that switch B-cells to either IgG3 or IgG4 production. This could either be because T-cells distinguish between various types of protein antigens and subsequently induce factors initiating this switch to IgG3 or IgG4, or that two distinct subsets of T-helper cells exist, predestined to produce either an IgG3 switch factor or an IgG4 switch factor. In the latter case we would predict that the T-cells would also be able to effectively induce IgE production, possibly by the same lymphokine, and the antigen receptor repertoire in the two subsets of T-cells must then be anticipated to differ markedly.

Antibodies to carbohydrate antigens are mainly restricted to IgG2 in adults. Theoretically, one would expect that high levels of specific IgM antibodies would also be present. However, this is most often not found and may be due to the relatively short halflife of IgM, or that B-cells responding to thymus-independent antigens in fact ultimately switch to IgG2 production To date, the IgG subclass distribution pattern of antibodies against a wide

variety of polysaccharides has been tested (for review see Ref. 3). Although these antibodies, as a rule, are restricted to IgG2, a number of interesting exceptions do occur. Thus, antibodies against selected Klebsiella polysaccharides may be almost exclusively restricted to IgG1. In addition, certain pneumococcal polysaccharides and meningococcal polysaccharides may, in addition to IgG2, give rise to high levels of anitgen-specific IgG1 antibodies. These findings are of importance for the understanding of the genomic organization of V_H genes and acquisition of the antibody repertoire during ontogeny. B-cells appear to start rearranging the V_H genes which are mostly 3′ to the D_H genes, and the sequential appearance of anti-protein antibodies in experimental animal systems supports this notion. The late appearance of antibodies against carbohydrate antigens would, therefore, seem to indicate that genes coding for antibodies giving rise to most anti-carbohydrate specificities are located 5′ to the genes coding for the V_H genes, resulting in antibodies reacting against protein antigens (for model see Figure 3.1). In the mouse there are seven distinct V_H gene families each encoding between 2–40 V_H genes. The organization of the corresponding locus in man is as yet not known, but based on serological data the existence of at least four distinct families has been suggested. The schematic view depicted in Figure 3.1 would thus suggest that most V_H genes used for the formation of antibodies with anti-protein specificity, would be encoded in the two most 3′ families. However, interspersed with these genes may be genes coding for selected carbohydrate antigens such as Klebsiella and pneumococcal polysaccharides. Since only the most 3′ V_H families would be accessable for utilization during

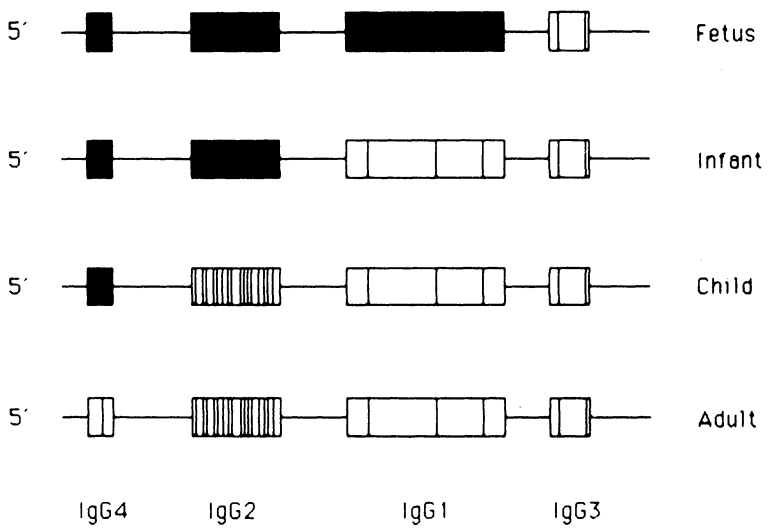

Figure 3.1 The maturation of the antibody repertoire. The V_H gene families are depicted as boxes containing numbers of genes coding for the variable part of immunoglobulins. Genes giving rise to antibodies against carbohydrate antigens are indicated as bars. Black boxes represent inaccessable V_H gene families. The restricted use of one V_H gene family by the respective subclass is suggested at the bottom of the figure

infancy, an ordered gene sequence would be of definite biological importance, inasmuch as even small children would be expected to respond to these antigens. This is in fact also seen and young children do respond to vaccination with pneumococcal polysaccharide type 3. In addition to the above-mentioned specificities, antibodies against a number of different blood group carbohydrate antigens have also been shown to be mainly or even exclusively of the IgG1 and IgG3 subclass. The genes encoding these specificities would therefore also be expected to lie within the two most 3′ V_H gene families.

AUTOANTIBODIES

Autoantibodies may normally be found in any individual since, due to the completeness of the antibody repertoire, the putatively random rearrangement of V, D and J segments would be expected to generate antibodies with reactivity against self-antigens. The production of a large amount of antibodies, however, probably requires breaking of the normally existing T-cell tolerance. Most autoantigens are of protein character and would thus require T-cell help in order to effectively induce antibody production, a fact which also influences the immunoglobulin class and subclass distribution of the antibodies.

Immunization against self-antigens appears to follow the general pattern of any protein antigen, i.e. initially IgM antibodies will be formed subsequently followed by a shift to IgG and eventually also IgA and IgE. The later phases of the autoimmune response/disease are characterized by an increasing level of specific IgG antibodies which eventually reach a plateau. The switching to IgG has, at least in experimental animal systems, been suggested to be of pathological significance as immune complexes deposited in the kidney leading to renal disease are not found until after the switch to IgG production has occurred. The IgG subclass distribution of autoantibodies is given in

Table 3.3 Suggested IgG subclass distribution of autoantibodies[a]

Antigen	*IgG1*	*IgG2*	*IgG3*	*IgG4*
Thyroglobulin	+	—	—	++
C3	++	—	+	—
RNP	++	—	—	+
DNA	++	—	+	—
Red blood cells[b]	++	—	++	+
Thrombocytes	++	—	++	+
Acetylcholine receptor	++	—	++	—
IgA	++	—	—	+
IgG	+	—	+	++
IgE				+

[a]Results are compiled from a number of different sources and are expressed as suggested by data in the respective papers. Negative –; positive +; strongly positive ++
[b]Multiple different antigens

Table 3.3. As can be seen, the majority of antibodies are of the IgG1 subclass. Again, there are implications that a given antigen may induce primarily IgG3 or IgG4 antibodies. The latter might possibly be a result from different routes of immunization as has been suggested in studies on vaccination with viral antigens such as rabies and hepatitis B. However, it may also reflect, as suggested above, the organization of the V_H genes.

Autoantigens thus resemble heterologous protein antigens with regard to the IgG subclass pattern induced. The complement fixing capacity by IgG1 and IgG3 is, therefore, of great pathological interest. Eventually, a shift to non-complement fixing subclasses such as IgG4 could reduce or even prevent binding of IgG1 and IgG3 antibodies to their target antigens, thus possibly preventing autoimmune disease. This has indeed been described in cases of haemolytic anemia where the spontaneous shift to IgG2 and IgG4 antibodies against red blood cells led to a remission of the disease. In view of the previous line of reasoning on the restricted combinatorial efficiency of certain V_H gene families and immunoglobulin subclass constant region genes, this is indeed an unexpected finding. One possibility would be the autoimmune response against two distinct epitopes where the induction of the non-complement fixing antibodies would block access to the primary antigen (eliciting IgG1 and IgG3 antibodies) thereby inhibiting the disease process. The shift to IgG2/IgG4 antibodies has also been suggested as leading to an improved clinical status in patients with other forms of autoimmune diseases, such as systemic lupus erythematosus induced nephritis. Therapeutically, the induction of this shift may, therefore, be of considerable potential interest in the future.

Autoantibodies against coagulation factors may occur spontaneously but may also arise as a consequence of coagulation factor therapy. In a majority of cases, these antibodies will be of the IgG1 and IgG4 subclasses and functionally impair the activation of coagulation factors. These antibodies were initially considered to be almost exclusively of the IgG4 subclass. Later studies, however, (Table 3.4) have suggested that antibodies may indeed be found in all subclasses although there is still a predominance of IgG4. In view of the protein nature of the coagulation factors and considering the prolonged exposure to the antigen, this subclass pattern is actually quite predictable.

Table 3.4 The IgG subclass distribution of antibodies against coagulation factors[a]

Factor	*IgG1*	*IgG2*	*IgG3*	*IgG4*
V	++	+	–	++[b]
VIII	+	—	+	++
IX	–	—	–	++
XIII[c]	+	+	–	+

[a]Results are compiled from a number of different sources and are expressed as suggested by data in the respective papers
Negative –; positive +; strongly positive ++
[b]Monoclonal IgG4/λ anti-V in one patient
[c]Limited number of reports

ROLE OF IgG4 IN RESPONSE TO ALLERGENS

Allergens are exclusively protein antigens and appear to induce the formation of IgE antibodies in genetically predetermined individuals. The response is clearly T-helper cell-dependent and has been suggested as resulitng from the secretion of a specific IgE helper factor from these cells. The role of IgE in allergic reactions of the immediate hypersensitivity type is undebated. Since IgG4 antibodies have been suggested as being able to sensitize basophils and mast cells, a considerable interest has also been focussed on the possible presence of IgG4 antibodies in allergic patients. Thus, a number of recent studies have demonstrated the presence of IgG4 antibodies against most offending allergens (Table 3.5). In view of the strong correlation between total serum levels of IgG4 and IgE both in normal, immunodeficient and allergic patients, this is not an unexpected finding. In the majority of the cited studies, IgG antibodies of non-IgG4 subclass have not been determined. Patients with IgE antibody mediated allergy to inhalent allergens or to insect venoms are often treated with allergen-specific immunotherapy (hyposensitization).

Table 3.5 The IgG subclass distribution of antibodies against selected allergens[a]

Allergen	*IgG1*	*IgG2*	*IgG3*	*IgG4*
Bee venom	++	—	—	+
Yellow jacket venom	++	—	—	+
Mould[b]				+
Grass pollen	+	—	—	+/++
Ovalbumin	+	—	—	++
Lactoglobulin	+	—	—	++

[a]Results are compiled from a number of different sources and are expressed as suggested by data in the respective papers
Negative —; positive +; strongly positive ++
[b]Limited number of studies testing IgG1, IgG2 and IgG3

Several previous studies have documented the clinical efficacy of immunotherapy with aqueous extracts of inhalent allergens and insect venoms. The present form of immunotherapy is, however, costly and time-consuming and in addition carries a significant risk of adverse reactions. Since some patients do not benefit from the immunotherapy it would be beneficial to have a test system whereby one could predict the clinical outcome of the treatment. Several studies have shown that a marked increase in IgG is the most consistent change during therapy. In addition, prolonged immunization regularly leads to the suppression of IgE responses and several studies have, however fruitlessly, attempted to correlate the IgG/IgE ratio with the symptomatic improvement. Several authors have suggested that the fall in IgE might actually be the most important mechanism underlying the symptomatic improvement in some patients. Still, however, the production of the high levels of allergen-specific IgG is often considered desirable, and a presumed beneficial effect of these antibodies has

traditionally been explained by their capacity to neutralize the allergen prior to its reaction with mast cell or basophil bound IgE. Subsequently, these IgG antibodies have often been considered as blocking antibodies. The understanding of the protective role of IgG has further been complicated by the recognition that some IgG molecules, possibly IgG4, may sensitize some patients (short term sensitizing antibodies). The clinical significance of these antibodies has been questioned but there is evidence that short term sensitizing antibodies actually do occur in some allergic patients. The influence of these antibodies on, or the induction by, immunotherapy still remains to be fully evaluated.

The IgG subclass distribution of specific antibodies developing during immunotherapy has been followed in a few studies (for review see Ref. 5). The antigens include grass pollen, yellow jacket venom and bee venom. As expected, the antibodies are primarily of IgG1 and IgG4 subclass. The initial response appears to be mainly of the IgG1 subclass whereas later phases are generally dominated by IgG4 antibodies. The balance between IgG1, IgG4 and IgE could possibly be modified by variations in the form of allergen presentation (aqueous or alum bound, glutaraldehyde or polyethylene glycol modified) or by variation in the dose regimen. During the early phases of immunotherapy a high level of IgG4 antibodies or a high IgG4/IgG1 antibody ratio has been suggested as being a highly sensitive and specific marker for a poor clinical outcome. In addition, high IgG1 antibody levels early in the course of immunotherapy are associated with the rapid decline in IgE antibody levels. In the case of insect venoms, some patients actually become permanently cured of their disease as a result of the suppression of the IgE antibody production. If a major increase in the IgG1 antibody levels is desirable, it has been considered likely that long intervals between the injections and possibly also a high dose regimen would favour this type of response.

The regulation of the IgE response is still poorly understood and markers predictive of a favourable response to immunotherapy are still largely missing. The suggestion that increased levels of IgG4 may predict a poor clinical outcome is, therefore, of great potential interest, an interest which is spurred on by the development of reliable anti-IgG subclass reagents. The assessment of clinical status and the tailoring of the antigens used for immunotherapy will undoubtedly contribute to symptom amelioration in some of these patients.

IMMUNODEFICIENCIES

It is quite clear from the reasoning above that antibodies against both protein and carbohydrate antigens may be restricted to certain IgG subclasses. As a consequence, the antibody repertoire may be expected to be drastically reduced in patients with various forms of immunoglobulin deficiencies. In the case of X-linked infantile hypogammaglobulinaemia (Bruton's disease) or common variable hypogammaglobulinaemia, both IgM, IgG and IgA levels are reduced. This leads to a profound immunodeficiency which is often, but

not always, accompanied by bacterial infections (for review see Ref. 6). Usually, there is a low level of IgG persisting although the subclass distribution of these antibodies may be markedly skewed. Possibly depending on compensatory mechanisms, hypogammaglobulinaemia may not lead to severe clinical complications until the level of IgG drops significantly below 2g/l. Common variable hypogammaglobulinaemia is normally defined as an acquired form of immunoglobulin deficiency where the major pathophysiological finding is a lack of immunoglobulin secreting cells. The actual acquisition, however, has only been documented in a few cases. The aetiology of common variable hypogammaglobulinaemia still remains elusive, and although additional immunoglobulin disorders are often found in family members, hypogammaglobulinaemia is not, in the strict sense, a hereditary disorder. However, taken together with the altered ratio of T-helper cells (OKT4 positive cells)/cytotoxic T-cells (OKT8 positive cells), the available data could suggest a transmissable agent as a cause of some forms of hypogammaglobulinaemia. The parallel to the acquired form of immunodeficiency induced by HTLV-III/LAV is in our view striking, and it is quite possible that selected cases of hypogammaglobulinaemia are in fact induced by B-lymphocytotropic retroviruses.

The therapeutically accepted approach in cases of hypogammaglobulinaemia is the substitution with gammaglobulin, i.e. purified IgG. A number of commercially available gammaglobulin preparations do exist and their IgG subclass distributions have been determined. In some of the products, IgG4 is missing which, in view of the IgG subclass restriction suggested above, may be of biological consequence. In addition, some of the preparations used for gammaglobulin substitution contain significant amounts of IgA. Since a considerable proportion of patients lacking IgA (either due to common variable hypogammaglobulinaemia or selective IgA deficiency) express anti-IgA antibodies, the purity of the product may certainly be of clinical importance in preventing anaphylactic reactions.

Immunoglobulin class deficiencies

Isolated IgM deficiency is a rare condition. It has been suggested as occurring mainly on a genetic basis but sporadic cases also exist (for review see Ref. 6), and has been noted to be associated with an increased incidence of meningococcal meningitis. However, in most of the described cases the IgM deficiency has been moderate, and considering that patients with hypogammaglobulinaemia normally do quite well on gammaglobulin replacement therapy (IgG only), it may be that IgM is not strictly necessary to combat infections. This clearly implies that patients with selective IgM deficiency and an increased frequency of bacterial infections may have an additional defect, possibly a failure to produce antibody to certain antigens such as meningococcal polysaccharides.

Selective IgG deficiency is also a rare condition. Isolated reduction of IgG due to an increased catabolism has been described in a number of patients, most often associated with autoantibodies against framework determinants

and thus affecting all IgG subclasses. In these patients, the moderately lowered IgG levels appear to be accompanied by an increased risk of bacterial infections. Two cases (both boys) with a total or near total absence of IgG which was not due to anti-immunoglobulin have previously been reported in the literature. Both of these children suffered from severe bacterial infections in spite of normal or even elevated concentrations of the remaining immunoglobulin classes. However, we have recently described a healthy adult who completely lacked IgG and displayed a compensatory increase in IgM and IgA levels. Thus, it appears as though IgG is not strictly necessary for survival, a finding which is also supported in experimental animal models by the longevity of a mutant strain of IgG deficient chickens.

Selective IgA deficiency, normally defined as below 0.05 g/l of serum IgA, is the most common immunodeficiency found in man. The prevalence ranges from 1/440 to 1/6500 among healthy blood donors, and an even higher frequency has been reported among patients with recurrent respiratory tract infections or autoimmune diseases (for review see Ref. 7). Levels of serum IgA rise slowly during childhood and a diagnosis of IgA deficiency should not be made before adolescence. It is, however, an indication of a delayed maturation of the immune system and could have clinical implications as it may affect the antibody repertoire. Genetic factors clearly influence the levels of IgA in man and IgA deficiency appears to be associated with genes within the major histocompatibility complex. From a genetic point of view, the two groups of individuals appear to be different since IgA deficiency in healthy subjects is associated with HLA B8/DR3 and in individuals with recurrent upper respiratory tract infections with HLA B40/CW3.

As expected, deficiency of IgA will lead to an increased frequency of respiratory tract and gastrointestinal infections. However, most of the individuals with selective IgA deficiency are quite healthy, and in these cases it appears as though compensatory mechanisms exist. Often, increased serum levels of IgG are found (mainly in IgG1 and IgG3) which could possibly account for the lack of disease susceptibility. The measurement of immunoglobulins found in secretions may possibly be more important than a mere analysis of serum immunoglobulin levels, and in IgA deficient donors there is often a compensatory increase in secretory IgM. Although the evidence is as yet rather incomplete, available data suggest that the levels of secretory IgM in IgA deficient individuals actually correlate with clinical status. Normally, there is a very strong correlation between lack of serum IgA and secretory IgA but exceptional cases have been described where a dichotomy has been observed. The two systems, therefore, appear to be distinct, a suggestion supported both by the recent finding of a mutant mouse strain lacking only secretory IgA and the different rates at which the two systems develop in children; the secretory system being apparently fully developed at a very early age.

The lack of protective antibody levels in the gastrointestinal tract has been suggested as leading to an enhanced uptake of dietery antigens, and increased amounts of immune complexes have in fact been found in serum from IgA deficient patients. Several workers have tried to link low levels of IgA in infancy with the subsequent development of infantile eczema and asthma.

However, it is likely that IgA deficiency merely exacerbates subclinical disease and may thus serve as a precipitating factor in atopy.

Antibodies to IgA develop in a substantial proportion of IgA deficient individuals and up to 40% display IgG antibodies (mainly IgG1 and IgG4) against IgA. Severe anaphylactic reactions may occur upon infusion of IgA containing products in these individuals. The procurement of gammaglobulin free of IgA has, therefore, been of considerable value therapeutically. By definition, serum levels of the other immunoglobulin classes should be normal in selective IgA deficiency. Similarly, the expected antibody repertoire of specific antibodies would also be expected to be normal. However, IgA deficient individuals often display low levels of IgD and IgE and may sometimes lack these immunoglobulin classes altogether. In addition, it has recently been acknowledged that immunoglobulin subclass deficiencies exist, and that there is an over-representation of IgG2/IgG4 deficiency in what has hitherto been described as 'selective' IgA deficiency. With regard to the repertoire of antibodies, a number of cases have been described where particular antibodies such as phosphorylcholine, *Helix pomatia* haemocyanin and tetanus toxoid, have been missing in spite of normal serum levels of IgM and IgG. The levels of specific antibodies against a number of different antigens appear to be lower in patients with high titres of anti-IgA, but the regulatory mechanism underlying this phenomenon is not yet understood.

No pathogenetic role has been ascribed to the low levels of IgD. Deficiency of IgD is preferentially found in HLA B8 positive individuals, and lack of IgD is a common finding in HLA B8 positive, healthy IgA deficient individuals.

Deficiency of IgE has also been described in a number of cases. Lack of IgE was initially described as giving rise to respiratory tract infections. However, most of these cases had an additional IgA deficiency and a selective deficiency of IgE does not seem to lead to disease in the western world. It is, however, quite probable that this type of deficiency may be of importance in geographic areas infested with parasites.

Immunoglobulin subclass deficiencies

Isolated IgG or IgA subclass deficiencies are rare in man. As indicated above, there is a strong correlation between levels of closely linked heavy chain constant region genes such as IgG1 and IgG3 and also IgG2 and IgG4. Most of the cases investigated to date appear to have a regulatory defect, i.e. the heavy chain constant region genes are retained in the genome but there is a defect in regulation of gene product expression. A few exceptional cases have, however, been described where the defect is due to a deletion of the corresponding heavy chain constant region gene, and DNA from these donors have helped illuminate the sequence of genes in the heavy chain locus on chromosome 14. Based on available data, one could calculate that the number of expected heterozygotes (with structural immunoglobulin gene deletions) in the general population would be around 1–3%. The deficiencies thus far described have been shown to encompass multiple subclasses (IgG1, IgA1, IgG2, IgG4; IgA1, IgG2, IgG4, IgE; IgA1, IgG2, IgG4) (for review see

Ref. 8). Surprisingly, the individuals lacking IgG and IgA subclasses due to genetic deletions have all been described as healthy, whereas a 'regulatory' IgG subclass deficiency has, in most cases, been associated with an increased frequency of upper and lower respiratory tract infections.

A few cases of selective IgG1 deficiency have previously been reported in the literature. In one of these families, one child suffered from chronic otitis media whereas two were healthy in spite of their IgG1 deficiency. A necessity of IgG1 in immune defence reactions is thus at present not established.

IgG2 deficiency is most often associated with IgG4 deficiency, although exceptions do occur. IgG2 constitutes only a small part of the total IgG and a deficiency is, therefore, easily misdiagnosed if only total IgG levels are measured. This type of defect has been suggested as leading to severe upper and lower respiratory tract infections often leading to an impaired lung function. The deficient individuals frequently have a concomitant deficiency of IgA and IgE.

Antibodies to carbohydrate antigens are mainly of the IgG2 subclass in adults. In children, however, IgG1 may be the dominant subclass expressed. Adult donors with IgG2 deficiency normally mount a satisfactory immune response towards protein antigens, and the majority of these antibodies are restricted to the IgG1 subclass. Vaccination of these donors also results in a normal immune response (for review see Ref. 4). Antibodies to carbohydrate antigens could, therefore, be expected to be present in the IgG1 subclass in these donors. However, as a rule, these individuals express low or negligible levels of antibodies against carbohydrate antigens. Vaccination with bacterial polysaccharides does not seem to increase the levels of specific antibodies (for review see Ref. 4). The lack of antibodies with a specificity for carbohydrate antigens in IgG2 deficient individuals with a 'regulatory' defect may help to explain their increased susceptibility to bacterial infections. However, a number of healthy IgG2 deficient individuals (including several normal blood donors) have recently been described. In addition, as stated above, adult individuals lacking IgG2 due to genetic deletion of the corresponding heavy chain constant region genes, have all been described as healthy. In the latter cases, antibodies against most tested carbohydrate antigens have been present in the remaining IgG subclass (IgG1 and IgG3 or only IgG3) (for review see Ref. 9). It, therefore, seems that lack of IgG2, although severely restricting the antibody repertoire, does not necessarily lead to infections in adults. However, it is worth pointing out that even moderately lowered IgG2 levels may apparently lead to frequent respiratory tract infections or otitis in children. In our view, it is quite possible that during childhood the IgG subclass pattern matures in parallel with yet another defence system, and immaturity of the complement system or the phagocytic system may thus, in combination with the relative IgG2 deficiency, contribute to the proneness to infections. If gammaglobulin replacement therapy is considered desirable, a preparation low in IgA should preferably be used (in patients with concomitant IgA deficiency), since a considerable proportion of these children prior to treatment, display high titres of anti-IgA.

Selective cases of IgG3 deficiency have been described, appearing both sporadically and in a hereditary form. As is the case in IgG2 deficiency, the

majority of cases described have exhibited a relative rather than a complete deficiency of IgG3. Again, it is questionable whether a selective decrease in IgG3 will lead to clinical consequences, and in the first family with selective IgG3 deficiency described, all individuals were healthy. The antibody repertoire in these individuals also appears to be normal (for review see Ref. 9) although, as expected, specific IgG3 levels are low or even absent. Recently, however, a number of patients with a relative deficiency of IgG3 have been described as suffering from frequent upper respiratory viral infections and short bouts of fever of unknown origin. Since IgG3 antibodies do play an apparently crucial role in the defence against at least some viral agents, it is possible that IgG3 deficiency may in fact predispose to viral infections. In this context, however, it should be kept in mind that the serum levels of IgG3 do not necessarily correlate to the number of IgG3 producing cells within the mucosa and marked discrepancies have been reported. Gammaglobulin treatment has been initiated in a few of these cases with limited success, and its use cannot at present be advocated. One important practical problem that arises is the difficulty in monitoring these patients, since the short halflife of IgG3, as compared to the other subclasses, may necessitate frequent gammaglobulin injections.

IgG4 constitutes a small minority of total IgG, although it may be more prominent in certain tissues such as the breasts of lactating women. In spite of this fact, patients lacking IgG4 have often been described as suffering from recurrent or chronic respiratory tract infections. It has, as yet, not been shown that selective IgG4 deficiency in any way affects the antibody repertoire and in view of the known correlation between IgG2 and IgG4 levels caution should be exerted in ascribing disease susceptibility to a mere lack of IgG4. Gammaglobulin replacement therapy has been tried in a number of these cases with reportedly good results. Before instituting therapy it should be born in mind that the IgG4 subclass matures late and low levels may be found normally during infancy, a 'defect' that does not seem to have any major influence on disease susceptibility.

CONCLUDING REMARKS

The immune system has evolved to help combat infections. During evolution a number of different defence mechanisms have evolved, each with a specialized function. In many cases, two different systems may display overlapping functions and a deficiency in one system may, therefore, be partially or completely compensated for.

In vertebrates five different immunoglobulin classes have evolved. B-cells secreting the various isotypes have a distinct tissue distribution. Most effector functions are carried out by the Fc portion of the immunoglobulin molecule. Depending on class/subclass, antibodies will fix complement either by the classical or the alternative pathway. Antibodies are also necessary for the effective phagocytosis of foreign antigens by binding immune complexes to Fc receptor bearing granulocytes and monocytes/macrophages. Due to the specialized function of each of the immunoglobulin classes, a deficiency may

not easily be compensated for and would thus result in an increased susceptibility to infections. In the case of IgG deficiency, substitution can, in most cases, readily be accomplished by the institution of gammaglobulin replacement therapy. There are no strict guidelines for the use of gammaglobulin with regard to serum level at which substitution should be started since, due to the complexity of the system, some patients may actually do well although exhibiting quite low IgG values (below 2g/l). On the other hand, some patients with moderately lowered IgG but with a clinical history of repeated infections, may indeed benefit from gammaglobulin replacement therapy.

Antibodies can theoretically be produced against any given antigen that an individual may encounter, and the completeness of the repertoire is probably a necessary prerequisite for the maintenance of an intact immune defence system. The finding of a marked subclass restriction of specific antibodies is, therefore, of considerable interest. Anti-carbohydrate antibodies are normally of the IgG2 subclass in adults, and donors lacking IgG2 also appear to lack the corresponding antibody repertoire. The missing specificities may of course be substituted by the introduction of gammaglobulin replacement therapy. However, in a number of cases, compensatory mechanisms do exist and a substantial number of healthy subclass deficient individuals have been described. Our present methods for the evaluation of the antibody repertoire in patients with suspected immunodeficiency disorders is still rather primitive. Although measurements of immunoglobulin classes and subclasses have been of great diagnostic value, there is a growing awareness that susceptibility to infectious diseases may also develop in patients who only lack particular antibody specificities. In this context it is interesting to note that deletions of parts of the V gene repertoire have recently been described in certain strains of mice, and it is quite possible that similar types of deletions have occurred in the human genome. The presence of such holes in our antibody repertoire will undoubtedly necessitate a re-evaluation of some of our current concepts of immunodeficiency, and the advent of molecular probes for human V genes may in the near future contribute to the solution of some of these problems.

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4
The Biological and Pathological Significance of Antibody Affinity

M. E. DEVEY

INTRODUCTION

A major role of antibody is to combine with antigens to form immune complexes which are then rapidly removed from the circulation by triggering various effector functions such as complement activation and phagocytosis. Antibody responses are often considered solely in terms of the amount of antibody produced, but it is important to realize that other factors may exert an even greater influence on its biological effectiveness. It has been known for many years that not only the amount of antibody increases with time after immunization, but also that there is a progressive change in the quality of the antibody produced. Thus, the ability of antibodies produced late in the immune response to react more rapidly with antigen and form less dissociable complexes as well as to show increased cross-reactivity can be explained, at least in part, by the progressive increase in antibody affinity that occurs with time[1]. It is, therefore, likely that antibody affinity has a fundamental role in governing the biological activities of antibodies, and may be of critical importance in the pathological processes mediated by the immune response.

AFFINITY OR AVIDITY?

Antibody affinity describes the strength of the interaction between an antibody combining site and an antigenic determinant. The term avidity has never been defined precisely in thermodynamic terms, and has come to mean different things to different people. Some use it synonymously with affinity, others use it to describe the enhanced binding power conferred by the multivalency of antibodies and, in addition, it has also been used to described the efficacy of antibodies in the performance of particular biological functions such as toxin or virus neturalization. For this reason, the term avidity is best avoided and, as suggested by Karush[2], it is preferable to use the

term *intrinsic affinity* to describe the primary binding energy of a single antibody combining site with a single antigenic determinant, while the term *functional affinity* may be used to describe reactions between multivalent antibodies and complex antigens (Figure 4.1). For IgM antibodies it may be seen that, despite a low intrinsic affinity for antigen, multivalent interaction results in a high functional affinity.

	ANTIBODY	K	ADVANTAGE OF MULTIVALENCE	DEFINITION OF K
	IgG	10^4	-	INTRINSIC AFFINITY
	IgG	10^7	$x10^3$	FUNCTIONAL AFFINITY
	IgM	10^{11}	$x10^7$	FUNCTIONAL AFFINITY

Figure 4.1 Definition of antibody affinity (*K*): for an antibody interacting with a monovalent antigen (intrinsic affinity) and the advantage, in terms of increased binding, conferred by multivalent interactions (functional affinity)

THE ANTIGEN COMBINING SITE: BINDING FORCES AND SPECIFICITY

Interactions between the antigen combining site and its corresponding antigenic determinant depend on a number of relatively weak non-covalent forces (Table 4.1). The strength of these forces falls off very sharply as the distance between the reactive groups increases. Thus the strength of interaction is highly dependent on the 'goodness of fit' of the antigen with its combining site. In situations when the combining site is exactly complementary to the antigenic determinant (Figure 4.2a) the forces holding them together become quite large. However, with a different but related antigen (Figure 4.2b), the opposing groups are not able to approach so closely and so the forces become very much weaker. An antibody combining site may, therefore, interact with a number of structurally related antigenic determinants, more strongly with some than with others, and this is the basis of both antibody *specificity* and *cross-reaction*.

Table 4.1 Major forces of attraction between antigen and antibody in the antibody combining site

	Strength (kcal)*	*Relationship between force and distance(d)*
Electrostatic	5-10	$1/d^2$
Hydrogen	2-5	$1/d^2$
Hydrophobic	1-5	$1/d^7$
Van der Waals	0.5	$1/d^7$

*Compare with covalent bonds: strength of interaction = 40–140 kcal

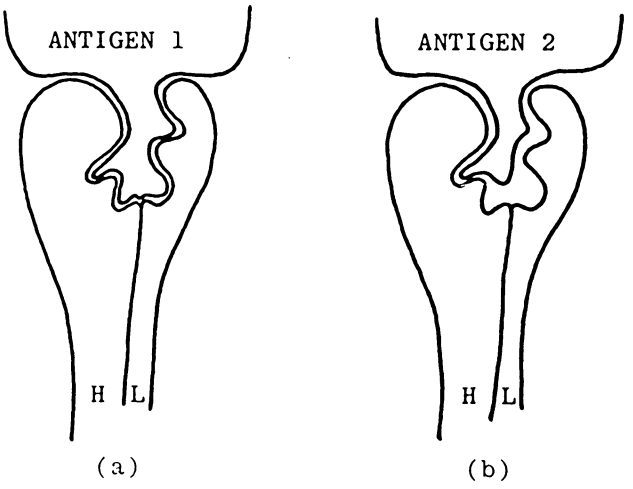

Figure 4.2 Interactions between an antibody combining site and an antigenic determinant: (a) a good fit, high affinity interaction, (b) a poor fit, low affinity interaction

ANTIBODY AFFINITY

All antigen–antibody reactions (however good the fit) are reversible:

$$Ab + Ag = AbAg \quad (1)$$

where Ab represents free antibody, Ag free antigen and AbAg the antigen–antibody complex. At equilibrium, the rate of formation of the complex will equal its rate of dissociation and the equilibrium constant (K) may be calculated from the ratio of the complexed to free reactants. Applying the Law of Mass Action:

$$K = \frac{(AbAg)}{(Ab)\,(Ag)} \quad (2)$$

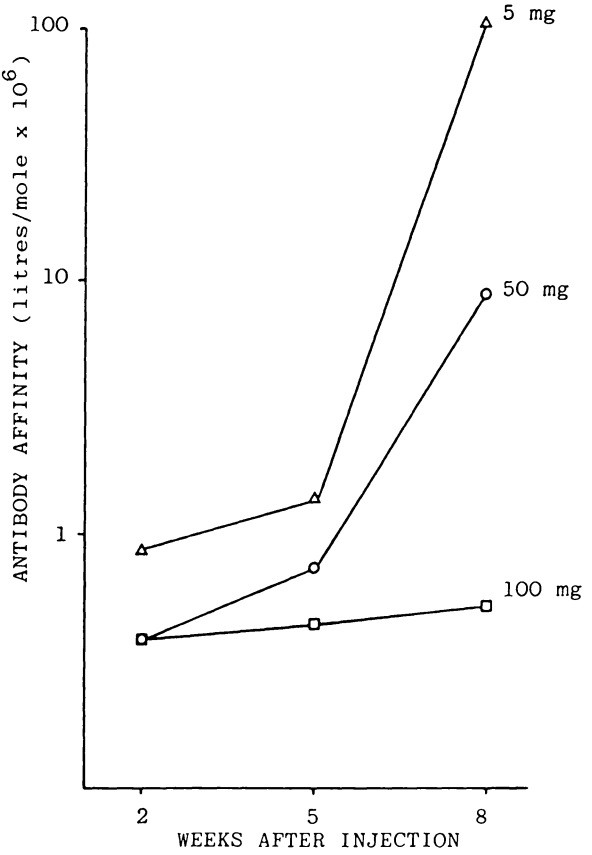

Figure 4.3 Affinity maturation of antibodies to DNP-lysine in rabbits receiving different doses of antigen. (Data from Eisen and Siskind[1])

K, therefore, is a measure of affinity and its value will increase with increasing immune complex formation, a situation that will be favoured by high affinity antibody, and will decrease with low affinity antibody, which will favour dissociation of the complex.

In classical experiments in 1964, Eisen and Siskind demonstrated that antibody affinity increases progressively with time after immunization[1]. This has been termed *affinity maturation* and has been subsequently confirmed in many different systems in both man and experimental animals where it appears to be a hallmark of the IgG response. Affinity maturation is most marked with small doses of antigen (Figure 4.3), and both phenomena can be explained by the *antigen selection hypothesis* of Siskind and Benacerraf[3]. This assumes that the antigen binding properties of B-cell immunoglobulin receptors are the same as those of the antibodies that will be eventually secreted by its progeny. Therefore, cells with high affinity receptors will give rise to clones of cells secreting high affinity antibodies, whereas those with low affinity receptors will give rise to cells secreting low affinity antibodies. At

high levels of antigen, there is enough antigen to stimulate both high and low affinity cells and, in addition, it is possible that high affinity cells may also be tolerated[4]. Thus, at the start of an immune response, more low affinity antibody will be produced than high. As antigen levels fall there will be competition between B-cells for the available antigen, resulting in preferential selection of high affinity cells and a gradual loss in the contribution made by low affinity cells. This hypothesis may be something of an oversimplification and certainly does not fully explain all the observations that have been made with regard to temporal changes in antibody affinity and the persistence of low affinity memory cells. Recent studies with hybridomas have shown that, as well as antigen selection, somatic mutation of germ line genes plays a major role in affinity maturation[5].

Paradoxically, low affinity antibody responses appear more specific and demonstrate less cross-reactivity than high affinity responses. This may be explained by the recognition of an increasing number of different antigenic determinants during affinity maturation and so the chance of cross-reaction with related antigens is maximized.

MEASUREMENT OF ANTIBODY AFFINITY

Equilibrium dialysis

The basis of most methods for determining antibody affinity is by measuring the relative amounts of bound and free antigen formed at equilibrium when antibody has been allowed to react with a range of antigen concentrations. The classical method is equilibrium dialysis[6]. Purified antibody is separated from a known amount of radiolabelled antigen (usually a low molecular weight hapten) by a dialysis membrane that is permeable to the antigen but not to the antibody. Antigen will diffuse into the antibody compartment until, when equilibrium is reached, the amount of *free* hapten is the same in both compartments. The antibody compartment will, however, contain more *total* hapten and the difference in the amount of total hapten between the two compartments will reflect the amount of hapten bound by the antibody (Figure 4.4). Several mathematical approaches can be used to analyse data

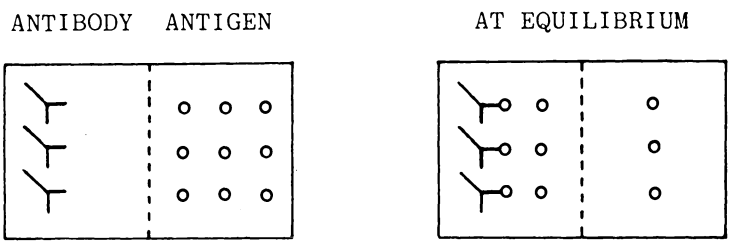

Figure 4.4 The principle of equilibrium dialysis

generated from such experiments, and one of three forms of equation are generally used:

1. The Scatchard Equation
2. The Langmuir Equation
3. The Sips Equation.

The mathematics of affinity calculations and the relative merits of the different approaches have been discussed in detail by Steward[7,8].

Ideally, measurement of antibody affinity should be performed using homogeneous reactants in terms of both the antigen and the antibody, for example: a monoclonal antibody and a monovalent hapten. Measurement of the affinity of antibody in whole serum is complicated by the heterogeneity of the normal antibody response in terms of amount, isotype and affinity as well as the fact that most antigens are large, complex and multivalent. Under such circumstances, and particularly if large numbers of serum samples need to be evaluated, equilibrium dialysis is probably not the method of choice.

Radioimmunoprecipitation

A relatively simple technique for measuring antibody affinity in whole serum is a modification of the Farr assay, in which ammonium sulphate precipitation is used to separate bound from free antigen[9,10]. This utilizes the fact that immunoglobulins are insoluble in 50% saturated ammonium sulphate (SAS) and that the equilibrium is not disturbed by the precipitation step. Antibody is mixed with radiolabelled antigen over a range of antigen concentrations. The reaction is carried out in antigen excess in order to obtain saturation of the antigen binding sites and allow both the high and low affinity populations to compete for antigen. The amounts of bound and free antigen are determined after SAS precipitation by counting the amount of radioactivity in the precipitate and supernatant (Figure 4.5) and, using the Steward–Petty modification of the Langmuir equation, a binding curve is

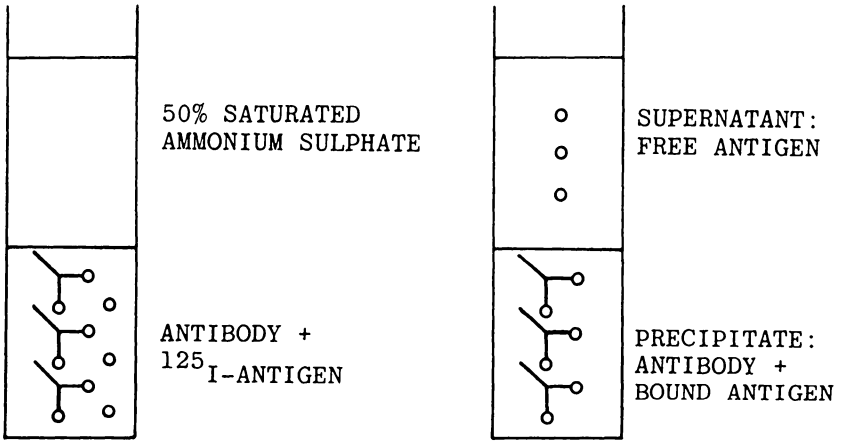

Figure 4.5 The principle of ammonium sulphate radioimmunoprecipitation (Farr assay)

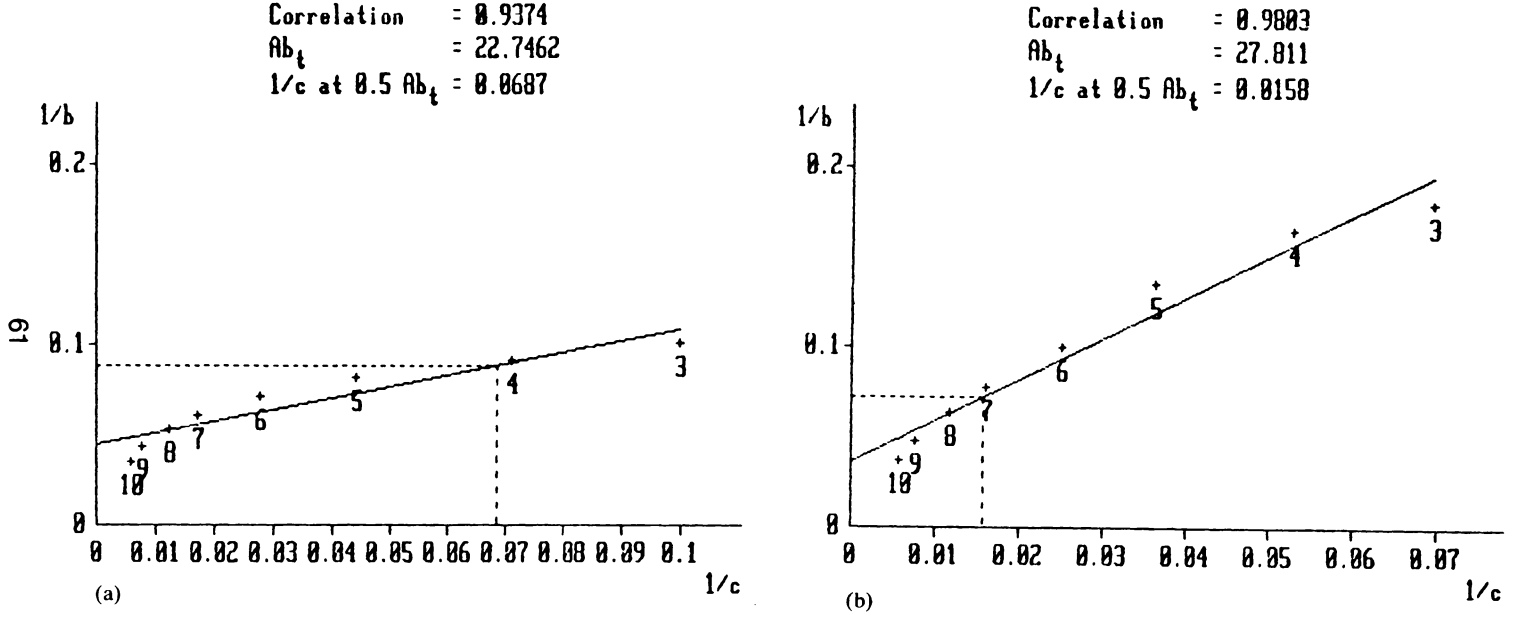

Figure 4.6 Binding curves for the interaction of ^{125}I *O*HSA with a serum containing high affinity antibodies (a) and a serum containing low affinity antibodies (b) with similar antibody levels

constructed by plotting the reciprocal of bound antigen against the reciprocal of free antigen at each antigen concentration (Figure 4.6). The slope of the line is dependent on the antibody affinity, and calculation of the reciprocal of the free antigen concentration when half the antibody binding sites are occupied by antigen gives the intrinsic binding site affinity. A value for total antibody (Abt) can be obtained by extrapolating the straight line to the intercept (where the reciprocal of bound antigen = 0). A limitation of the method is that the antigen must be soluble in 50% SAS, although with antigens that are not, the problem may be overcome by using other selective precipitating agents such as polyethylene glycol or a second antibody. This method is ideal for the rapid estimation of the overall affinity of an antibody response in whole serum, and has been shown to rank sera in the same order as equilibrium dialysis[11], although it cannot give any information about the distribution of different antibody populations.

Enzyme-linked immunosorbent assay (ELISA)

The finding that ELISA may be highly dependent on antibody affinity[12-14] has led to suggestions that such assays may be used to measure antibody affinity[15],[16]. These methods are based on analysis of the shape of the dose–response curve when optical density is plotted against dilution of antibody. At low dilutions of sera (antibody excess) it would be expected, from the Law of Mass Action, that high affinity antibody would bind preferentially to antigen on the solid phase, whereas at high dilutions of sera (antigen excess) low affinity antibody should also bind. Therefore, antibody affinity should, theoretically, influence the slope of the linear part of the curve, which should be steeper for high affinity antibodies compared to low. This has been shown, experimentally, to be the case using monoclonal antibodies of defined affinity to haptens (Figure 4.7). Whether this can be utilized as a reproducible, reliable and sensitive method for measuring antibody affinity in whole serum has not yet been established.

An alternative approach, which may have greater practical application, has been applied to the measurement of the affinity of antibodies to viral antigens[17]. This is based on ELISA performed in the presence and absence of low concentrations of a protein denaturant, which should prevent binding of low affinity antibody to the solid phase antigen but which should not affect high affinity antibody binding. Thus, the relative amounts of high and low affinity antibody can be obtained from a comparison of the binding curves in the presence and absence of the denaturant.

ANTIBODY AFFINITY AND ANTIBODY ASSAYS

Probably all antibody assays are influenced by antibody affinity to a greater or lesser extent[18]. In particular, solid phase assays such as ELISA would be expected to be poor at detecting low affinity antibody, which may be more easily dissociated from the solid phase antigen during the numerous washing steps. The extent to which this occurs has been shown to depend on the

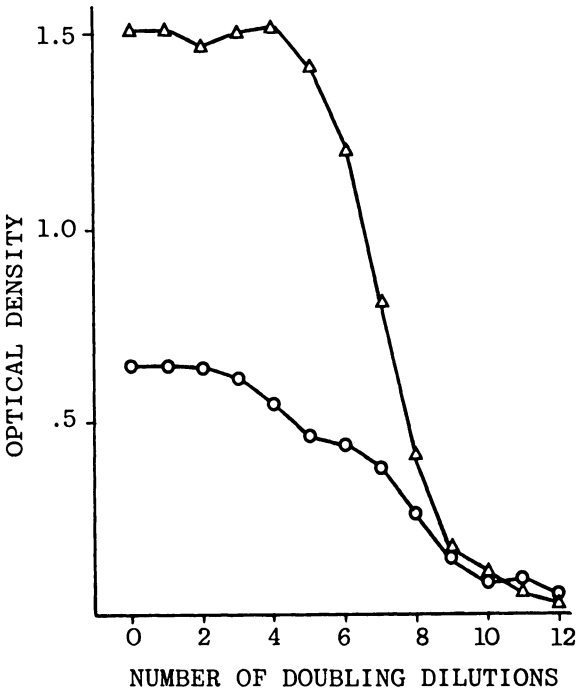

Figure 4.7 ELISA binding curves of a high affinity IgG1 (Δ–Δ) and a low affinity IgG1 (o—o) mouse monoclonal antibody to DNP. (From Lew[13])

epitope density of the antigen on the solid phase[13], and so it should, theoretically, be possible to increase sensitivity for low affinity antibodies by increasing the epitope density. The controversy as to whether ELISA is primarily a measure of antibody concentration or antibody affinity[12] probably reflects the different ways in which the data can be expressed (as an end point titre or as an optical density value at a single serum dilution), and stresses the need for careful analysis of dose–response curves[14]. Another possibility for significant error has been shown in the use of second, isotype-specific monoclonal antibodies in such assays, which may themselves differ in their affinities and thus cause erroneous assumptions as to the relative amounts of the different isotypes present in an antibody population[19]. Other assays in which the effect of antibody affinity on titre has been assessed are passive haemagglutination, complement-mediated haemolysis, precipitation and the Farr test[18,20]. Clearly, an increasing awareness of the influence of antibody affinity in the commonly used antibody assays is of great importance, particularly when assessing the responses of individuals who may, for a number of different reasons, be prone to produce low affinity antibody responses.

FACTORS THAT AFFECT ANTIBODY AFFINITY

Genetic control

Studies with experimental animals have demonstrated that antibody affinity is under genetic control which is similar to, but independent of, the genetic control of antibody levels[21–23]. It seems likely that affinity is controlled by several genes but, unlike most of the genes involved in the control of immune responses, these do not appear to be linked to the major histocompatability loci[23,24]. The cellular basis at which this control operates has not yet been fully elucidated, but it is likely to be expressed either at the level of antigen presentation by the macrophage or at the level of T-cell control of B-cells or, perhaps, at both of these levels. There is evidence to suggest that suppressor T-cells have an important regulatory role in the maturation of antibody affinity by preferentially suppressing B-cells that have high affinity antigen receptors[25]. Recent studies in experimental animals, in which low doses of cyclophosphamide (thought to selectively affect suppressor T-cells) have been shown to result in increased antibody affinity, support this view[26].

Age

Immunological competence is known to vary with age, increasing during postnatal development to a maximum in adulthood and then decreasing with old age. A number of studies in experimental animals have shown that the ability to form high affinity antibody responses broadly follows this pattern[27,28]. This probably results from age-related changes in T-helper and suppressor cell populations as well as to possible changes in B-cells.

Environmental factors

A number of other factors have been shown to affect antibody affinity. Protein deprivation results in reduced antibody affinity in experimental animals[29], and this has also been demonstrated in children with protein-energy malnutrition[30]. Parasitic infection, such as malaria and trypanosomiasis, reduces antibody affinity and delays affinity maturation[31,32]. Treatment with immunosuppressive drugs reduces affinity[33], whereas a wide range of substances which act as immunological adjuvants increase affinity[34].

ANTIBODY AFFINITY AND DISEASE

It is a common observation that the *in vitro* measurement of antibodies to toxins or micro-organisms often fails to correlate with their protective activity *in vivo*. One of the factors that may explain these discrepancies are differences in antibody affinity. There is experimental evidence from several studies showing that high affinity antibodies mediate a number of different biological effector functions very much more effectively than do low affinity antibodies (Table 4.2). In many cases only a small difference in affinity produced a significant change in biological reactivity, and up to four times more low

Table 4.2 Biological reactions that are performed more effectively by high affinity antibodies compared to low affinity antibodies

Reaction	*Reference*
Passive cutaneous anaphylaxis	35, 36
Complement fixation	37
Virus neutralization	38
Enzyme inactivation	39
Protection against bacteria	40
Immune elimination	41, 42

affinity antibody was required to obtain comparable activity to that of high affinity antibody. However, the differences between high and low affinity antibodies were less marked for certain reactions (complement fixation and passive cutaneous anaphylaxis) when higher antigen concentrations were used. Thus, low affinity antibody activity can be compensated for either by increasing the amount of antibody or, in some cases, by increasing the concentration of the antigen.

Antibody affinity and immune complex diseases

There are two major mechanisms by which antibodies play a role in the aetiology of renal disease. These involve either antibodies that are specifically directed to kidney antigens or, and by far the most common, antibodies that form immune complexes with non-glomerular antigens in the circulation which are then deposited in the glomeruli. Much of the evidence for the immunological mechanisms in immune complex mediated glomerulonephritis has come from the study of animal models which have certain similarities to the human disease. One of the most important factors in determining the pathogenic potential of immune complexes is their size. Very large complexes formed in antibody excess are usually rapidly removed from the circulation by the mononuclear phagocyte system without causing disease while, on the other hand, very small complexes formed in large antigen excess are unable to initiate inflammatory processes and have little pathogenic potential. However, it is the soluble complexes of intermediate size that appear to have the ability to localize in filtering membranes such as the renal glomeruli and thus cause disease. A number of factors are likely to influence the size of circulating immune complexes and, in addition to the relative amounts of antigen and antibody and the nature of the antigen, there is now a great deal of evidence to suggest that antibody affinity plays a key role.

An early indication that low affinity antibodies were of immunopathological significance in immune complex disease came from the finding that certain inbred mouse strains that were susceptible to glomerulonephritis after neonatal infection with lymphocytic choriomeningitis virus were the strains that produce low affinity antibody responses after injection of protein

Table 4.3 Induction of chronic serum sickness by repeated injections of a foreign protein antigen in mice selectively bred to produce either high or low affinity antibodies. (Data from Devey *et al.*[44-46,48])

	Low affinity mice	*High affinity mice*
Proteinuria	+++	±
Renal immune complex deposition	+++ (diffuse capillary)	+ (focal mesangial)
Death from renal failure	+++	±
Circulating immune complexes	+++	++
Clearance of immune complexes	++	+++
Size of immune complexes	12-17S	>19S

antigens in saline[43]. This gave rise to the hypothesis that susceptibility to immune complex diseases might result from the genetically controlled production of low affinity antibody, which would be poor at elimination of antigen from the circulation and which would result in the production and tissue deposition of antigen-excess immune complexes[41]. This may be considered as a form of immune deficiency and, as such, is likely to be antigen non-specific.

Studies in mice selectively bred for several generations into two lines (high and low affinity) on the basis of the affinity of their antibody responses to protein antigens injected in saline[21], have confirmed the association between the production of low affinity antibodies and susceptibility to immune complex disease[44] (Table 4.3), and have shown that disease is related to an inability to show affinity maturation[45]. Low affinity antibody may, therefore, have immunopathological significance, not only because it fails to eliminate antigen and favours the formation of intermediate sized immune complexes, but also because it affords the opportunity for the *in situ* formation of immune complexes. An interesting observation in low affinity mice is that, if they can produce very high levels of antibody, they are protected from disease[45]. These mouse lines have provided an extremely useful animal model for studying the role of antibody affinity in disease and other factors that influence the formation, clearance and tissue deposition of immune complexes[46-48].

It is paradoxical that, although complement-mediated inflammation has a major role in many immune complex diseases, there is also an association between complement deficiencies and such diseases[49]. This has given rise to the suggestion that complement plays an important role in immune complex handling and removal from the circulation, possibly via its ability to 'solubilize' complexes. It has been shown that the rate of immune complex solubilization, by the alternative pathway of complement activation, is highly dependent on antibody affinity[50]. However, as immune complexes are almost always formed *in vivo* in the presence of complement, the biological importance of this phenomenon is not clear, and it seems more likely that the complement-mediated *inhibition* of immune complex precipitation, which

occurs mainly via the classical pathway, may be of greater relevance, but the role of affinity in this reaction is as yet unknown.

Antibody affinity and autoimmune disease

Although it would seem to be generally biologically advantageous to develop high affinity antibody responses, a major exception may be in the production of autoantibodies, particularly in the organ-specific autoimmune diseases, where high affinity antibodies may have greater immunopathological significance than low. There are two pieces of experimental evidence obtained from animal models to support this view. Firstly, it has been demonstrated in the rat that high affinity antibodies to the glomerular basement membrane in Masugi nephritis cause greater renal damage than low affinity antibodies[51]. Secondly, it has been reported that several autoimmune disease-prone mouse strains demonstrate restricted heterogeneity with regard to the affinity of their antibody responses and may lack the capacity to produce low affinity antibodies[52]. However, in the very few studies carried out in patients with organ-specific autoimmune diseases there is, as yet, little convincing evidence for an association between antibody affinity and disease. In a study in patients with generalized idiopathic myasthenia gravis, there was no correlation between the affinity of antibodies to the acetylcholine receptor and the severity of disease, although affinity was significantly higher than in patients with penicillamine-induced myasthenia[53,54].

Antibody affinity in systemic lupus erythematosus

The role of antibody affinity in SLE is the subject of some debate, and there have been a number of conflicting reports of the association of both high and low affinity antibodies to ds-DNA with disease activity and nephritis. This lack of agreement on the binding characteristics of DNA antibodies may be due to the rather unusual nature of DNA as an antigen; its multideterminant and polyanionic nature make it notoriously 'sticky', which may result in interaction with immunoglobulins that has little to do with primary binding site affinity and which undoubtedly causes methodological problems in affinity assays. In addition, there is evidence that nephritis in SLE may not be due to deposition of circulating immune complexes but rather to prior interaction of DNA with the glomerular basement membrane which will then act as a target for circulating antibody in the *in situ* mechanism of immune complex formation[55]. If this is the primary mechanism of complex deposition, then high affinity antibody might be expected to have greater immunopathological significance than low in SLE.

Antibody affinity, infection and vaccination

Studies in experimental animals suggest that low affinity antibody is less protective than high against bacterial infections in mice[40]. In a recent study, the affinity of antibodies to tetanus toxoid was measured in patients with chronic or recurrent acute chest infections but with normal immunoglobulin levels, following immunization[56]. A number of patients failed to respond to

Table 4.4 Mean levels (Abt) and affinity (*Kr*) of antibodies to tetanus toxoid (±SD) following vaccination in responding patients with chronic or recurrent chest infections and in age and sex matched controls

	n	*Abt* (pmol/10μl)	*Kr* ($l/mol \times 10^6$)
Controls	32	39.9 ± 9.2	5.04 ± 8.74
Patients	25	39.4 ± 12.4	3.08 ± 3.94
t		0.13	1.04

immunization, suggesting that, in some, systemic antibody deficiency may have been an aetiological factor in disease. In the patients who did respond to immunization, mean antibody affinity was lower than in a group of age and sex matched controls, although the differences failed to reach significance (Table 4.4). These results did not provide convincing evidence that low affinity antibody responses were a major factor in susceptibility to chest infections, although they did not exclude the possibility that it may have been a factor in at least some of the patients.

The hypothesis that defective or low affinity antibody responses may result in persistent virus infection leading to chronic disease[57] is supported by the finding of significantly lower affinity antibody to hepatitis-B surface antigen in patients with chronic liver disease compared to those that had recovered from acute hepatitis-B infection[58]. In addition, the importance of assessing the efficacy of vaccination in terms of antibody affinity as well as titre has been demonstrated in a recent study in healthy individuals receiving a plasma derived hepatitis-B vaccine[59].

Antibody affinity and the IgG subclasses

Of the four subclasses of human IgG, IgG4 is present at the lowest concentration in normal sera. Despite this, a surprisingly large amount of antibody activity has been described in association with this subclass. In particular IgG4 antibodies to pollen have been found in hayfever patients undergoing hyposensitization[60], and IgG4 responses have also been described to inhaled and ingested antigens in normal individuals, leading to the suggestion that they are produced in response to chronic antigenic stimulation[61]. Antibodies to tetanus toxoid are usually restricted to the IgG1 and IgG4 subclasses. In the study in patients with chest infections described above[56] it was found that 35% of the patients and controls immunized with tetanus toxoid produced an IgG4 antibody response (usually together with IgG1) while the remainder produced a predominantly IgG1 response. An unexpected observation was that those individuals with IgG4 antibodies to tetanus toxoid had significantly lower antibody affinity than those with IgG1 antibodies (Figure 4.8). In addition, there was a significant correlation between IgG1 antibody levels and the amount of high affinity antibody. These

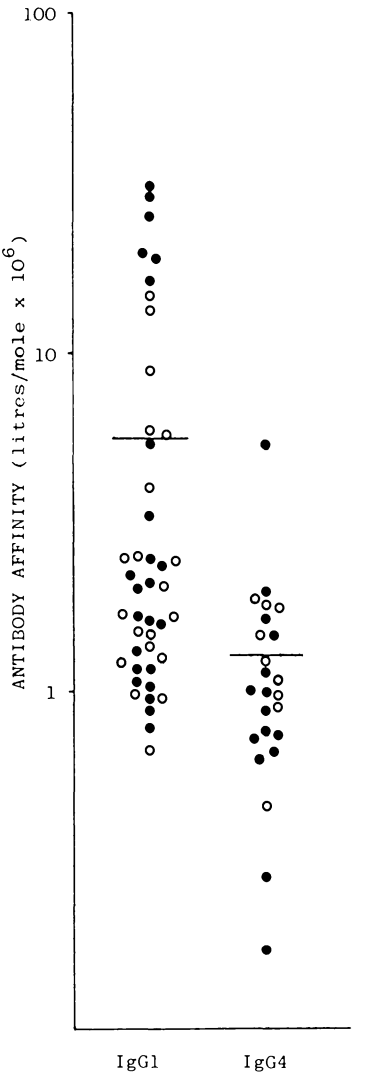

Figure 4.8 Association between antibody affinity and the IgG subclass of antibodies to tetanus toxoid in patients with chest infections (o) and in controls (•). IgG1 antibody responders: Kr = 5.57 ± 7.85; IgG4 antibody responders: Kr = 1.28 ± 1.01 (p = 0.01). (From Devey *et al.*[56])

findings can be interpreted in two ways, either that IgG4 antibodies are themselves of low affinity while IgG1 antibodies are high affinity, or that IgG4 antibodies are produced when low affinity antibody responses occur in any of the IgG subclasses.

The association between IgG4 responses, low affinity antibodies and the presumptive T-cell suppression that may exist during chronic antigenic stimulation, mucosal exposure to antigen or hyposensitization suggest the hypothesis that IgG4 antibodies are an associated marker for T-cell

suppression. This suppression may result in preferential toleration of high affinity antibody producing cells and may thus result in a predominantly low affinity response.

CONCLUSIONS

Antibody affinity must be considered as an important parameter of the immune response. It is evident that, under normal circumstances, high affinity antibody responses are more advantageous to the host than low affinity responses. The superiority of high affinity antibody in mediating various biological functions has clear implications for the handling of infectious micro-organisms. Inability to produce high affinity antibodies may result in poor elimination of foreign antigens from the circulation, persistent infection and the formation of phlogistic immune complexes. Preliminary evidence suggests that, in man, the IgG4 subclass is associated with low affinity antibody responses.

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5
Physiology and Clinical Significance of Secretory Antibodies

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Secretory immunoglobulins are specialized molecular forms of immunoglobulins, adapted to be transported into the moist environment of mucosal surfaces and external secretions. Most secretory immunoglobulin is of the IgA class. The development of this specialized molecular form of antibody is the most striking adaptation made in the immune system to the specialized circumstances of the body's mucosae, but it is not the only one. The immune system at the different mucosal surfaces - gut, respiratory, urogenital, mammary, etc., - is linked so that a common mucosal immune system can be recognized, which is in many ways separate and distinct from the systemic immune system of blood, bone marrow, lymph nodes and spleen[1]. This chapter will outline some of the characteristics of the mucosal immune system, relying largely on illustrations from the gastrointestinal mucosa, before describing secretory immunoglobulins in detail, and describing the abnormalities in mucosal immunity that lead to or arise in disease.

MUCOSAL IMMUNITY

The greatest external antigenic challenge to an individual lies at the mucosal surfaces. The skin excludes most antigens physically, whilst mucosal surfaces are relatively permeable, and many are colonized by bacteria. The antigenic challenge is greatest in the gastrointestinal tract, which contains over 10^{10} bacteria, and daily receives a complex macromolecular diet. The prime function of the gut is absorption, implying permeability. Even though the permeability of the small intestine to large and thus potentially antigenic molecules is limited, there is compelling evidence that small amounts of antigens are absorbed[2]. A protective immune system that responded to these antigens in the way that the systemic immune system does, by generation of complement-fixing and precipitating antibodies and cytotoxic lymphocytes, would be an inappropriate means of responding to this normal antigen load.

There are two major adaptations of the immune system to this dilemma – secretory antibody and tolerance.

Secretory antibodies

The response to a mucosally presented antigen involves the development of local specific antibody which is present in the mucosa and at the mucosal surface, but not necessarily in the circulation. The secretory IgA lining the surface of the gut mucosa has been described as an 'antiseptic paint', emphasizing its role against microbial antigens. However, under most circumstances the combination of an IgA molecule and its specific antigen does not lead to complement fixation, so the layer of protective antibody does not result in inflammation within the mucosa[3]. Rather, the protective action of the antibody is mediated in other ways. For example, IgA antibody at the luminal surface of the gut prevents the combination of the *Vibrio comma* enterotoxin with the enterocyte surface: without this attachment, the toxin cannot initiate the secretory processes in the enterocyte which lead to the clinical manifestations of cholera[4]. With regard to food antigens, the development of specific IgA antibody and formation of an antigen–antibody complex results in a larger molecular form of antigen, less likely to penetrate the mucosa. Antigen immobilized in this way at the mucosal surface is also exposed to proteolytic enzymes, and more likely, therefore, to be degraded.

Oral tolerance

The phenomenon of mucosal tolerance was first described at the gut mucosa, with the demonstration that feeding an antigen was associated with systemic non-responsiveness to that specific antigen if an attempt was later made to immunize an animal by a parenteral injection[5]. Teleologically this seems highly desirable: the mechanism avoids the generation of damaging systemic immune responses to small amounts of antigen which may be absorbed intact from the gastrointestinal tract. Perhaps because it seems so desirable, the generation of oral tolerance as a normal occurrence has been widely accepted, although in fact experimentally only certain well-defined feeding regimes appear to have this effect. The mechanisms for development of oral tolerance lie outside the scope of this chapter, but will be briefly discussed in the section of induction of secretory antibody responses below.

MUCOSAL ANTIBODIES

Historically the first description of secretory antibody was made by Davies in 1922, who showed that antibodies appeared in the faeces (coproantibodies) of patients with dysentery before they could be demonstrated in the circulation. IgA was described by Heremans in serum, but its significance in mucosal immunity became apparent when it was found to be the predominant immunoglobulin in mucosal secretions[6].

Structural aspects

Monomeric, m-IgA

This is the predominant (approximately 90%) form in the circulation in man. Its structure is entirely analogous to IgG, consisting of a single Y-shaped molecule formed (for IgA) from two α heavy chains and two light (κ or λ) chains. It has two antigen binding sites per molecule. The molecular weight of m-IgA is approximately 160000 daltons. The majority of circulating m-IgA is spleen and bone-marrow derived.

Dimeric, d-IgA

The combination of two monomers of IgA via a J (joining) chain results in dimeric IgA. This is the minority of serum IgA, but the majority of IgA synthesized in plasma cells adjacent to mucosal surfaces and destined to appear on secretory surfaces in the form of secretory IgA. The J chain is a short 15000 dalton chain which links the two monomers via disulphide bonds at the 'foot' of the two Y-shaped molecules. Like IgA, the J chain is a plasma cell product, and J chain synthesis is largely distributed in the sites from which d-IgA is derived, adjacent to mucosae. IgM producing plasma cells also synthesize J chain, for it also links the five monomers that make up the pentameric IgM.

Secretory, S-IgA

S-IgA is a combination of d-IgA, covalently linked to a secretory component (SC), the total molecular weight being approximately 395000 daltons. A small amount of higher molecular weight polymers is also detectable. SC is not a product of plasma cells but of epithelial cells, and in rodent species, hepatocytes. SC is intimately linked with the transport of d-IgA through epithelial cells to reach the mucosal surfaces as discussed later. The secretory form of IgA is significantly more resistant to proteolysis than the native IgA, which is clearly an advantage for a protective immunoglobulin lying within the intestinal lumen[7].

Initiation of a secretory antibody response

The first step in the initiation of an immune response is the uptake and processing of antigen. Macromolecular uptake can take place via various routes: in the gut for example, endocytosis of whole proteins can take place through enterocytes, or persorption of particulate matter can occur through epithelial extrusion zones at the tips of villi. Particular attention has been paid to the specialized structures of the gut associated lymphoid tissue (GALT) which has counterparts in other mucosae, for example the bronchus associated lymphoid tissue (BALT)[8].

In the gut mucosa, the Peyer's patch of the distal small gut, and the smaller lymphoid follicles found elsewhere in the lamina propria, are overlaid by a specialized cell – the M-cell, or follicle-associated epithelial cell[9]. These are

ultra-thin cells, which rapidly transport antigen from the gut lumen to the follicle beneath, where uptake into macrophages and interaction with lymphocytes occurs. Initially described in the human small intestine, analogous cells have now been shown to be also present in the colon. In experimental infections, bacteria preferentially attached to the follicle-associated epithelial cell, which thus acts as a rapid portal of entry for foreign antigens, presumably permitting a rapid initiation of an immune response[10].

Within the lymphoid aggregate, macrophages can phagocytose and presumably process antigen. Whilst there is good evidence that both humoral and cell-mediated immune responses are initiated within Peyer's patches, full development to effector cells does not occur at this site, and IgA plasma cells and IgA release are not seen. A cardinal event in the lymphoid aggregate, however, is the commitment to IgA as the predominant isotype of immunoglobulin, which may be linked with the initiation of systemic tolerance.

The processes leading to the occurrence of IgA as the major isotype are controversial, but might be related to isotype switching. Isotype switching is the process in committed B-cells which, for example, leads to the predominance of IgG production in a secondary systemic antibody response after initial IgM production (see Chapter 3). This reflects the sequential expression of the genes controlling the constant region of the Ig molecule. One school of thought argues that, as in mice, terminal deletion of genetic material leads to sequential encoding of μ, γ, ϵ, and α chains and, therefore, the predominance of α chains in Peyer's patches merely reflects the longer maturation time of plasma cells in that site[11]. There is, however, probably more convincing data, based on *in vitro* analysis of the functional effects of cloned T-cells from Peyer's patches, to suggest that a distinguishing feature of mucosal associated lymphoid tissue is the presence of T-cells which directly switch IgM producing B-cells to IgA production – the T 'switch' cell[12].

In either case, the subsequent elaboration of IgA is strongly T-cell dependent, as is shown not only by *in vitro* analysis of lymphocyte behaviour, but by the IgA deficiency found in T-cell deficient mice, and by the deficient IgA production noted in some clinical T-cell deficiencies in man.

Analysis in rodents of the T-cell dependency of IgA expression offers some clues to the mechanisms underlying the development of oral tolerance. It is now widely accepted that the generation of antibody is closely modulated by T-cells, both helper and suppressor T-cells being involved. Suppressor T-cell activity can be stimulated in culture by certain mitogens such as concanavalin A, and T-cells from the spleen stimulated in this way can be shown to suppress the generation of all classes of immunoglobulin by polyclonally stimulated B-cells. In contrast Con-A-activated T-cells from Peyer's patches of mice, while similarly suppressing IgG and IgM, were shown to substantially enhance IgA synthesis[13]. This differential effect, suppressing IgG and IgM but stimulating IgA, can be shown to affect the specific antibody responses following feeding of an oral antigen. In principle, therefore, the differential help and suppression of immunoglobulin classes offers a mechanism by which antigen-feeding induces a local immunoregulatory cell that helps the predominant immunoglobulin of the local immune system, but suppresses those that predominate systemically.

As already indicated, plasma cells do not mature to produce IgA and hence export this isotype in the local aggregates of the mucosal system. The IgA cell precursors traffic to the local mesenteric lymph nodes, then via thoracic duct lymph to the systemic circulation, and finally back home to the lamina propria. However, this homing is not only to the immediate area in which the immune response was initiated, but throughout the mucosal surfaces of the body. Thus an immune response initiated in response to a luminal antigen in the distal small intestine is eventually distributed, in the form of IgA plasma cells making specific antibody, to other areas of the intestine and also the mucosae of the respiratory and other tracts. This wide distribution indicates that the presence of antigens overlying the mucosa cannot be solely responsible for the homing of antigen to these sites, although it plays some role[14].

IgA transport

The process of transport of d-IgA from the lamina propria to the surface of the gut was initially inferred from immunofluorescent studies in the mouse which localized the immunoglobulin and secretory component, and this has been subsequently confirmed in colonic cancer tissue culture studies. In brief, polymeric J chain-containing IgA combines with a secretory component, which is a product of the epithelial cell, and transcellular vesicular transport of the resulting S-IgA leads to the appearance of S-IgA at the cell surface[15].

The combination of d-IgA and SC occurs irrespective of the subclass of α-chains that are present. The SC is synthesized within the rough endoplasmic reticulum of the enterocyte, and transported to the basal–lateral membrane of the cell via the Golgi apparatus. In its initially synthesized form the molecular weight of SC is approximately 95000, but an 80000 molecular weight moiety ends up in combination with d-IgA at the mucosal surface, part of the original molecule being lost after fulfilling its role either in presentation of SC at the enterocyte surface, or in transcellular transport[16].

This local transport of d-IgA to the adjacent epithelium is well established. In the case of the rodent gut, recent years have highlighted an alternative means by which S-IgA appears within the gut lumen. The liver has been shown to function as an 'IgA pump', due to the expression in this species of SC on the surface of hepatocytes. Dimeric-IgA in rats is the predominant circulating form of IgA, in contrast to man. The d-IgA combines with SC on the hepatocyte surface, and by a transcellular vesicular transport mechanism analogous to that seen in the enterocyte, is transported into bile, and thence to the upper gastrointestinal tract[17]. So potent is this mechanism in rats that over 50% of an injection of labelled d-IgA can be recovered from the biliary tract within 4 hours, and as much as 50% of the intraluminal IgA in the gut may be derived from this route. In man there is good evidence that a similar transport system exists, but it is much more limited. The hepatic expression of SC is limited to the biliary epithelium, so that only relatively small quantities of d-IgA can be extracted from the bloodstream, and secreted into bile. For example, less than 2% of injected labelled d-IgA was recovered from bile after 24 hours in patients with total external biliary drainage[18]. This route in man

can make an important contribution to biliary defence, contributing approximately half of biliary IgA, but must make only a minor contribution to gut defences.

The attention given to the trans-hepatic transport of IgA has focused attention on two functional aspects of the IgA system. The first is the transport not only of uncomplexed IgA, but of antigen–antibody complexes. Experimentally, immune complexes of polymeric IgA and dietary antigens in mice have been shown to be taken up by hepatocytes and to be excreted into bile, a mechanism which would appear to offer a valuable means of clearing small quantities of gut-derived antigens from the circulation[19]. The second aspect is the significance of secretory component at any site – biliary, mammary, urogenital, respiratory or gastrointestinal – in transporting dimeric immunoglobulin to the local mucosal surface that was synthesized adjacent to a different mucosa, but which has diffused thence into the bloodstream. This is thus a further mechanism, reinforcing that of the initial migration of plasma cell precursors to differing mucosal surfaces, which ensures that there is a wide distribution of the effector arm of the humoral immune response to all mucosal surfaces.

Other mucosal antibodies

When mucosae are inflamed, serum immunoglobulins can reach the surface, probably via intercellular pathways. The dilated lymphatics of intestinal lymphangiectasia can weep all classes of immunoglobulins on to the gut surface. However, IgM can also function as a true secretory immunoglobulin, reaching the mucosal surface by the identical SC-mediated transcellular pathway that transports IgA, reflecting the presence of J chain within the polymer. Little IgM, however, usually takes this pathway, as the diffusion characteristics and relative abundance of the smaller d-IgA make it a successful competitor for the system, but in conditions such as selective IgA deficiency secretory IgM can be of importance.

CLINICAL IMPLICATIONS OF SECRETORY ANTIBODIES

Diffuse inflammation in the mucosal surface is associated with enhanced numbers of plasma cells, with increased numbers not only of IgA producing cells, but of plasma cells of other classes. In chronic inflammatory conditions an increase in IgG plasma cells may be most striking. Any inflammatory process, therefore, may be associated with increased production of secretory antibody, although epithelial damage, transudation of serum derived proteins, and the difficulties of quantitating IgA in secretions (due to the effects of proteolytic enzymes, different molecular forms, etc.) make assessment of this difficult. Studies of secretory antibody in respiratory mucosal washings have demonstrated the anticipated specificities to respiratory pathogens such as *Haemophilus influenzae* and *Pseudomonas aeruginosa*[20].

The IgA producing plasma cell within the mucosa can be the site of

malignant change in α chain disease. Considerable interest attaches to this proliferative condition, which predominantly affects the small gut, although respiratory forms of the disease are reported. The spectrum of immunoproliferative small intestinal disease ranges from a benign proliferation to a disseminated lymphomatous process[21]. The condition has a strong geographic distribution, being initially reported from the Middle East and Mediterranean, with a rural preponderance. It appears that there is a benign proliferation of IgA plasma cells in the early stage which, however, usually progresses to the full picture of Mediterranean lymphoma, with diffuse lymphomatous infiltration in the small gut and elsewhere. The disease is characterized by an abnormal α chain which is present both in serum and secretions. The abnormal IgA can be delivered from the mucosa to the lumen via the secretory component transport mechanism, being linked to SC in jejunal secretion. Some epidemiological and pathological observations suggest that over-stimulation of the mucosal immune system by repeated enteric infections may underlie the hyperplasia and eventual neoplasia of the IgA system that α-chain disease represents[22].

SECRETORY ANTIBODY DEFICIENCY

The purest form of secretory antibody deficiency would be that which would follow a deficiency of secretory component, resulting, therefore, in an inability to transport J chain containing Ig to the mucosal surface. There is a single report of a patient with SC deficiency, and chronic intestinal candidiasis, but the follow-up of this patient suggested that the SC deficiency was not permanent. There are, however, many patients in whom mucosal antibody deficiency reflects a relative or absolute deficiency of either IgA alone or IgA with other immunoglobulin classes, in some cases with additional cell-mediated immune deficiencies. A brief classification of these antibody deficiency syndromes is shown in Table 5.1.

Table 5.1 Immune deficiency syndromes in which lack of secretory antibody may occur

Antibody Deficiency Syndromes
Humoral deficiencies
X-linked agammaglobulinaemia
Common variable hypogammaglobulinaemia
Selective IgA deficiency
(Secretory component deficiency)
Immunodeficiency with elevated IgM
Combined cellular and humoral deficiencies
Severe combined immunodeficiency
Wiskott-Aldrich syndrome
Ataxia - telangiectasia
Immunodeficiency with thymoma

SELECTIVE IgA DEFICIENCY

This is the commonest immunodeficiency seen in clinical practice, the incidence in the general population being between 1 in 500 and 1 in 700. Clearly many selectively IgA-deficient individuals are normal, and the commonest explanation for this is that an effective secretory antibody function is provided by IgM in these circumstances. Immunofluorescent evidence supports this. It is interesting that the use of IgM antibody in this way, despite its strong complement fixing propensities, does not result in a chronic inflammatory state in the gut epithelium. This presumably reflects the lack of availability of complement at the luminal surface, and argues against one of the major theoretical justifications for the development of the IgA system outlined above, i.e. its relative lack of complement fixing ability. Another explanation of the disease susceptibility of only a proportion of patients with IgA deficiency is that an additional systemic immune abnormality, a deficiency of IgG2, may be required to predispose to disease[23] (see Chapter 3).

The deficiency of IgA is normally defined on the basis of circulating levels, and it can be either relative or absolute, with either low or absent IgA levels, although clinical manifestations may occur in either case. These manifestations are outlined in Table 5.2.

Table 5.2 Clinical manifestations of IgA deficiency

Acute and chronic sinopulmonary infections
Atopic disorders
Autoimmune disorders
Pernicious anaemia
Coeliac disease
Crohn's disease
Gastrointestinal infections

Atopy

Amongst both small groups of children and large populations of adult blood donors, there is suggestive evidence that selective IgA deficiency is associated with an increased incidence of atopic disorders, which reflects an enhanced tendency to form IgE antibody to common extrinsic allergens. The suggested mechanism is that in the absence of a normal first line of mucosal defence, a local IgE-mediated response takes place in that site. This may account for the development of many childhood allergic diseases, particularly as colonization of the gut lamina propria with IgA bearing cells is progressive after birth, with relatively low densities of these cells and low serum IgA levels for a few months until normal adult levels are achieved. It is suggested that withdrawal of potential allergens, particularly cow's milk, during this period of immaturity, may prevent genetically at-risk infants developing systemic atopic disorders such as atopic eczema[24].

Infections

Predictably IgA deficiency is associated with infections at mucosal surfaces. Of the immune deficiencies predisposing to recurrent sinopulmonary infections, selective IgA deficiency is the most common, accounting for about 75%[20]. These recurrent infections may occur without obvious sequelae in the respiratory tract, or be associated with chronic sinusitis or bronchiectasis. It is of interest that a proportion of patients with advanced chronic bronchitis show a relative lack of abundance of IgA plasma cells compared with normal[25].

In the gastrointestinal tract, recurrent or persistent infections are associated with IgA deficiency, including giardiasis and bacterial overgrowth. However, these are relatively rare, and screening unselected patients with giardiasis for selective IgA deficiency is an unrewarding process.

Autoimmune disorders

A high incidence of autoantibodies in patients with IgA deficiency is recorded with about 5% of patients having an autoimmune disorder. In Ammann and Hong's series[26], 26 had rheumatoid arthritis, 10 systemic lupus erythematosus, 8 thyroiditis, 6 pernicious anaemia and 5 pulmonary haemosiderosis (an alternative explanation for the pernicious anaemia is given below).

Other gastrointestinal diseases

Pernicious anaemia in association with gastric atrophy occurs in some families with IgA deficiency. Coeliac disease, and arguably Crohn's disease, are relatively more common in patients with selective IgA deficiency – 1 in 50 patients with these diseases being IgA deficient. It is tempting to ascribe this in both cases to the gut being the site of expression of an immune response directed against gut antigens (wheat protein in the case of Coeliac disease, and a variety of bacterial or other gut antigens in the case of Crohn's disease) which have developed in the absence of the antigen-excluding secretory antibody. Even in the apparent absence of gastrointestinal disease, patients with selective IgA deficiency have a higher than normal incidence of circulating anti-food antibodies.

PANHYPOGAMMAGLOBULINAEMIA

A variety of diseases can be associated with a decrease in all classes of immunoglobulin, and a combined loss of IgA and IgM theoretically leaves the mucosa bereft of secretory antibodies. In fact these deficiencies tend to be relative rather than absolute, but nonetheless patients with panhypogammaglobulinaemia have a variety of complications that seem directly attributable to a loss of secretory antibody. Clearly, however, the loss of systemic immunity is also a major contributing factor, and in severe antibody deficiency states such as X-linked agammaglobulinaemia, recurrent severe systemic infections, including pneumonia, meningitis and septicaemia, will predomin-

ate. The manifestations of local immune deficiency tend to be of more importance in patients with common variable hypogammaglobulinaemia, (otherwise known as late onset hypogammaglobulinaemia). In these patients, there is normally a substantial depression but not a complete absence of systemic and local antibody.

There are several manifestations in these patients which reflect the absence of mucosal defences.

Respiratory infections

These account for approximately half the episodes of illness in patients with antibody deficiency, with the lower respiratory tract being more frequently affected than the sinuses[20]. Chronic respiratory tract changes include bronchiectasis, pulmonary fibrosis and chronic purulent bronchitis.

Gastrointestinal disease

Almost any part of the gastrointestinal tract can be abnormal. Progressive elucidation of the inflammatory disorders occurring in the gastrointestinal tract in association with an absence of secretory antibodies has identified new pathogens, or the persistent carriage of pathogens which normally give rise to self-limited disease.

Gastric complications

Gastric atrophy can occur which has been attributed to the development of autoimmune cell-mediated damage to the gastric mucosa[27], and this damage certainly takes place in the absence of antibodies to gastric parietal cells or intrinsic factor. The gastric atrophy can be associated with vitamin B12 deficiency, but B12 deficiency may also occur as a result of malabsorption from the intestine in the presence of bacterial overgrowth or giardiasis. There is a high incidence of gastric malignancy, possibly reflecting bacterial overgrowth in the absence of protective antibody (and acid), and favouring the carriage of these bacteria that generate carcinogens from ingested food.

Small intestinal disease

Chronic diarrhoea is common, often reflecting a giardia overgrowth that may be difficult to eradicate. Giardial infestation can cause mucosal damage, or small intestinal atrophy may occur in the absence of identifiable infection (hypogammaglobulinaemic sprue)[27]. Some patients have diffuse nodularity of the small intestine, corresponding anatomically to markedly exaggerated submucosal lymphoid aggregates.

In addition to giardiasis, other chronic infections occur with organisms such as coccidi and cytomegalovirus.

Colonic disease

A chronic inflammatory infiltrate in the colon, for some time attributed to an

'immune' disorder, has recently been attributed to persistence of infection with the normally self-limited pathogen Campylobacter.

CONCLUSIONS

Secretory antibodies play a vital protective role at mucosal surfaces. Whilst common infectious diseases, particularly of the gastrointestinal tract, remain the major killers in the world, further work is required to enhance our understanding of their production, and particularly to allow the development of immunization schedules which offer firm protective immunity at mucosal surfaces.

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6
Methodological Aspects of Serum Immunoglobulin Assays

P. A. E. WHITE AND A. MILFORD WARD

INTRODUCTION

Immunoglobulins can be assayed by any standard immunochemical technique. The choice of method for any particular immunoglobulin analyte depends on the expected concentration range in the biological fluid, the physicochemical characteristics of the analyte and the logistics of the workload in the specific laboratory environment. This latter factor, in particular, may dictate the choice between manual and machine methodologies.

The mass concentration range encompassed by the immunoglobulins in serum is large; from mean values of 25 μg/l for IgE to 10.00 g/l for IgG. Second or third order reaction methods, either in gel or fluid phase, are applicable at the higher concentration ranges but labelled assays are essential for the lower concentration analyte such as total and allergen-specific IgE. The choice of method may also be influenced by the availability of equipment acquired primarily for other analytical purposes. This is of particular relevance in the choice of turbidimetric assays for IgG, IgA and IgM when a laboratory has available analytical capacity on a centrifugal analyser primarily purchased for general clinical chemistry.

REVIEW OF AVAILABLE METHODS

Single radial immunodiffusion

The quantitation of protein analytes by radial immunodiffusion[1] requires diffusion in the gel phase to proceed to completion. This is time-consuming, taking some 2–3 days for smaller molecular weight proteins such as IgG, and 6–7 days for the larger molecular weight proteins such as IgM. A modification of the technique[2] allows the diffusion rings to be measured after

a limited time, usually 18 hours, which makes the technique more applicable to the clinical situation but which introduces elements of imprecision in that the high concentration samples do not reach completion and the resultant calibration graph is flattened at the upper extremity.

Methodology

Monospecific antibody to the appropriate protein analyte is uniformly dispersed through an agar or agarose gel. A measured volume of serum or calibrant is placed in wells cut in the gel and allowed to diffuse radially in a moist atmosphere. As the antigen diffuses into the antibody containing gel, the antigen–antibody complexes precipitate at the point of equivalence. The advancing front of antigen causes the precipitate to redissolve and the leading edge advances progressively until completion when all free antigen is consumed. A ring of precipitation is formed, the area of which is proportional to the concentration of antigen. In practice the diameter of the precipitin ring is measured, and a calibration curve constructed by plotting the square of the diameter against antigen concentration. If the reaction is allowed to proceed to completion the resultant calibration plot is a straight line. In the time-limited reaction the plot shows variable degrees of flattening at high antigen concentration[3].

Despite advances in methodology single radial immunodiffusion remains the universally accepted reference method for the measurement of serum IgG, IgA, IgM and IgD. It is also applicable, with modification, to the measurement of IgG subclasses using monoclonal antibodies. However, some enhancement of precipitation is required, and this may be achieved by the incorporation of polyethylene glycol (PEG 4000) into the agarose gel[4].

Commercial kits comprising prepared gels and ready-to-use calibrants are available from numerous sources. Using these kits immunoglobulin quantitation can be performed by any laboratory with an acceptable standard of precision and at a reasonable cost. With between six and twelve wells per plate, these kits are ideally suited to those laboratories with a relatively small clinical workload.

Despite the universal acceptance of the method, certain limitations and potential problems must be borne in mind.

Measured volumes of serum should be added to the wells using an accurate microlitre syringe. Imprecision will result from attempts to fill the wells to the brim because of inconsistencies in reading the resultant meniscus.

Antigen excess situations which may result from grossly elevated immunoglobulin levels may cause precipitation to occur in the reverse slope of the Heidelberger curve (Figure 6.1) where ring diameters *decrease* with increasing antigen concentration.

Single radial immunodiffusion is also influenced by the molecular radius of the antigen. Problems may be encountered in cases where there is a marked discrepancy in molecular radius between the test samples and the calibrants. Such a situation may be seen in respect of IgM where pathological monomeric (7S) IgM will be overestimated due to the increased diffusion in relation to the normal pentameric (19S) calibrants. Similar problems will be

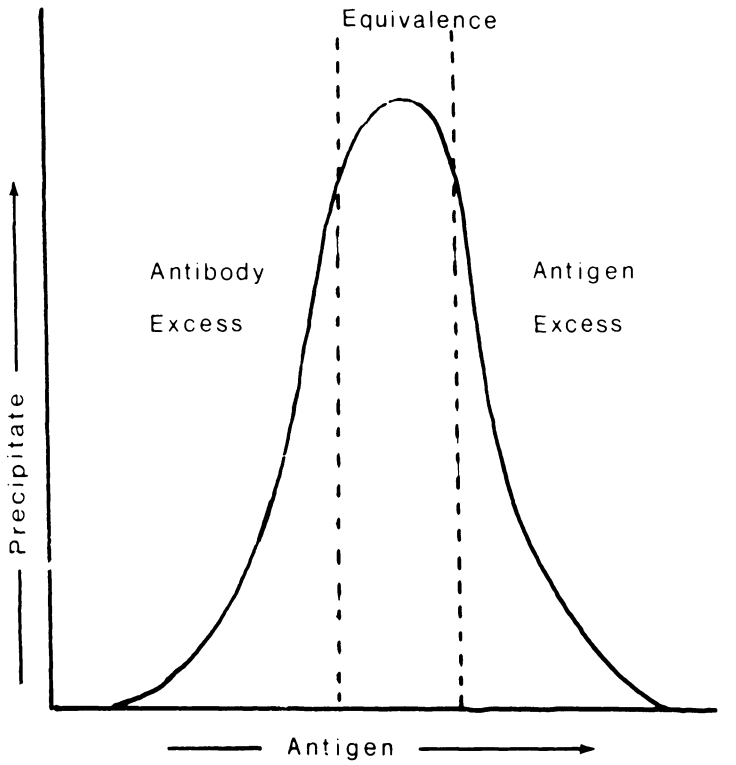

Figure 6.1 Precipitation curve for increasing antigen concentration in the presence of constant antibody concentration

encountered in samples with a significant proportion of high molecular weight species (26S) where values will thus be underestimated.

Monoclonal immunoglobulins, by virtue of their idiotypic restriction, are always liable to be in relative antigen excess even when the notional concentration is within the limits of the calibration curve. It is for this reason that immunochemical methods should never be used for the quantitation of monoclonal components, and their presence should be excluded by zone electrophoresis prior to immunoglobulin quantitation.

Limiting factors of the method are precipitin ring diameter and density. Enhancement of the rings by immersion of the gel in 1% tannic acid or drying and staining can improve the sensitivity, whilst ring diameters may be modified by adjustment of antiserum concentrations. For practical purposes the lower limit of sensitivity is in the order of 5–10 mg/l. In practised hands precision of ±2% can be achieved, but values of ±8–10% are more usual.

Electroimmunodiffusion

Electroimmunodiffusion or 'rocket' immunoelectrophoresis is an extension of single radial immunodiffusion, the migration of antigen into the antibody-

containing gel being assisted by an electrophoretic field. The technique was originally described for the quantitation of albumin[5], but can be used for any anodal migrating protein. The quantitation of immunoglobulins by electro-immunodiffusion requires some modification of their normal cathodic migration. This can be achieved by carbamylation[6] or formylation[7].

Methodology

Antigen is caused to migrate into an antibody-containing gel under the influence of an electrophoretic field. The advancing front of antigen forms a visible precipitate of antigen-antibody complexes which gradually advances through the gel and can be likened to a vapour trail. At completion the precipitate converges to a point. Peak height is proportional to concentration. For optimal performance and precision antigen and antibody concentrations should be adjusted to give final peak heights of between 10 and 40 mm. Accurately measured volumes of sample should be added to the wells with the current switched on, in order to limit radial diffusion – a potential source of imprecision.

Measurement of peak heights is prone to error. Considerable imprecision will be produced if peak heights achieved are less than 10mm. Problems are also encountered with cigar-shaped precipitates extending on both the anodic and cathodic sides of the application well, usually due top inadequate carbamylation or formylation of cathodic migrating proteins. The effective lower limit of sensitivity is in the order of 5 mg/l but the technique will allow identification of the absence of a protein. Precision of ±3% can be achieved but ±8–10% is more usual except in the most practised hands.

Nephelometry

Quantitative immunoassay of proteins in the fluid medium began with the quantitative precipitin technique[8]. Libby[9] applied the principles of nephelometry to the antigen-antibody reaction, but it was Shultze and Schwick[10] two decades later who described the first clinically applicable method. Ritchie[11] adapted the technique to the Technicon(R) continuous flow methodology, and the method was further refined[12] by the addition of polyethylene glycol to augment and accelerate the immune reaction. The adoption of the helium-neon laser as a stable light source[13,14] led to the development of 'laser nephelometry'.

Nephelometry is the measurement of light scattered at an angle from the beam path by particles in a clear medium[15]. The optimum wave length is selected according to the size of the particles to be measured, and the degree of light scatter is proportional to the antigen concentration provided antibody concentrations are maintained at a constant level. The initial systems[16,17] were based on the measurement of light scattered at 90° to the beam path and suffered from a lack of sensitivity. The current generation of nephelometers have resolved this problem by changing to measurement at a forward angle of 30–35°.

Methodology

(1) *Automated immunoprecipitation*

The method is based on the Technicon Autoanalyser II system. Serum samples and saline are aspirated into the system alternately at a rate of up to 120 samples/hour and injected into an antiserum stream. The antigen–antibody complexes formed in a mixing coil during a 3 minute reaction time are measured as light scattering units in a fluoronephelometer. Because of the inherent 90° light scattering properties of serum, a test blank is required.

(2) *Laser nephelometry*

The amount of light scatter, and hence the electronic signal, in a nephelometer is highly dependent on the initial light energy. The high light intensity that can be generated by a monochromatic laser beam offers a substantial theoretical increase in sensitivity. This is further enhanced by the mounting of the optical detection system at the 30–35° forward angle.

Test samples and appropriate blanks are incubated with appropriate concentrations of antiserum for 15–60 minutes; they are then introduced into the beam path and the scattered light converted into an instrument reading. Most systems have the ability to electronically subtract blank from test readings and the more sophisticated will compute results from the calibration curve. Both discrete and automated systems are available.

(3) *Reaction rate nephelometry*

The Beckman ICS™ is an example of kinetic nephelometry. The instrument measures the maximum rate of change of light scattering at a forward angle of 70° during the first 60 seconds after mixing of antigen and antibody. Under antibody excess conditions a mathematical relationship exists between rate of change of light scatter and antigen concentration. The non-linear maximal rate data is converted into a linear concentration plot to allow single point calibration. The filtered tungsten light source is claimed to provide a better signal–noise rate monitoring than a laser source.

Nephelometric assays can be used for routine laboratory measurement of IgG, IgA, IgM and IgD. The early systems, with 90° acceptance optics, had limitations in terms of low range sensitivity and relatively high serum blank effect, which made it impractical to measure concentrations of less than 200 mg/l. This lower limit could be reduced to 5–10 mg/l in relatively clear fluids such as CSF. The forward angle instruments suffer less from the blank effect but various interfering constituents of serum will produce artefactual light scattering, the intensity of which is concentration-dependent. Chylomicrons induce considerable light scattering but this can be reduced by prior filtration of the sample. Dust or bacterial contamination of reagents will also cause high blank values and all reagents should be subjected to 0.2 μ filtration before use. Heparin plasma is unsuitable for any method that employs polyethylene glycol enhancement because of a complex formation between the heparin and PEG with a resultant artefactual light scattering.

All nephelometric systems require antiserum of high avidity as well as high titre. For optimal performance antisera should be evaluated in the particular system prior to use in clinical assays. An antiserum which performs well in gel phase assays will not necessarily be suitable for the nephelometric mode.

Antigen excess

The major problem encountered in all fluid phase methods is the identification of antigen excess. In constant antibody concentration, the amount of precipitate increases in relation to antigen concentration until the point of equivalence is reached. Further addition of antigen leads to the production of smaller, soluble, complexes and a *reduction* in the amount of precipitate. Any measured amount of precipitate, or light scattering, can, therefore, be equivalent to two concentrations of antigen. In practice the fluid phase assays are devised to give a linear response in the antibody excess limb of the Heidelberger curve. In the clinical assay it is essential to be able to identify those samples that fall outside the linear response range and give a light scattering response on the antigen excess limb of the curve.

In the Automated Immunoprecipitation assay antigen excess is comparatively easy to identify, the condition being characterized by a bifid peak on the chart recorder. The bifid nature of the peak is produced by the leading and trailing edges of the sample bolus being in antibody excess. In contrast, the laser nephelometric systems must rely on relatively high antibody concentrations to minimize the risk of antigen excess. The automated versions will 'flag' samples whose analytical data falls in the zone of equivalence or in antigen excess, and demand retesting at dilution. The kinetic system is programmed to reject over range peak rate values and indicate that samples should be retested at dilution. Values derived from the zone of equivalence are verified by the addition of more antibody. In the manual systems identification of antigen excess can prove more problematical and demands an element of clinical suspicion by the technician.

In serum the effective lower limit of sensitivity of the nephelometric assays is in the order of 100 mg/l and 5 mg/l in clear fluids such as CSF. Precision is in the order of ±5–6% for the laser nephelometric techniques and ±1–4% for the reaction rate assay.

Turbidimetry

The determination of specific proteins by turbidimetry has proven to be rapid, precise and accurate. Although most photometric analysers can be used to measure the absorption of light by immune complexes, with the introduction of centrifugal fast analysers into the routine clinical biochemistry laboratory has come the realization of how easily immunochemical analyses can be performed. Methods for quantitation of IgG, IgA and IgM have been adapted for use in all centrifugal fast analysers using either end-point or kinetic chemistries[18,19].

Methodology

This method involves the pipetting of calibrants or samples and diluted antiserum into chambers arranged concentrically in a cuvette or transfer disc. During the spin-phase antigen and antibody are expelled into optical cuvettes by centrifugation at approximately 1000 r/min, mixing of the reactants being augmented by agitation or simultaneous application of a vacuum. The spinning cuvettes pass through a focused beam of light, some of which is absorbed by the antigen–antibody complexes. Light passing through the cell to a photomultiplier tube generates a signal proportional to intensity.

The increase in turbidity, and hence optical density, with increasing antigen concentration is not linear (Figure 6.2). There is an initial lag phase (T_o–T_i) followed by a steep rise (T_i–T_m) and a plateau (T_m–T_f).Reaction rate data (T_i–T_m) is used in the kinetic assays, whereas terminal optical density (at T_f) is used in the end-point assays. Immunoglobulin assays are usually treated as end-point assays with antigen excess identification based on the reaction kinetics (T_i–T_m). Standard curves and sample values can be calculated manually from optical density data but are more usually produced on-line by on-board or external computer with multiple curve-fitting programmes.

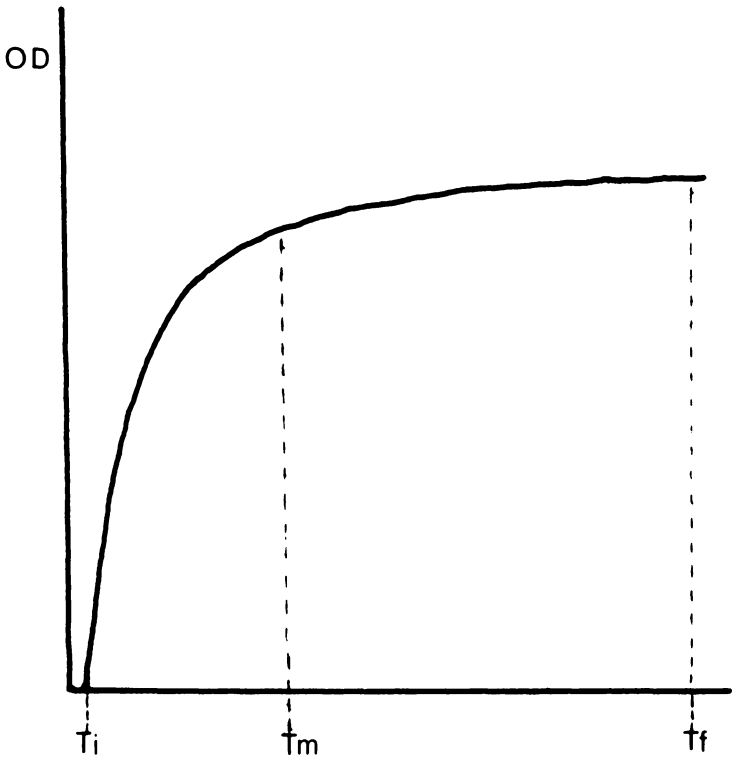

Figure 6.2 Reaction curve in turbidimetric immunoassay. T_i, time of initiation of reaction; T_m, time of maximal response; T_f, time of end-point reaction with no further increase in absorbence

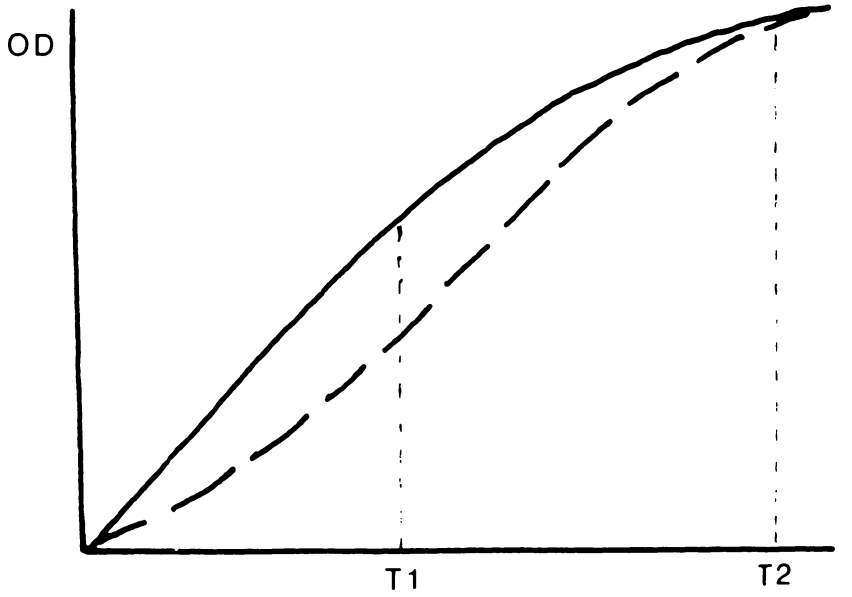

Figure 6.3 Antigen excess check - turbidimetry. Solid line represents sample (standard) in antibody excess; dotted line represents sample in antigen excess

Antigen excess

The rate of formation of antigen-antibody complexes shows subtle differences when either antigen or antibody is in excess. Figure 6.3 shows the kinetic curve profile for two samples, one in antibody excess and one in antigen excess. Whilst both achieve identical maximum optical densities at T_2, that in antigen excess shows a pronounced lag phase and a lower optical density at T_1. The most sophisticated antigen excess check available at the present is that in the Baker Encore Specific Protein Analyser. The immunoglobulin assays are run as end-point chemistries, but the individual reactions are monitored in relation to two adjacent standards at 12 second intervals throughout the spin phase. Antigen excess is identified as a deviation in the relationship between optical density of test sample and standards which should remain constant throughout the reaction (Figure 6.4). The effective lower limit of sensitivity for the turbidimetric assays is 100 mg/l in serum and 5 mg/l in CSF. Precision is in the order of ±2–4% and, in contrast to the manual techniques, does not vary with the workload.

Immunofluorometric assays

Immunofluorometric assays have the potential of offering a wide analytical range with the increased low range sensitivity which is inherent in the labelled immunoassay systems, the assays no longer being reliant on relatively large reaction masses of antigen and antibody to produce a visible precipitate. Immunofluorometric assays for IgG and IgM are available but have not

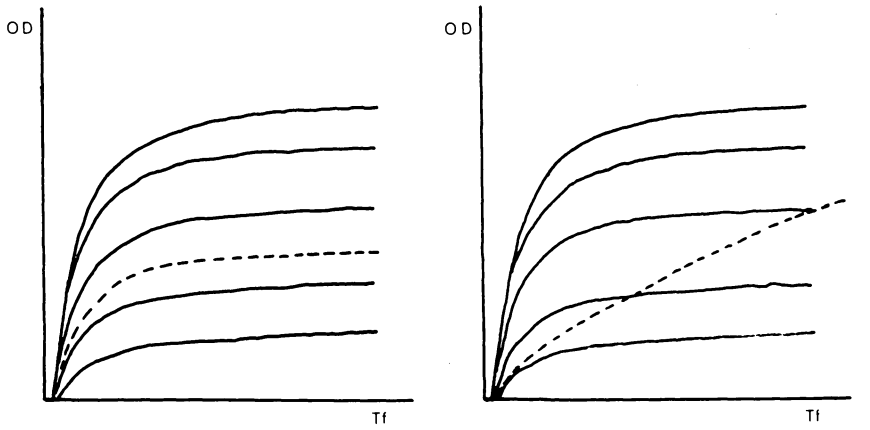

Figure 6.4 Antigen excess check (Baker Encore system). Solid line, absorbent curves for standard(s); dotted line, absorbent curve for sample; (a) antibody excess, (b) antigen excess

achieved any significant degree of acceptance, mainly due to the relative scarcity of available instrumentation.

Methodology

(1) *Non-homogeneous assays*

Two commercial systems, FIAX and Immunofluor, are available and have been shown to produce results comparable with single radial immunodiffusion[20] and nephelometric or turbidimetric techniques[21]. In the FIAX assay purified antigen bound to a sampler surface is reacted with diluted sample and fluoroscein conjugated antibody in a conventional competitive binding assay. Fluorescence intensity on the solid phase is proportional to analyte concentration. The analytical system comprises a modular series of instruments including a fluorimeter which is programmed with a magnetic card and included in each reagent kit. The immunofluor assay works on the same principle, but is not dependent on integral components. The solid phase polyacrylamide bead is processed in a tube assay which requires relatively long incubation periods and a centrifugation step to separate the free and bound fractions. The method is adaptable to continuous flow automation using antibody coated magnetizable particles[22].

(2) *Homogeneous assays*

The main advantage of the homogeneous assay is the avoidance of a separation phase which simplifies the procedure and removes the major area of imprecision. Fluorescence quenching assays, commonly used in drug estimations, depend on the decrease in signal from labelled antigen as a result of antibody binding. Assays of this type are not applicable for large protein molecules.

Nargessi[22] described an indirect quenching assay for the measurement of serum albumin which could be adapted for immunoglobulin determination.

Anti-fluoroscein antibody is added to the conventional reaction mixture of sample, antibody and fluoroscein conjugated antigen. The fluoroscein radicals of the free labelled antigen are quenched by binding anti-fluoroscein antibody whilst the bound fraction is protected from similar quenching. The degree of quenching in the reaction mixture is proportional to the relative concentration of free and bound fractions.

Despite their theoretical increased sensitivity, the immunofluorometric assays offer little advantage for the measurement of serum immunoglobulins.

Enzyme linked immunosorbent assays (ELISA)

Enzyme linked assays present another method of potential sensitivity to rival that of fluorochrome or isotope conjugated assays. Pioneered by Engvall and Perlmann[23] and Van Weemen and Schuurs[24], enzyme linked immunosorbent assays are commonly used for the measurement of specific antibody to infectious agents. They are, however, equally applicable to the measurement of antigen. The sensitivity of the method is not necessary for the measurement of serum IgG, IgA and IgM, but does offer advantages for the quantitation of IgG subclasses and IgE. Commercial application is, at present, restricted to the measurement of total and allergen-specific IgE (see Methods for IgE).

Methodology

Antibody is passively adsorbed to a solid phase. The solid phase may be either polystyrene, polypropylene or polyvinyl beads, tubes or microplates. The mechanism of passive adsorption is ill understood and may lead to unexplained batch-to-batch variation. The principles of immunoglobulin assay are those of a double antibody sandwich or immunometric assay. The diluted sample is first incubated with the precoated solid phase antibody. After washing, enzyme conjugated antibody is added for a second incubation period. The assay is completed by a colour development reaction utilizing specific chromogenic substrates. Colour development is proportional to the concentration of antigen.

The enzymes used in this methodology include β-galactosidase, peroxidase, alkaline phosphatase and glucose oxidase. It is usual to use affinity purified antibodies in conjugates but a total immunoglobulin fraction may perform adequately in many assay systems.

The lower limit of sensitivity which may be achieved in the immunometric mode equates well with that of the isotopic assays at 5–10 μg/l. With the exception of batch-to-batch variability of the adsorption of antibody to the solid phase, precision is in the order of $\pm 5\%$.

IgE assay methods

IgE differs from the other serum immunoglobulins in that the serum levels are below the sensitivity limits of conventional immunoassay methods. Quantitation of total serum IgE has traditionally been performed by isotopic labelled assays, radioimmunoassay or immunoradiometric assay. More recently, concern over the possible environmental hazards of radioactivity has led laboratory workers and manufacturers to research alternative labelled immunoassays which can give the same degree of sensitivity and precision without the associated potential hazards. This has led to the increasing popularity of enzyme and fluorescent labelled assays.

The first practical assay for serum IgE[25] was a competitive binding radioimmunoassay, separation of free and bound fractions being achieved by covalently binding the antibody to a Sephadex particle. This assay has acceptable precision and an effective lower limit of sensitivity of 40–50 units/ml. There is now a veritable plethora of available commercial kits, which fall into two main groups, competitive binding assays and two-site immunometric assays. Isotope, enzyme, and fluorochrome labels are all available in one or other assay type.

The two-site immunometric assays depend on antibody bound to a solid phase, tube, bead or paper disc, which binds antigen in the sample. A second, labelled, antibody is added. After incubation the solid phase is washed and the bound label measured using appropriate instrumentation.

All of the commercial assays have a lower limit of sensitivity in the order of 1–10 units/ml and precision in the order of ±2–10%. There are no significant differences in analytical performance between the isotype, enzyme and fluorochrome labelled assays. All assays bar one are standardized in relation to the International Reference Preparation and give comparable results.

Allergen-specific IgE assays

The available methods for the estimation of allergen-specific IgE are modifications of the two-site immunometric assay. Allergen bound to a solid phase is allowed to react with the test sample, the bound antibody being detected by addition of labelled anti-IgE. Isotope, enzyme and fluorochrome labelled systems are available as for total serum IgE. There does not appear to be any particular analytical advantage in any one of the test systems. Specificity is essentially related to the purity of the allergens applied to the solid phase, a situation which has, on occasion, yielded some interesting and unexpected results. Although it is possible to generate results in terms of units of IgE, it is more usual to report data by grade 0–4 in relation to standards included in the kits. The clinical situation rarely calls for a more precise reporting system. All systems have a lower limit of sensitivity which is equivalent to approximately 0.5 units/ml of IgE.

Estimation of monoclonal immunoglobulins

Monoclonal immunoglobulins, as the product of a neoplastic cell line, exhibit extreme isotypic and idiotypic restriction. For this reason they should never be assayed by immunochemical techniques in relation to normal immunoglobulin calibrants. Because of their restricted antigenic heterogeneity, monoclonal immunoglobulins are always liable to be in antigen excess even when the nominal concentration is within the range of the calibration curve. The widely discrepant, and misleading, results that can be generated by the inappropriate use of immunoassay techniques in such a situation were illustrated by Kohn[26].

Methodology

The quantitation of monoclonal immunoglobulins is of importance in both the diagnosis and the monitoring of B-cell malignancy. The recommended method of assay is by densitometric scanning of the electrophoretic separation. This is a compromise methodology in that it lacks the absolute specificity of immunoassay and shows relatively poor sensitivity in the presence of significant amounts of 'normal' immunoglobulin. It does, however, provide useful data for the management of the individual patient, where serial values for the monoclonal immunoglobulin are compared with previous values in the same patient.

Serum samples are subjected to electrophoresis at pH 8.6 on a suitable support medium, usually cellulose acetate or agarose, and the proteins visualized by staining with Amido Black or Ponceau S. Both dyes have a reasonably linear protein-binding realtionship between albumin and the immunoglobulins. Densitometric scanning of the electrophoretic separation allows the monoclonal immunoglobulin to be expressed in mass concentration terms in relation to the total serum protein as determined by the Biuret reaction.

Standardization

The major causes of interlaboratory variation in analytical results in all immunoassays are differences in primary calibration. In this respect the immunoglobulins do not differ from any other serum protein. In an attempt to resolve this problem various international professional and regulatory bodies: World Health Organisation (WHO), International Union of Immunological Societies (IUIS) and International Federation of Clinical Chemists (IFCC), have produced reference preparations. These International Reference Preparations (IRP) are intended to provide base reference points for the designation of national reference preparations and for commercial or local working calibrants (Table 6.1). The IRPs are expressed in terms of a unitage – the International Unit (IU) – in the absence of agreement on the molecular mass of an antigen or protein.

National reference preparations also exist, assigned a unitage in relation to the IRP. These are usually developed as candidate preparations during the evaluation and acceptance process involved in designating the IRP.

Table 6.1 Reference preparations and calibrants for serum immunoglobulins

	International Reference Preparations			*National Reference Preparations*	*Working Calibrants*
IgG, IgA and IgM	WHO	1st reference preparation	67/86	1st British working standard for human serum immunoglobulins 67/99	Protein Reference Units (PRU) SPS-01
	WHO		67/97		CAP † protein standard
	IFCC		74/1	US National reference preparation	Various commercial calibrators
IgD				British research standard 67/37	CAP protein standard
					Various commercial calibrators
IgE	WHO	1st reference preparation	(69/204)*	British research standard 75/502	Various commercial calibrators
		2nd reference preparation	1980		

*No longer available

†College of American Pathologists

Working calibrants, designed for day-to-day use in the laboratory for the construction of calibration curves are produced by commercial or national organizations. These are usually assigned values in mass concentration terms as well as International Units. The conversion of units to mass concentration produces the first of a series of potential errors in that these are 'best estimate' relationships. All commercial and national working calibrants are nominally assigned values in relation to the IRP and most use the mass equivalence factors of Reimer[27]. Despite this, differences in antigenic potency can be seen between calibrants from different sources and these may be batch-dependent. Care should be taken when changing source or batch of working calibrant and rigid attention paid to internal quality control. Even with this degree of standardization and cross-calibration, methodological differences may occur, particularly in relation to high molecular weight proteins and proteins which have a tendency to form aggregates in the biological medium[28,29].

Quality control and external quality assurance

As with all laboratory tests, immunoglobulin assays should be subjected to continual scrutiny in terms of both accuracy and precision.

Precision is monitored by the inclusion of control sera in each assay batch. The results obtained from the control sera should always be plotted to give a graphical demonstration of performance. The choice between Shewhart, Youden or Cusum plots is a matter of individual preference as all will demonstrate changes in accuracy and precision over a period of time.

In addition to internal quality control, laboratories should also participate in appropriate External Quality Assurance, or Proficiency testing, programmes. Such schemes are available in many countries, including the UK, USA and France. External Quality Assurance is not ideally suited to the assessment of precision, but gives essential information on accuracy and allows the laboratory to compare their own performance with that of others using the same, and other, methods.

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7
Physiology and Pathology of Immunoglobulins in the Fetus and Child

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The human fetus normally becomes immunocompetent long before birth, although it remains relatively inactive immunologically. The present chapter considers the normal development of humoral immunity and the consequences of antigenic stimulation of the fetus and child at various stages of development. Important therapeutic implications in a variety of paediatric diseases will also be discussed.

DEVELOPMENT OF HUMORAL IMMUNITY

Production of immunoglobulin by the fetus

At approximately 7 weeks gestation, pre-B-cells appear in the fetal liver. These early precursor cells display no surface immunoglobulins but contain small amounts of μ chain (the heavy chain of IgM molecules) in their cytoplasm. By 9 to 10 weeks gestation, the first B-cells with surface μ chains can be found in the fetal liver. The sequence of appearance of immunoglobulin heavy chains on the surface of B-cells in the human fetus is (1) μ (9–10 weeks), (2) γ (10–11 weeks), (3) α (11–12 weeks), (4) μ plus δ (12–13 weeks)[1-3]. IgE synthesis has been demonstrated in lung and liver tissue cultures from 11 week fetuses[4].

By 15 weeks gestation, B-cells with adult-type surface markers are found in the blood and spleen. Generation of B-cells is largely relocated to the bone marrow by 30 weeks gestation; at this point, there are no remaining pre-B-cells detectable in the liver[5].

Despite the presence of B-cells containing various classes of immunoglobulins, the fetus does not ordinarily liberate a significant amount of immunoglobulin into its circulation until much later[6]. At 20–22 weeks gestation, active transport of IgG from the maternal to the fetal circulation by way of the placenta begins. At term, all IgG subclasses are present in umbilical cord serum at levels comparable to maternal levels. IgG1 is

transported to the fetus in sufficient amounts to result in cord blood levels which are generally greater than those of maternal blood. Some investigators have suggested the existence of a 'selective barrier' to placental IgG2 transport, resulting in maternal levels at term which are considerably greater than those in cord blood[7]. However, other studies have not substantiated this[8]. Differing techniques or precautions in assaying IgG subclasses may have led to these contradictory results.

Placental transfer of immunoglobulins

The only class of immunoglobulin which crosses the placental 'barrier' in significant amounts during pregnancy is IgG. The most important basis for selectivity appears to be immunoglobulin class, with the Fc portion of IgG playing a major role in the transport process[9]. Most of the transfer of IgG occurs in the third trimester, presenting a potential problem for the prematurely born infant who does not obtain the full benefit of maternal IgG antibodies to help protect against potentially life-threatening infections. Studies of IgG levels in premature infants and abortuses[10] reveal that fetuses of less than 20 weeks gestational age have IgG levels under 100 mg/dl. At 16 weeks, IgG1 may be detected in fetal serum, followed by IgG2 and IgG3; IgG4 is detectable by 22 weeks[11]. The fetus or premature infant of less than 32 weeks gestational age usually has an IgG level of less than 400 mg/dl.

Premature infants starting with a low level of IgG are likely to experience a decline in IgG to clinically important hypogammaglobulinaemic levels (less than 100–150 mg/dl) before the age of 6 months. For this reason, they are at an increased risk of serious infection, a situation which continues until synthesis compensates for the decreased immunoglobulin acquired from the mother.

There is an interesting correlation between low levels of IgG and intrauterine growth retardation. Presumably, this results from inadequate placental function, both in transporting IgG normally from mother to fetus and in transferring adequate nutrients to the fetus[12]. The level of IgG found in small-for-gestational-age infants is significantly less than that of gestational age-matched infants who are not growth retarded, but still greater than that of weight-equivalent infants who are premature (appropriate-for-gestational-age).

Ongoing studies are seeking to clarify the role of exogeneous gammaglobulin therapy in prophylaxis or treatment protocols for premature infants[13,14], since sepsis is a major source of morbidity and mortality in these infants. Preliminary results suggest value in this form of therapy, which apparently causes no lasting suppression of the infants' natural development of humoral immunity.

Amniotic fluid

While several workers have reported an absence of IgM and IgD in amniotic fluid, all five major immunoglobulin classes have been detected by sensitive methodology such as hemagglutination inhibition[15,16]. Studies to date suggest there is little, if any, transport of maternal immunoglobulins to the fetal

circulation via the amniotic fluid[17]. Although the fetal respiratory and gastrointestinal tracts are bathed in amniotic fluid which may contain protective antibodies, absorption of immunoglobulins from the amniotic fluid through the fetal gastrointestinal tract appears to be minimal.

Amniotic fluid levels of IgA, G, and D peak at mid-gestation, and decrease thereafter, as do total protein levels. However, IgM remains constant at low levels until 35 weeks gestation, then increases as the fetus approaches term; this has been interpreted as possibly reflecting increased fetal synthesis of IgM in the last trimester. IgM, G, and D are found to be more concentrated in cord blood samples than in amniotic fluid[16]. On the other hand, IgE is found in amniotic fluid after 13 weeks gestation, often in higher concentrations than in simultaneous cord blood samples; it rises steadily with increasing gestational age. Similarly, the concentration of IgA is over twice as high in amniotic fluid as in cord serum. It is still not clear how much of this IgA is derived from the mother and how much from the fetus[18,19].

Selective increases in amniotic fluid IgM have been reported in occasional cases of neural tube defects, even in the presence of normal fetal serum levels[20]. Additional information is needed before definite conclusions can be drawn.

Prenatal immunization

Prenatal active immunization of the fetus may possibly be accomplished by administering various vaccines to the mother. Early stimulation of specific antibody production in the fetus may well afford protection to the infant during an otherwise vulnerable period in life. During early extrauterine life, there is normally a decrease in maternally-derived IgG antibodies. At the same time, the newborn infant's own immunoglobulin synthesis slowly increases, with a lag period during which there is a transient but significant drop. If the infant had been previously immunized via the mother in prenatal life, the first extrauterine exposure to the infective agent could be expected to elicit an effective secondary immune response.

The studies of Gill *et al.*[21] demonstrated that immunization at 5 and 9 months of pregnancy with tetanus toxoid produced active immunization of the fetus. It may be that immunization only at 5 months or at another time in pregnancy would be equally effective. Other schedules should be studied, as should the effect of pre-immunization antibody levels in the mother. Would there be an inadequate fetal antibody response if certain quantities of maternal antibody were already present in the fetal circulation?

One concern is the potential for induction of tolerance rather than sensitization to the infectious agent. Although rarely seen in naturally acquired congenital infections, induced tolerance might render the infant more susceptible to overwhelming infection than would have been the case without maternal immunization during pregnancy.

Postnatal humoral responses

The sequence of synthesis and secretion of the various immunoglobulin classes follows a predictable pattern. Synthesis of IgM progresses steadily

during the first weeks after birth, and adult serum levels are attained by 3–5 years. After the age of 7 years, IgM levels tend to be higher in girls than boys, for reasons that are not clear[22].

The second immunoglobulin class to be produced by the fetus is IgD. It is not transmitted via the placenta but is present in small amounts in cord blood, and by 6 weeks can usually be detected by radial immunodiffusion at 0.1–0.3 mg/dl. Adult levels of 1–8 mg/dl are generally reached at 4–6 years of age.

Rapid synthesis of IgG normally starts within the first few weeks of postnatal life. IgG1 and IgG3 synthesis probably accelerate most rapidly[23]. The serum IgG3 level may reach a nadir at around one month of age, presumably because its halflife ($T^1/_2$) in the bloodstream is only about 7 days[24], and production is inadequate to replenish the rapidly diminishing IgG3 passively acquired from the mother. IgG1 levels ($T^1/_2$ = 21 days) also decrease rather rapidly during the first month, then decrease more slowly as synthesis increases in the infant. The nadir of serum IgG1 is reached at 2–3 months, as is the case with IgG2 and IgG4. Synthesis of IgG2 and IgG4 accelerates at 2–3 months of age, but low levels often persist during the first year or two of life. Final adult levels of all subclasses are generally attained between 8 and 10 years of age, but the values in most children are within the normal adult range by 3 years, there being a slow gradual increase in all subclasses between 3 and 10 years of age[23,25–28].

The last class of immunoglobulin to reach adult serum levels is IgA. Generally, production begins 2–3 weeks after birth, but adult levels are not achieved until early adolescence[29].

IgE can be detected in cord serum in various amounts, usually in very small amounts but at times even exceeding maternal levels. On occasion, allergen-specific IgE has been found in cord but not maternal serum, suggesting that IgE antibodies may be synthesized *in utero*. Cord blood IgE levels above 1–1.5 IU/ml appear to predict subsequent allergy in the newborn infant, and may be useful in identifying infants for whom special precautions should be taken to minimize the development of allergy[30]. The circumstances which predispose infants to develop allergic symptoms will be addressed later in this chapter.

Table 7.1 summarizes the catabolic rates and the approximate time sequence of attaining adult levels of the various immunoglobulin classes and subclasses.

IMMUNOGLOBULIN RESPONSE TO INFECTION

Chronic intrauterine infection

Infection of the fetus may result from placental infection via the maternal bloodstream or from ascending infective organisms from the cervix or birth canal. The most common transplacentally acquired infections are cytomegalovirus (CMV), toxoplasmosis, rubella, syphilis, hepatitis B, and HTLV-III (the virus causing AIDS).

Table 7.1 Estimated rates of catabolism and ages at maturation of immunoglobulin synthesis*

Ig class or subclass	$T\frac{1}{2}$ (days)	*Age when mean serum level reaches final adult value* (years)
IgM	5	3-5
IgD	3	4-6
IgE	3	5-7
IgG	20	7-9
IgG1	21	5-7
IgG2	21	8-11
IgG3	7	7-9
IgG4	21	9-11
IgA	6	12-14

*Figures derived from data in the literature[31-38], and those of our own laboratory

Other agents generally cause more acute infections of the fetus. Herpes simplex infection may be acquired by either the transplacental or the ascending route. In the latter instance, the fetus may become infected by ingesting the infective agent in amniotic fluid (e.g. in amnionitis), or by direct transfer of organisms into the fetal bloodstream (if placentitis is present). Viruses such as varicella zoster, influenza, measles, mumps, variola-vaccinia, and enteroviruses (polio, coxsackie, and ECHO viruses) occasionally cause intrauterine infections[39], as may certain fungi, parasites and bacteria. The fetal response to each of these agents depends in some measure on the stage of gestation and maturity of the fetal immune system at the time of infection[40]. Many of these infections are self-limited, but some may lead to abortion in early gestation.

Humoral immune responses in congenital rubella illustrate what may occur in chronic intrauterine infections. Rubella virus appears in the maternal bloodstream 8–10 days following exposure. Approximately one week later, the mother may experience the onset of symptoms or she may have an inapparent infection. In either event, by this time, multiple maternal organs have been infected, including the placenta. Now (16–18 days post-exposure), the first specific IgM antibodies appear in maternal serum, and there is clearing of maternal viraemia but continued viral excretion for a few weeks. The initial antibodies, being of the IgM class, seldom cross the placental barrier and enter the fetus. The next antibodies formed in quantity are of the IgG class; they are transported to the fetal circulation. However, if infection has occurred in the first 20 weeks of gestation, placental transfer is minimal since at this gestational age, fetal IgG levels rarely exceed 5% of maternal levels. During the first trimester, fetal antibody synthesis is minimal as well; therefore, the infective organism has a prime opportunity to invade multiple

organs in the infant and to establish a chronic disseminated infection.

Fetal antibody acquired passively results in a rising titre from 20 weeks gestation onward. At term, fetal and maternal levels of IgG antibody are approximately equal. At 20–30 weeks gestation, a significant rise in fetal IgM occurs. This increase in IgM continues, often becoming the major specific antibody. An increase in specific IgA also may be noted at birth and in early life[41–44].

Subclinical cases of chronic intrauterine infection may cause only mild elevations of total IgM and/or IgA in the fetus. In general, the severity of infection tends to correlate with the degree of IgM antibody response. Also, different infectious agents may stimulate varied degrees of response. Thus, one is apt to find higher IgM levels in infants with congenital rubella or syphilis than with congenital CMV or toxoplasmosis.

The pattern of serum immunoglobulin level increases in infants chronically infected *in utero* is an acceleration of the normal pattern occurring in uninfected infants. IgM is the first class to respond and elevated levels are detectable *in utero* as well as in the neonate. Enhanced synthesis of IgG may begin *in utero* but accelerates shortly after delivery, elevated levels becoming evident in asymptomatic infants with congenital CMV, rubella or toxoplasma infection at about one month of age, increasing steadily thereafter until adult levels are attained at about one year of age. Development of serum IgA levels is more like that in normal infants, reaching adult levels only after several years. The more severe the infection, and presumably the larger the antigenic load, the more rapidly serum immunoglobulin levels reach the normal adult mean. Thus, in symptomatic infections, IgM may achieve or exceed normal adult levels at birth, and there may be rapid increases in IgG and IgA to adult values soon thereafter.

Measurement of IgM can be used as a screening test to evaluate newborns for congenital infection. More definitive diagnostic procedures are often indicated as well: (1) specific IgM antibody in cord blood, (2) serial antibody determinations and (3) isolation of pathogenic organisms.

The production of specific IgM antibodies continues in the infant until antigen production by the pathogen ceases. In congenital CMV or toxoplasmosis, antigenic stimulus from viral or parasite antigens may persist for years. In congenital rubella, there is generally a decrease in excretion of virus at around 6 months of age with a concomitant decrease in total and rubella-specific IgM. Likewise, once syphilis is treated, the level of *Treponema pallidum*-specific IgM falls as a result of elimination of the pathogen. In each instance, serum IgG antibodies gradually become predominant and a relatively high level of total antibody persists for a year or more[39].

Neonatal infection

Bacterial

Neonates and infants during the first few months of life usually encounter a multitude of bacteria. Most handle the encounter quite well while others,

particularly premature infants, develop severe, overwhelming infection. There appears to be a correlation between low levels of type-specific serum antibody and risk for sepsis with type III group B Streptococcus[45,46], a major cause of perinatal infection. This may be the result of either a lack of type-specific IgG antibodies in maternal serum and in the term infant or an inadequate placental transfer of such antibodies (as in premature infants). Oxelius *et al.*[47] found 13 of 19 mothers of infants with severe group B streptococcal infection to have significantly lower IgG1, 2 or 3 subclass levels than mothers of healthy infants. These and other protective factors must be considered when studying neonatal infections.

In vitro opsonization of micro-organisms by the classical pathway of complement activation is similar in neonatal and adult blood with regard to group B Streptococci and *Staphylococcus aureus*. However, resistance to organisms such as *E. coli*, which activate the alternate pathway (C3–C9) via endotoxin, may be compromised due to decreased concentrations of important factors involved in the alternate pathway and poor opsonization by cord serum[48].

Secretory IgA antibodies are found in human milk which have activity against various bacterial antigens, such as *E. coli* O and K antigens, enterotoxins of *E. coli* and streptococcal antigens. These appear to be present in maternal milk as a result of antigenic stimulation of maternal intestinal lymphoid cells, some of which home to the mammary gland. Comparable serum antibodies may be absent. Secretory IgA (predominantly IgA2 subclass) from human milk can pass through the length of the intestinal tract of the breastfed baby with minimal destruction of the immunoglobulin molecule, enhancing specific local antibody activity in the gut. In this way, breastfeeding can protect the infant from a variety of intestinal infections. Breast milk similarly may play a role in protection against respiratory tract infections with influenza and respiratory syncytial viruses, since neutralizing antibodies to these agents have been found in human colostrum[49].

Colostrum has been found to contain IgM and IgG as well, with preferential contributions of certain IgG[50] and IgA subclasses[51]. The particular role of these antibodies in the immunity of breastfed children has not been fully elucidated. IgG4 has been found to comprise about 15% of the total IgG in colostrum, whereas in plasma it comprises only 3–4%. It is likely that IgG4 plays a special role in defence against pyogenic infections of the respiratory tract[50]. Colostral IgG probably is important in protecting the neonate from infection, but further research is necessary before details will be known.

Viral

Mucosal antibodies in the nasopharynx and the gastrointestinal tract comprise an important line of defence against viruses entering the body. These antibodies are largely secretory IgA (sIgA) but may include IgM, IgG (perhaps certain subclasses) and IgE. IgA plasma cells in the lamina propria of the intestine and the subepithelial region of the bronchopulmonary tree appear to differentiate into antibody-producing cells upon exposure to

specific antigens[51]. Virus-neutralizing sIgA has been demonstrated to be particularly important in protection against polio virus, as well as against various viruses causing respiratory and possibly exanthematous diseases.

Circulating anti-viral antibodies are predominantly of the IgG class, stimulated by various structures on the virion once the virus has entered the regional lymphatics. IgM, IgA and perhaps IgE may also be produced. Viruses that infect by cell to cell spread, without an extracellular phase, stimulate little antibody synthesis; those which infect by incorporation of nucleic acids into the genetic structure of the cell (e.g. Epstein–Barr virus and retroviruses) are little influenced by antibodies produced against their coat antigens[52].

Antibodies active against a wide variety of viruses have been found in higher concentration in cord blood than in maternal blood[53]. These may play an important protective role in neonates infected with herpes simplex, measles, rubella or respiratory syncytial virus, and possibly other viruses[54].

Hemophilus influenzae

H. influenzae type b is responsible for a significant number of serious illnesses and deaths in children younger than 5 years. Its most common serious manifestations are meningitis and epiglottitis, and it remains the leading cause of acquired mental retardation in the United States, despite antibiotic therapy. Why are young children so susceptible to this infection? All the answers are not yet known, but several points can be made: first, antibodies to the *H. influenzae* type b capsular polysaccharide antigen are thought to be important in immunity. IgG2 subclass antibodies have been thought to be particularly important in responses to polysaccharide antigens[55]. Studies in our laboratory suggest that antibodies to the polyribose phosphate antigen can also belong to other subclasses, particularly IgG4. Since maturation of IgG2 and IgG4 production occurs later than that of IgG1 and IgG3, the child under 2 years of age may be especially susceptible to infection with encapsulated bacteria.

There is an inverse correlation between mean serum antibody level and the incidence of bacteremic *H. influenzae* disease[56]. Children less than 18 months old produce less antibody, and it is primarily of the IgM class which is deficient with regard to immunologic memory. The recently developed *H. influenzae* type B (Hib) capsular polysaccharide vaccine provides protection in children 18 months of age or older. Children with a high level of prevaccination antibody (presumably resulting from 'natural' exposure to Hib itself or to antigens cross-reacting with Hib) are able to sustain lasting high antibody levels post-vaccination, suggesting that the vaccine is a good stimulus of memory B-cells in children over a year and a half of age[44].

Selective immunoglobulin class and subclass deficiencies

Isolated absence in serum of an IgG subclass is often associated with disease, as summarized in Table 7.2[57–62]. Severely deficient levels of IgG2 and/or IgG4 are typically associated with pyogenic sinopulmonary infections. Healthy

Table 7.2 Selective IgG subclass deficiencies

Subclass deficiency	*Clinical findings*
IgG1	Often healthy May have chronic/recurrent otitis media
IgG2	Occasionally asymptomatic Recurrent respiratory tract infections Often coexists with IgA or IgG4 deficiency May have abnormal lung function[61]
IgG3	Usually healthy May be associated with abnormal lung function[61] in some IgA deficient patients
IgG4	Occasionally asymptomatic Recurrent respiratory tract infections Commonly associated with low IgG2, IgA or IgE

subjects may have a wide range of values, sometimes overlapping with 'abnormal' values.

IgA-deficient individuals with frequent infections often have co-existing IgG2 and/or IgG4 deficiency[62]. Allergic individuals with IgA deficiency tend to have an increased incidence of gastrointestinal complaints, upper respiratory infections and asthma which may be resistant to treatment[63]. A few instances of isolated IgM deficiency (<20 mg/dl)[64] have been described in the literature to date. Each had severe recurrent bacterial infections. There are no known effects of selective IgE or IgD deficiency, though each is an occasional finding.

EFFECTS OF MATERNAL ANTIBODY ON THE FETUS

Table 7.3 summarizes the known effects of a variety of maternal antibodies on the fetus[57], including antibodies to blood components and autoantibodies present in the maternal circulation. The most common maternal antibody-mediated disease in the fetus is haemolytic disease of the newborn due to blood group incompatibility.

Passively acquired anti-Rh, anti-A, or anti-B blood group antibodies comprise over 99% of maternally derived antibodies to fetal erythrocyte antigens. The severity of the resulting neonatal haemolytic process is dependent on the nature of the maternal antibody, the capacity of such antibodies to act as opsonins for red blood cells (RBCs), and the degree of placental transfer of these antibodies.

Haemolytic disease due to anti-Rh blood group antibodies (most importantly anti-D) is generally not a problem in a primiparous pregnancy, since initial 'leakage' of fetal Rh+ RBCs into the circulation of the Rh− mother stimulates mainly an IgM response. On second exposure, however, a

Table 7.3 Effects of maternal antibodies on the fetus

Antibody	*Effect on fetus*
Anti-D (Rh incompatibility)	Haemolytic disease of the newborn
Anti-A or anti-B (ABO incompatibility)	Haemolytic disease of the newborn (usually less severe than Rh disease)
Anti-leukocyte antibodies	Usually no detectable adverse effect Occasional transient neonatal neutropaenia and susceptibility to infections
Anti-platelet antibodies (largely IgG1 and IgG3)	Transient neonatal thrombocytopaenia Occasional neonatal purpura
Maternal lupus erythematosus with IgG anti-nuclear antibodies	Generally no clinical effect
Rheumatoid factor (usually IgM)	Generally no effect
Long-acting thyroid stimulator (LATS) or LATS-P	Transient neonatal thyrotoxicosis
Maternal myasthenia gravis: IgG anti-ACh* receptor antibodies	Transient neonatal myasthenia gravis in 10–15% of cases

*ACh = acetylcholine

large and rapid rise in anti-D IgG antibodies occurs, with resultant transport of these across the placenta to the fetus. The anti-D antibodies are mainly of the IgG1 and IgG3 subclasses, both of which are efficient opsonins[65]. Damage to fetal RBCs is most likely to be due to these antibodies causing RBCs to adhere to macrophages, which then engulf the cells and are subsequently sequestered in the spleen. Since the D antigen is found exclusively on RBCs, the damage is confined to these cells.

ABO haemolytic disease can affect first-born infants, due to IgG 'natural' blood group antibodies possessed by the mother; however, the majority of maternal 'natural' anti-A and anti-B iso-agglutinins are of the IgM class and do not cross the placenta. Nevertheless, maternal 'immune' or 'hyperimmune' iso-antibodies of the IgG class may be stimulated by exposure to fetal RBCs bearing A or B blood group antigens, or by other substances containing A or B antigenic determinants. These may pass easily to the fetus, causing haemolysis. Because A and B antigens are present in several body fluids and tissues, much of the transferred maternal antibody is diverted from binding to RBCs by first combining with non-red cell fetal A and B antigens[17]. In addition, fetal RBCs exposed to anti-A *in vitro* exhibit less than half the amount of lysis shown by adult RBCs with similar amounts of anti-A[66]. These factors combine to explain the milder nature of ABO haemolytic disease.

It should be noted that haemolytic disease of the newborn due to ABO incompatibility is much more likely in infants of O mothers than of A or B

mothers (37.9% versus 0.8%)[67]. This may be due to more frequent production of IgG 'immune' blood group iso-antibodies in group O mothers than in those who are group A or B. The stimulus resulting in these antibodies is unclear, but it is known that certain vaccinations during pregnancy (e.g. tetanus or smallpox) can increase maternal anti-A and B titres, with a resultant increased incidence of haemolytic disease of the newborn[68]. It is also known that certain parasites contain A- and B-like substances which can stimulate high levels of antibody in humans[69,70].

IMMUNOGLOBULINS IN SELECTED PAEDIATRIC ILLNESSES

Infectious mononucleosis

The acute phase of Epstein–Barr virus (EBV) induced infectious mononucleosis is associated with a polyclonal increase in production of all immunoglobulin (Ig) classes. However, sequential changes occur in each class in a characteristic pattern somewhat different than generally noted in acute viral infection.

There is a striking early peak in serum IgE during the first week of illness, with a rise in IgM at the same time or slightly afterward. IgG peaks at 2–3 weeks, IgG4 reaching a later and more marked peak than total IgG[71,72]. IgD may peak at widely varying times during the first 2 months of illness. After the acute phase of illness, IgE and IgM levels drop to well below pre-illness levels, with a nadir at 3–6 months post-onset of illness[72].

Specific antibodies against EBV-associated antigens probably comprise only a small percentage of the increase in total immunoglobulins. There has been no apparent relationship between the intensity of Ig responses and the clinical severity of the disease.

The impressive IgE response early in this illness is not unique to EBV-induced infectious mononucleosis. Other acute viral illnesses, including cytomegalovirus mononucleosis, have been found to be associated with elevated serum IgE[73]. Although the exact significance of the IgE response is unclear, it is interesting to note that acute viral infections may precipitate clinical evidences of allergy, especially during early childhood[74]. Perhaps activation of IgE productivity during certain viral infections plays a role in the pathogenesis of allergy.

Kawasaki syndrome (mucocutaneous lymph node syndrome, MCLS)

This disease has a peak incidence between 6 months and 2 years of age. It is characterized by prolonged fever ($\geq$ 5 days), conjuctival infection, oral mucous membrane erythema or fissuring, 'strawberry tongue', an erythematous urticaria-like rash, erythema of palms and soles, with firm oedema of the hands or feet, and subsequent desquamation. There is often enlargement of cervical nodes. A dangerous complication is coronary artery inflammation leading to aneurysms in 17–40% of patients and a 1–2% incidence of sudden death. Non-specific signs of carditis may also be found.

Sterile pyuria, arthralgias, and central nervous system irritability may accompany the above[75].

Acute and convalescent sera studies in 20 Japanese children with non-fatal MCLS[76] revealed elevation of all five classes of Igs during active disease, most remarkably IgE and IgM. IgE rose within 1–2 weeks of onset, then declined over the next 1–2 months. IgM reached a peak somewhat later, with IgG, IgA and IgD generally following. Circulating immune complexes have been elevated in some cases, often appearing in association with thrombocytosis during the third week of illness[77,78].

High-dose intravenous gammaglobulin (IVGG) therapy has recently been advocated for use in the early stages of clinically recognized Kawasaki syndrome[79]. This appears to cause a significant reduction in the frequency of coronary artery abnormalities in patients given 400 mg/kg daily for 5 days. The reasons for success with this mode of therapy are yet to be elucidated, as is the pathogenetic mechanism of this disease. If immune complexes play a role in pathogenesis, perhaps IVGG offers competition for Fc receptor sites on blood vessel walls; on the other hand, if the aetiology is infectious, IVGG may be providing antitoxin or neutralizing antibody. Intact gammaglobulin may also lessen thrombus formation by inhibiting the adhesion of platelets to vessel walls.

Paediatric acquired immune deficiency syndrome (paediatric AIDS)

Over 95% of adult acquired immune deficiency syndrome (AIDS) in the United States occurs in homosexual or bisexual men, intravenous drug abusers, Haitians, recipients of blood or blood products, or sexual partners of infected persons. The aetiologic agent for this disease has been recently established to be human T-cell lymphocytotropic virus III (HTLV-III). The same virus independently discovered in France is called LAV. In children, infection with HTLV-III usually follows receipt of infected blood or blood products, or occurs in a child whose mother has AIDS or is at risk for AIDS. It is likely that transplacental infection occurs, especially in infants who are symptomatic within the first month or so of life[80].

Although many clinical and immunologic manifestations of AIDS are similar in adults and children, paediatric AIDS is more frequently associated with recurrent parotitis, interstitial pneumonia without *Pneumocystis carinii*, and frequent bacterial infections prior to the development of opportunistic infections. Recurrent otitis media is common, and serious bacterial infections such as pneumonia and meningitis also occur[81]. Children with HTLV-III infection almost always have increased serum IgG levels and may exhibit elevations in IgA and IgM as well. Their increased susceptibility to bacterial infection may be due to a number of factors. Undoubtedly infection and destruction of helper T-cells by the AIDS virus is a critical factor in the failure of patients to produce antibodies to neoantigens[82]. It is of interest that many children with AIDS have been found to have a selective deficiency of one or more IgG subclass[83]. This in itself may make them more susceptible to bacterial infections and perhaps to the AIDS virus as well. Subclass deficiencies are not nearly so common in adult patients with AIDS.

Preliminary studies with intravenous gammaglobulin therapy[84,85] (200 mg/kg biweekly for 3 months to 3 years) in infants and adults with AIDS or AIDS-related complex are somewhat encouraging. A significant reduction in bacterial infections has been noted. Many patients also show improvement in mitogen induced lymphocyte blastogenesis and in the T4/T8 ratio during treatment.

ALLERGY

Although immunoglobulins are important in protection against infection, there are also implications for the pathogenesis of allergic disease. There is ample evidence that atopy is inherited[86]. If both parents are atopic, the risk of atopy in each child is estimated to be two chances in three. If only one parent is atopic, roughly one of three children will become allergic and if neither parent has atopic disease, only one in ten will develop allergy[87,88]. Neither specific atopic manifestations (e.g. allergic rhinitis, eczema, asthma) nor hypersensitivity to specific allergens appears to be inherited directly, only a tendency to be atopic. Several findings have been considered to be strongly predictive for the development of allergic symptoms in infancy and childhood: (1) family history of allergy[30], (2) elevated cord serum IgE, and (3) T-suppressor cell deficiency[89,90].

Several investigators have indicated that a combination of family history and cord serum IgE offers good prediction[91,92]. Hamburger *et al.* (1974)[93] demonstrated a significant correlation between an increase in serum IgE early in childhood and the onset of allergic disease; 10 or 11 babies who developed serum IgE levels greater than 20 IU/ml before 12 months of life went on to have definite or probably allergic symptoms.

Certain ethnic groups in modern society appear to have an increased mean serum IgE compared to that of Caucasians. These include immigrants of southeast Asian, Japanese and African origin. Intermediately elevated levels of IgE persist into at least the first generation of offspring born in America. It is presumed that they decrease with succeeding generations until a levelling off occurs at a value still somewhat above that in Caucasians of long-standing American lineage. There may be a genetic selection for subjects with high IgE levels in areas where parasitic diseases are endemic since IgE may play an important role in host defence against parasites[94].

Early detection of atopy is of importance in the immediate postnatal period, since general preventive measures may be helpful to 'high-risk' infants. When possible, the infant should be breastfed for at least 6 months; when this is not possible, the infant at risk may do best if fed a hypoallergenic (preferably predigested or elemental) formula. Introduction of solid foods should also be delayed until 6 months of age in seeking optimal prevention of sensitization. After 6 months of age, introduction of solid foods one at a time at weekly intervals will generally permit identification of foods that cause untoward responses[95–97]. Attention should also be given to minimize unfavorable environmental exposure for the infant at risk. This includes eliminating insofar as possible cigarette smoke, animals, birds, dust, aerosol

deodorants, perfumes, insecticides, etc. from the household.

The atopic state of children whose mothers received maintenance immunotherapy for allergic disease during pregnancy was assessed in 109 instances by Metzger *et al.* (1978).[98]. About 25% of the children developed asthma or allergic rhinitis, and more than 42% showed some form of atopic disease. These percentages are comparable to those expected for children born into allergic families and whose mothers do not receive immunotherapy during pregnancy, suggesting that the fetus is neither more susceptible to allergic disease nor protected from developing atopy when a pregnant woman is treated with immunotherapy.

CONCLUSIONS

Several aspects of immunoglobulin physiology and pathology in the developing human have been considered. A spectrum of neonatal difficulties can be encountered as a result of passively acquired maternal antibodies, but in general, this is heavily outweighed by the all-important protection against infection. The ability of the fetus to mount an immune response is dependent on its developmental stage. Consequently, the timing of an antigenic stimulation may be critical in determining the presence or absence of clinical disease. The infant and young child remain uniquely susceptible to certain infections not ordinarily affecting adults. Sucessful immunization programmes can change the course of many of these diseases, and new vaccines will likely protect children of the future from currently devastating infectious diseases. Development of such vaccines depends in large measure on knowledge concerning the normal immune response to specific antigenic determinants or infectious agents.

With the current availability of safe intravenous gammaglobulin preparations, therapeutic successes have been achieved for a variety of diseases. While intravenous gammaglobulin has been beneficial in the treatment of immunodeficiency diseases and shows promise in favourably modifying thrombocytopaenic purpura[99,100], Kawasaki syndrome and paediatric AIDS, some of its actions are poorly understood. As more is learned about the aetiology and immunopathology of these and other diseases, more specific therapeutic interventions will surely become possible.

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Serum Immunoglobulins in Disease Diagnosis and Management

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INTRODUCTION

Serum immunoglobulins were one of the first components of the immune system to be comprehensively described, and routine assays of them have been available in clinical chemistry and clinical immunology laboratories for many years. Consequently, abnormalities of serum immunoglobulin concentrations have been demonstrated in many diseases and the measurement of serum immunoglobulin concentrations has become a commonly used laboratory investigation. However, in an era in which the value of laboratory investigations is being critically analysed[1], it has become clear that this investigation is overused and often misused. There is no doubt that there are clinical situations in which the measurement of serum immunoglobulin concentrations is of value, and even essential, for disease diagnosis and management but these need to be more clearly defined than they are at present. It is the aim of this chapter to go some way towards doing this.

Abnormalities of serum immunoglobulin concentrations will be discussed in terms of those commonly encountered in clinical practice, that is low or high, and IgE and IgD will be considered separately from the other immunoglobulin isotypes.

As is the case with many plasma proteins, serum immunoglobulin concentrations in healthy individuals vary for a variety of reasons and, although this may not be of great importance in an individual patient, it could be a consideration when comparing serum immunoglobulin concentrations in different populations and when calculating normal reference ranges. It is, therefore, appropriate to discuss this topic before proceeding to a discussion of serum immunoglobulin abnormalities.

SERUM IMMUNOGLOBULINS IN HEALTH

Several genetic, environmental, age-related and sex-related factors influence serum immunoglobulin concentrations in healthy individuals.

Serum concentrations of all immunoglobulin isotypes increase at various rates throughout childhood (see Chapter 7) but adult values for all are achieved by puberty. Thereafter, serum IgG and IgA concentrations increase with age[2], the high concentrations of IgG in the elderly[3] being mainly due to IgG1 and IgG3. In contrast, there is a rapid decline of IgE concentrations[4] and a slight decline of IgM concentrations[2].

Both genetic and environmental factors influence serum immunoglobulin concentrations throughout life. Serum IgG, IgE and, to a lesser extent, IgM, but not IgA nor IgD, are influenced by genetic factors[5] which, in the case of IgG subclasses, include the Gm allotypic genetic markers of IgG[6]. Racial differences in serum immunoglobulin concentrations[4,7,8] are apparently due to a combination of environmental and genetic factors.

Sex-related differences in serum immunoglobulin concentrations are most notable in the case of IgM and IgG4 and probably also affect IgE. Serum IgM concentrations are higher in females than males, apparently an effect of a gene or genes on the X chromosome[8], but the reverse is true for IgG4[9] and probably for IgE[10].

Normal reference ranges

Because of the various factors which can influence serum immunoglobulin concentrations in health, it is advisable to use a large number of sera from healthy individuals of both sexes and from the appropriate geographical area when calculating reference ranges. Age-related differences are important in children but not in adults, although the high serum concentrations of IgG and IgA in some elderly adults should be noted. Reference ranges will vary somewhat between laboratories because of differences in assay technique but, as a guide, the values used by the Protein Reference Unit in Sheffield are shown in Table 8.1.

IMMUNOGLOBULIN DEFICIENCY

Immunoglobulin deficiency is a characteristic feature of most antibody deficiency syndromes and, consequently, the assessment of patients with an increased susceptibility to infections is the major indication for measuring serum immunoglobulin concentrations. However, the demonstration of serum immunoglobulin deficiency is not always indicative of an immunodeficiency syndrome, there being several diseases and even some drugs which cause a reduction of serum immunoglobulin concentrations. It is, therefore, of some practical value to consider immunoglobulin deficiency as being either primary, when due to an intrinsic defect of the immune system, or secondary, when due to another disease or drug therapy. Not all cases can be conveniently classified as such because some disease-related causes of im-

Table 8.1 Reference ranges for serum immunoglobulin concentrations in adults

Immunoglobulin	*Reference range*
IgG	5.4-16.1 g/l
IgG1	3.2-10.2 g/l
IgG2	1.2-6.6 g/l
IgG3	0.15-1.95 g/l
IgG4	<0.03-1.30 g/l
IgA	0.8-3.0 g/l
IgM	0.5-2.0 g/l
IgE	<5.0-120 kU/l
IgD	5.0-200 μg/ml

munoglobulin deficiency, for example chronic lymphocytic leukaemia, primarily affect the immune system. However, this approach to classification is useful in clinical practice.

Various patterns of serum immunoglobulin deficiency may occur, the most extreme being hypogammaglobulinaemia. Hypogammaglobulinaemia describes the reduced density of the gammaglobulin band found on zone electrophoresis of a serum which is severely deficient in immunoglobulins. It mainly reflects deficiency of IgG. Although zone electrophoresis was the original method used to demonstrate serum immunoglobulin deficiency, the usual current practice is to measure serum immunoglobulin concentrations directly by immunochemical methods. By so doing, it is possible to demonstrate less severe degrees of immunoglobulin deficiency, involving one or more immunoglobulin isotype, which would not cause hypogammaglobulinaemia. Immunoglobulin deficiency which is not severe enough to cause hypogammaglobulinaemia is sometimes called dysgammaglobulinaemia. A numerical classification of the several types of dysgammaglobulinaemia has been devised, but this is now rather outdated and it is more usual to classify them in terms of the pattern of immunoglobulin deficiency.

Primary immunoglobulin deficiency is often associated with antibody deficiency, as may be some types of secondary immunoglobulin deficiency. But a direct relationship does not always occur; some patients with immunoglobulin deficiency are able to produce antibody responses normally and, occasionally, antibody deficiency occurs in patients with normal or high serum immunoglobulin concentrations. It, therefore, follows that an antibody deficiency syndrome cannot always be confirmed or excluded by measuring serum immunoglobulin concentrations alone.

Primary immunoglobulin deficiency

There are a number of primary immunodeficiency syndromes in which serum immunoglobulin deficiency is a characteristic feature (Table 8.2). Classifica-

Table 8.2 Congenital and acquired primary immunoglobulin deficiencies

Type of immunoglobulin deficiency	*Congenital*	*Acquired*
Hypogammaglobulinaemia	Sex-linked hypogammaglobulinaemia Transient hypogammaglobulinaemia of infancy As part of a severe combined immunodeficiency syndrome	Common variable hypogammaglobulinaemia Hypogammaglobulinaemia with thymoma
IgG and IgA deficiency with normal or high IgM	Yes	Yes
Selective IgA deficiency	Alone or in ataxia telangiectasia	Probable
Selective IgM deficiency	Yes	Unknown
Selective IgG deficiency	Yes	Unknown
IgG subclass deficiency	Alone or with selective IgA deficiency	Probable

tion of these is generally based on the pattern of immunoglobulin deficiency and, in some, associated clinical and immunological features. Both congenital and acquired forms occur, the acquired forms presenting at any age, even as late as the sixth and seventh decades of life. Although the classification of primary immunoglobulin deficiencies, based as it is on patterns of immunoglobulin deficiency, suggests a homogeneity of immune defects, *in vitro* studies on lymphocytes from patients with these syndromes have demonstrated a variety of immune defects affecting B-cell maturation or regulator T-cell function (excess T-cell suppression or reduced T-cell help)[11].

Hypogammaglobulinaemia

Hypogammaglobulinaemia occurs in several primary congenital or acquired antibody deficiency syndromes (Table 8.2) but the degree and pattern of immunoglobulin deficiency varies somewhat between them. In sex-linked hypogammaglobulinaemia serum IgG concentrations are less than 2g/l and often undetectable, with undetectable or extremely low concentrations of IgA and IgM, whereas in common variable hypogammaglobulinaemia the serum IgG concentration is often higher, although usually less than 3g/l, and IgA and IgM may be absent or present in significant amounts. In contrast, the hypogammaglobulinaemia associated with a thymoma is very rarely due to a serum IgG concentration of less than 1.5g/l and IgA is almost always present. Transient hypogammaglobulinaemia of infancy is mainly an effect of IgG deficiency with normal or slightly reduced serum concentrations of IgA and IgM, although in some children there is deficiency of all immunoglobulin

isotypes. The different patterns of immunoglobulin deficiency in the various types of primary hypogammaglobulinaemia presumably reflect differences of underlying immune defects.

Most patients with primary hypogammaglobulinaemia are abnormally susceptible to infections, particularly pneumococcal and *Haemophilus influenzae* infections of the respiratory tract. However, there are a small number of patients who, for unexplained reasons, do not suffer from recurrent infections at all and many children with transient hypogammaglobulinaemia of infancy who are only prone to minor infections.

It is important to differentiate primary from secondary hypogammaglobulinaemia and to classify cases of primary hypogammaglobulinaemia because this has implications for the patient in terms of long-term management, associated diseases and complications, and genetic counselling. The causes of secondary hypogammaglobulinaemia are usually apparent (see later) but not always so. It may be particularly difficult to differentiate between primary and secondary hypogammaglobulinaemia when there is associated diarrhoea. Primary hypogammaglobulinaemia may be complicated by diarrhoea but a protein losing enteropathy, which is associated with diarrhoea, can cause hypogammaglobulinaemia. The serum albumin is often helpful here, being normal in primary hypogammaglobulinaemia but low in a protein losing enteropathy. The history, clinical examination and other immunological investigations usually provide sufficient information for a classification of primary hypogammaglobulinaemias.

Both sex-linked hypogammaglobulinaemia and transient hypogammaglobulinaemia of infancy present at around 6 months of age when placentally transferred maternal IgG has reached a nadir. A low serum IgG concentration (approximately 3.5 g/l) is common in normal babies at this age and some may experience recurrent infections. However, the serum IgG concentration is much lower in congenital hypogammaglobulinaemia and infections are more severe. By definition, children with transient hypogammaglobulinaemia of infancy eventually develop normal serum immunoglobulin concentrations, usually before 2 years of age. Before that age, other laboratory and clinical investigations should help to differentiate sex-linked hypogammaglobulinaemia from transient hypogammaglobulinaemia of infancy. Numbers of peripheral blood B-cells, lymphoid tissue and serum IgM and IgA concentrations are absent or markedly reduced in the former but not in the latter. In addition, hypogammaglobulinaemia in a girl generally excludes sex-linked hypogammaglobulinaemia, although cases of a similar syndrome have rarely been reported in girls.

Congenital hypogammaglobulinaemia may also be associated with cell-mediated immunodeficiency in one form of severe combined immune deficiency (Swiss type agammaglobulinaemia). Unlike the other types of congenital hypogammaglobulinaemia, infections occur earlier than 6 months of age because of the cell-mediated immunodeficiency which also results in recurrent viral, fungal and protozoan infections in addition to those infections which are typical of antibody deficiency syndromes. Severe combined immune deficiency can also be differentiated from the other types of congenital hypogammaglobulinaemia by demonstrating deficient T-cell

numbers and/or function. An indicator to the presence of reduced T-cell numbers is a lymphopaenia.

Acquired primary hypogammaglobulinaemia is classified as that presenting after 2 years of age, so as to differentiate it from the congenital types, but can occur at any age. Most cases are due to common variable hypogammaglobulinaemia. Approximately one third of patients with common variable hypogammaglobulinaemia also have defects of cell-mediated immunity, but these do not result in an increased susceptibility to the types of infection associated with cell-mediated immunodeficiency. In addition to recurrent infections, patients may present with or develop several other diseases, including autoimmune diseases, malabsorbtion syndromes, arthritis and malignancies, particularly carcinoma of the stomach.

Whereas common variable hypogammaglobulinaemia usually presents between the ages of 15 and 35, the hypogammaglobulinaemia associated with a thymoma, presents after the age of 40. Hypogammaglobulinaemia is only one of a number of diseases associated with thymoma, the others including myaesthenia gravis, systemic lupus erythematosus, haematological cytopaenias and chronic mucocutaneous candidiasis. The nature of the relationship between the thymoma and the hypogammaglobulinaemia is not known, but may involve autoimmunity in view of the association between thymoma and other autoimmune diseases. Patients presenting with primary hypogammaglobulinaemia after the age of 40 must be examined for a thymoma and if this is not demonstrated, regular follow-up is mandatory. Although removal of the thymoma does not correct the hypogammaglobulinaemia, thymectomy is usually indicated because some thymomas are malignant. If a thymoma is not present at presentation, differentiation from common variable hypogammaglobulinaemia can usually be made by the pattern of immunoglobulin deficiency (see earlier) and by quanititating B-cells in peripheral blood. These are absent or very low in hypogammaglobulinaemia with thymoma and normal, high or low, but rarely absent, in common variable hypogammaglobulinaemia.

IgG and IgA deficiency with a high IgM

Otherwise known as the 'hyper-IgM' syndrome, this is a rare antibody deficiency syndrome which is sex-linked in the congenital form but can affect either sex in the acquired form. This is also associated with antibody deficiency and recurrent infections.

Selective IgA deficiency

By convention, IgA deficiency is usually defined as a serum IgA concentration of less than 0.05 g/l because this is the lower limit-of-detection of radial immunodiffusion, the original method used to measure serum immunoglobulin concentrations. However, some diseases appear to be associated with serum IgA concentrations of greater than 0.05 g/l but less than the first or even fifth centile of normal, and the use of the term partial IgA deficiency is valuable in this context. Serum concentrations of IgM

and/or IgG are sometimes high, the increase in IgG being due to IgG1 and IgG3. Serum IgA deficiency is almost always associated with secretory-IgA deficiency and cases of secretory-IgA deficiency with normal serum IgA are rare, if they occur at all. Therefore, the measurement of secretory immunoglobulin concentrations provides no further information.

IgA deficiency may be found in patients who are investigated for recurrent respiratory tract infections but may also be found coincidentally during the investigation of other problems. Unlike the types of immunoglobulin deficiency so far discussed, selective IgA deficiency is not necessarily associated with an increased susceptibility to infections. Indeed, it has been found that many IgA deficient blood donors are completely asymptomatic. This is probably because secretory-IgM and systemic-IgG antibodies compensate for the lack of secretory-IgA antibodies. Those individuals who are abnormally susceptible to respiratory tract infections may only develop frequent 'viral' upper respiratory tract infections, but some have lower respiratory tract infections and occasionally bronchiectasis. There is growing evidence that patients with severe infections sometimes have additional defects of IgG antibody responses despite a normal or raised serum IgG concentration. Low serum IgG4 and/or IgG2 concentrations are common in such patients (see Chapters 2 and 5) and some have low systemic antibody responses following immunization.

Several other diseases have also been associated with IgA deficiency (Table 8.3) which may, therefore, be a coincidental finding during the course of routine investigations of those diseases. These associations have always been conjectural because it has been argued that chance associations might occur due to the high prevalence of selective IgA deficiency. However, it is clear to those who study such patients that the associations are real but that the mechanisms of association are probably different.

A low serum IgA concentration has been shown to be a predisposing factor in infants who subsequently develop allergic diseases[12] and it is suggested that deficient secretory-IgA antibody responses against potential allergens

Table 8.3 Disease associations with selective IgA deficiency

Recurrent respiratory tract infections
Allergic diseases
Autoimmune diseases
e.g. systemic lupus erythematosus juvenile onset autoimmune thyroiditis type I diabetes mellitus
Coeliac disease
Anaphylactic reactions to blood transfusions
Other possible associations
polyarthritis - malignancies of stomach and lymphomas - post-gastrectomy diarrhoea

increases the degree to which those allergens are absorbed across mucous membranes, thereby increasing the chances of IgE antibody responses against them. Similar mechanisms may be operating in older children and adults with established allergic diseases who have IgA deficiency. It is said that the management of allergic diseases is more difficult than usual in IgA deficient patients. This may, in part, be because recurrent infections are a frequent 'trigger' factor in addition to allergen exposure. Indeed, it is often difficult to differentiate between allergic and infective respiratory tract symptoms. Partial IgA deficiency is not uncommonly found in patients with asthma and rhinitis 'triggered' by allergies and infections. Measurement of serum IgA concentrations will identify those patients with partial or 'complete' IgA deficiency in whom particular care should be taken to reduce the effects of allergies and recurrent infections.

Deficiency of secretory-IgA antibodies also results in the excess absorption of dietary macromolecules from the gut. Hence, IgA deficient individuals have a much higher than normal incidence of serum antibodies to bovine proteins and, consequently, circulating immune complexes[13]. It has been suggested that such circulating immune complexes might be the cause of the polyarthritis and vasculitis which are occasionally seen in IgA deficient individuals, but the relationship is far from conclusive.

The relationship between coeliac disease, some autoimmune diseases and IgA deficiency is not understood and the demonstration of IgA deficiency in patients with these problems has no practical significance. The relationship may, in fact, be through common linkage with human leukocyte antigens (HLA). Coeliac disease and some autoimmune diseases are associated with certain HLA antigens and haplotypes, in particular HLA A1, B8, DR3. This same haplotype has recently been found to be more prevalent than normal in IgA deficient individuals.

The serum of between 30 and 50% of IgA deficient individuals contains antibodies against human IgA. The reason for their presence is not known but might reflect cross-reacting antibodies to bovine immunoglobulins. When present in high titre, anti-IgA antibodies may cause anaphylactic reactions following the transfusion of normal blood containing IgA. It is, therefore, important to search for IgA deficiency in patients who have unexplained reactions to blood, particularly if the patient also has clinical problems associated with IgA deficiency. If it is known that a patient has anti-IgA antibodies, anaphylactic reactions can be avoided by giving washed packed red blood cells or whole blood from IgA deficient blood donors.

Selective IgG deficiency and selective IgM deficiency

Both are rare but can be a cause of recurrent pyogenic infections. Patients with selective IgM deficiency are particularly susceptible to meningococcal meningitis and septicaemia, and IgM deficiency must be considered in any patient with recurrent or unexplained meningococcal sepsis.

IgG subclass deficiency

Complete or partial deficiency of one or more IgG subclass may be associated

with an increased susceptibility to infections, although rare asymptomatic individuals with severe IgG subclass deficiencies have been described (see Chapter 3). Deficiency of IgG2, often with undetectable IgG4, is most common and is found in association with selective IgA deficiency or as an isolated finding. The IgG2 deficiency is probably associated with low antibody responses against polysaccharide antigens because patients are susceptible to infections by bacteria with polysaccharide capsules such as *Haemophilus influenzae* and pneumococci. Immunization studies in the small number of reported cases have demonstrated deficient antibody responses against polysaccharide antigens. Total serum IgG concentrations are usually normal and the diagnosis of an IgG subclass deficiency can only be made by measuring the serum concentrations of each subclass. The assay methods for doing this are established but not widely available, and there are still problems with the standardization of results.

There is little information on the prevalence of IgG subclass deficiencies, but two studies have shown that approximately 5% of patients with chronic chest infections have IgG2 deficiency, and that the prevalence is even higher in patients with recurrent chest infections mainly because many such patients have IgA deficiency[14] (French and Harrison, unpublished). Isolated IgG4 deficiency has been associated with recurrent chest infections, but the significance of this finding is difficult to determine because a proportion of normal individuals have undetectable serum IgG4 using the assay methods currently available.

Where clinical findings are suggestive of an antibody deficiency syndrome, but serum immunoglobulin concentrations are normal, IgG subclass concentrations should be measured because gammaglobulin therapy can be effective in some symptomatic patients with IgG subclass deficiency.

Other causes of primary immunoglobulin deficiency

Immunoglobulin deficiency may be one manifestation of several congenital syndromes in which the immune defect is only one of a number of congenital anomalies (Table 8.4). In the appropriate clinical setting, measurement of serum immunoglobulin concentrations may aid in diagnosis. Various degrees of immunoglobulin deficiency may also occur in other rare and poorly classified syndromes.

Antibody deficiency with normal immunoglobulins

It is well established that an antibody deficiency syndrome can occur in individuals with normal or high serum immunoglobulin concentrations, although at present it is not known how common a problem this is. It, therefore, follows that an antibody deficiency syndrome cannot be absolutely excluded by demonstrating normal concentrations of serum immunoglobulins or even IgG subclasses. In this situation the diagnosis can only be made by measuring systemic antibody responses.

Table 8.4 Primary immunoglobulin deficiency in congenital syndromes

Syndrome	*Clinical features*	*Serum immunoglobulins*	*Other immune defects*
Ataxia telangiectasia	Ataxia, telangiectasia and recurrent respiratory tract infections	IgA deficiency in 40% often with IgE and/or IgG2 deficiency	Cell-mediated immunodeficiency in some cases
Bloom's syndrome	Short stature, photosensitivity and recurrent respiratory tract infections	Low serum immunoglobulins but not hypogammaglobulinaemia	*In vitro* T-cell defects
Wiskott–Aldrich syndrome	Thrombocytopaenia, eczema and recurrent pyogenic infections	Low IgM with high IgE and sometimes IgA	Cell-mediated immunodeficiency in some cases
Short-limbed dwarfism	Short-limbed dwarfism and ectodermal dysplasia	Hypogammaglobulinaemia	Usually part of a combined immunodeficiency syndrome

Secondary immunoglobulin deficiency

Immunoglobulin deficiency is more often secondary to an underlying disease process than due to a primary immune defect. In addition, there is a steadily increasing number of drugs which can cause various degrees of immunoglobulin deficiency. Such causes must, therefore, be considered whenever immunoglobulin deficiency is demonstrated.

Disease-associated secondary immunoglobulin deficiency

In most instances the cause of disease-associated secondary immunoglobulin deficiency is obvious but it is not always so. Several diseases may be responsible and can be conveniently considered in six categories (Table 8.5).

Table 8.5 The major causes of disease-related secondary immunoglobulin deficiency

Marrow disorders	Hypoplastic anaemia Metastatic infiltration Myelosclerosis Paroxysmal nocturnal haemoglobulinuria
Excessive loss	Nephrotic syndrome Protein losing enteropathy Burns
Reduced synthesis	Uraemia Severe viral infections, e.g. rubella
Hypercatabolism	Familial idiopathic hypercatabolic hypoproteinaemia Dystrophia myotonica Myeloma Anti-immunoglobulin antibodies Wiskott-Aldrich syndrome
B-cell lymphoproliferative disease	Chronic lymphocytic leukaemia B-cell lymphoma Waldenström's macroglobulinaemia Myeloma Heavy chain disease
Miscellaneous	Systemic lupus erythematosus

Marrow disorders

The bone marrow is the site of formation of stem cells from which B-cells and plasma cells are produced, and it also contains large numbers of plasma cells which synthesize a large proportion of serum antibodies, particularly IgG antibodies. Therefore, any disease which causes bone marrow 'failure' or infiltration can result in reduced immunoglobulin production and serum immunoglobulin deficiency, sometimes hypogammaglobulinaemia.

Excessive loss

Loss of immunoglobulins from the body, due to whatever cause, mainly affects IgG because it has the longest halflife and is, therefore, resynthesized at a slower rate than IgM and IgA. In the nephrotic syndrome it is predominantly IgG alone which is affected. The IgG deficiency is partly due to to a generalized loss of small molecular weight proteins through 'leaking' glomerular basement membranes damaged by various glomerulopathies, but also due to hypercatabolism of IgG. Immunoglobulin loss in a protein-losing enteropathy is less selective than in the nephrotic syndrome, and may be severe enough to cause hypogammaglobulinaemia. There are many causes of protein losing enteropathy, including primary disorders of the gastro-intestinal tract such as ulcerative colitis, Whipple's disease, Menetrier's disease and primary intestinal lymphangiectasia, and secondary causes such as tricuspid regurgitation and various causes of lymphatic obstruction.

Other serum proteins, particularly albumin, are lost in both the nephrotic syndrome and protein losing enteropathies. Therefore, a low serum albumin concentration provides evidence that serum immunoglobulin deficiency is secondary rather than primary.

Reduced synthesis

The 'toxic' effect of uraemia on the immune system is well recognized, and results in reduced immunoglobulin synthesis as well as depressed cell-mediated immunity. Because IgM and IgA have a shorter halflife than IgG they are affected first. In severe uraemia, immunoglobulin deficiency may be associated with a reduced capacity to produce antibodies and an increased susceptibility to infections. Severe infections, particularly viral infections such as intra uterine rubella infection, may affect the immune system resulting in the reduced synthesis of immunoglobulins. The mechanisms involved are poorly understood. Hypogammaglobulinaemia is usually transient but IgA deficiency may persist.

Hypercatabolism

This is an uncommon cause of low serum immunoglobulin concentrations which may affect immunoglobulins alone or as part of a generalized increase in the catabolic rate of serum proteins. Because of its longer halflife, IgG is affected more than IgM and IgA. The catabolic rate of several serum proteins is increased in the rare condition of familial idiopathic hypercatabolic hypoproteinaemia, but in dystrophia myotonica, one of the best characterized causes of immunoglobulin hypercatabolism, only the catabolic rate of IgG is increased. Hypercatabolism of immunoglobulins due to various causes has been described in other diseases (Table 8.5).

B-cell lymphoproliferative disease

Abnormalities of serum immunoglobulins are, not surprisingly, a common

finding in patients with the various types of B-cell lymphoproliferative disease. Apart from monoclonal paraproteins, which are characteristic of macroglobulinaemia, myeloma and heavy chain diseases, immunoglobulin deficiency is also a frequent finding.

Serum immunoglobulins may be normal in chronic lymphocytic leukaemia and B-cell lymphoma, particularly in the early stages, but immunoglobulin deficiency is a common occurrence. This may be associated with an antibody deficiency syndrome which can be the presenting problem. A low serum IgM concentration is the commonest finding, but isolated IgA deficiency is occasionally seen. Hypogammaglobulinaemia may also develop and is associated with the greatest susceptibility to infections.

The synthesis of normal immunoglobulins is often decreased in myeloma and macroglobulinaemia by mechanisms which have been extensively investigated but are poorly understood. This also results in an increased susceptibility to infections. In some patients the low serum concentration of normal IgG is due, in part, to hypercatabolism, which probably explains why gammaglobulin therapy is often ineffective.

Drug-induced immunoglobulin deficiency

Several drugs may cause immunoglobulin deficiency (Table 8.6), usually IgA deficiency but occasionally hypogammaglobulinaemia. The mechanisms whereby drugs do this are unknown but may involve suppressor T-cells in some instances[15]. There is sometimes an associated increased susceptibility to infections. Serum immunoglobulin concentrations usually return to normal on ceasing the drug but IgA deficiency sometimes persists.

Table 8.6 Drugs which may cause immunoglobulin deficiency

Fenclofenac
Gold
Levamisole
Penicillamine
Phenytoin
Sulphasalazine

Serum immunoglobulin assay as a means of monitoring immunoglobulin therapy

Antibody deficiency syndromes are usually treated with gammaglobulin therapy in the form of intramuscular injections, intravenous infusions or by plasma infusions. By far the most common indication is hypogammaglobulinaemia. Serial measurement of serum IgG concentrations is of value in monitoring the adequacy of therapy, but the results of serum immunoglobulin assays must be assessed in the context of the overall clinical picture, and regular informed clinical follow-up of patients receiving immunoglobulin therapy is important. Some patients with apparently adequate serum IgG

concentrations continue to have repeated infections, and require intravenous immunoglobulin therapy or plasma infusions to obtain higher serum IgG concentrations than can be achieved with intramuscular injections. Furthermore, isolated measurements of serum immunoglobulin concentrations may not be the best way of determining the optimum dose of immunoglobulin therapy, at least in those patients receiving intravenous immunoglobulin therapy[16].

DISEASES ASSOCIATED WITH HIGH SERUM IMMUNOGLOBULIN CONCENTRATIONS

The serum concentration of gammaglobulins may be increased above normal in a large number and variety of diseases, reflecting a monoclonal or polyclonal increase of one or more immunoglobulin isotype. The differentiation of a monoclonal from a polyclonal increase of serum immunoglobulins is of obvious diagnostic importance and is discussed elsewhere in this volume (see Chapter 9). A polyclonal increase of serum immunoglobulins is a feature of several acute or chronic infective and inflammatory diseases, and in some of these diseases measurement of serum immunoglobulin concentrations can be of diagnostic value. However, it is in this situation that this investigation is often overused or misused. When used alone as a diagnostic investigation, the predictive value of high serum immunoglobulin concentrations is very poor, but when used in conjunction with other immunological, biochemical and clinical investigations such abnormalities can be of value in a limited number of diseases.

Occasionally, the pattern of elevation of serum IgM, IgG and IgA may provide a clue as to the cause of an an unexplained high ESR or incidentally found high serum gammaglobulin fraction. Several patterns of serum immunoglobulin elevation have been described in a variety of diseases[7], some common examples of which are shown in Table 8.7. However, the large number of diseases in which such abnormalities are encountered indicates the low diagnostic specificity of this investigation and, furthermore, in many of these diseases the occurrence of immunoglobulin elevation is variable, further reducing the diagnostic value. In most instances the measurement of serum immunoglobulin concentrations adds very little, if anything, to the use of other clinical and laboratory investigations, including other immunological investigations. There are, however, a small number of particular clinical situations in which patterns of immunoglobulin elevation are of value in disease diagnosis and classification and others in which measuring serum immunoglobulin concentrations may be of use in monitoring disease activity or predicting complications.

Liver diseases

An increased serum concentration of one or more class of immunoglobulin is a frequent finding in patients with all types of liver disease. Although not absolute, the patterns of serum immunoglobulin elevation are sufficiently

Table 8.7 Patterns of serum immunoglobulin elevation in some diseases

Predominant elevation of serum immunoglobulin(s)	*Disease*
IgG	Connective tissue diseases — systemic lupus erythematosus — mixed connective tissue disease — Sjöegren's syndrome — polymyositis — unclassified Autoimmune thyroiditis 'Lupoid' chronic active hepatitis Sarcoidosis Acquired immune deficiency syndrome (some cases)
IgM	Acute viral infections — hepatitis-A — infectious mononucleosis — others Primary biliary cirrhosis Congenital and neonatal infections (see Chapter 7) Malaria Trypanosomiasis
IgA	Acute gastrointestinal or respiratory tract infections Gluten enteropathy IgA glomerulopathies Ankylosing spondylitis Rheumatoid arthritis
IgG and IgA	Cirrhosis Chronic respiratory tract infections — bronchiectasis — tuberculosis Acquired immune deficiency syndrome (some cases)
IgG, IgM and IgA	Chronic bacterial infections — infective endocarditis — subphrenic abscess — osteomyelitis — empyema

distinctive to be of diagnostic use, particularly when considered in conjunction with other immunological and biochemical investigations[17]. Serum IgG concentrations may be increased in several types of liver disease but the highest concentrations occur in the serum of patients with chronic active hepatitis. Serum IgA concentrations may also be elevated in most liver diseases, except primary biliary cirrhosis, but abnormalities are greatest in micronodular cirrhosis. There is a disproportionate increase of polymeric IgA in the serum of patients with cirrhosis, perhaps reflecting impaired clearance by the liver. Concentrations of serum IgA tend to correlate with the degree of liver damage. In addition, increased serum IgA concentrations may also be seen in extra-hepatic obstruction. In primary biliary cirrhosis, serum IgM

concentrations are increased in approximately 80% of patients for reasons which are not understood. A high serum IgM concentration is also a frequent finding in acute hepatitis due to hepatitis-A virus infection but not that due to hepatitis-B virus infection.

It can be seen, therefore, that the measurement of serum immunoglobulin concentrations can have great practical value in the differential diagnosis of acute or chronic jaundice and other liver diseases.

Systemic rheumatic diseases

Serum immunoglobulin concentrations are also increased in many patients with connective tissue diseases and some other systemic rheumatic diseases. But, unlike liver disease, patterns of elevation are rarely distinctive enough to be of diagnostic use. Other clinical, radiological and immunological investigations are usually sufficient for diagnostic purposes. However, abnormalities can sometimes be of use in disease classification and monitoring disease activity or complications.

A high serum IgG concentration is a common finding in several of the connective tissue diseases, particularly when active, but seems to be most characteristic of sera containing antibodies against some of the extractable nuclear antigens, such as RNP and La, and cytoplasmic antigens, such as Ro. Hypogammaglobulinaemia is, therefore, a frequent finding in patients with systemic lupus erythematosus (SLE) whose sera contain these antibodies and patients with Sjöegren's syndrome or mixed connective tissue disease. The serum of some patients with typical connective tissue diseases, such as SLE and Sjöegren's syndrome, and, more importantly, atypical connective tissue diseases or related disorders, such as 'lupus-like' syndromes and recurrent abortion or fetal loss, do not contain antinuclear antibodies but do contain anti-Ro antibodies. The demonstration of high serum immunoglobulins during the course of investigations in such patients or even as an incidental finding can be a pointer to this situation.

Variable patterns of serum immunoglobulin increase occur in rheumatoid arthritis, with the most marked changes occurring in patients with concomitant Felty's or Sjöegren's syndrome.

The polyclonal increase of serum immunoglobulin concentrations in Sjöegren's syndrome appears to be related to the polyclonal lymphoproliferation which is characteristically present in exocrine glands, including salivary and lachrymal glands. Lymphoproliferation may eventually extend to tissues other than the exocrine glands and can become malignant. The onset of malignant lymphoproliferation is often accompanied by a reduction of previously high serum immunoglobulin concentrations and the development of hypogammaglobulinaemia or a monoclonal IgM κ paraprotein. Serial measurements of serum immunoglobulin concentrations may, therefore, be of value in the long-term management of this condition.

In general, the serial measurement of serum immunoglobulin concentrations is of little value in monitoring the activity of systemic rheumatic diseases. However, one exception may be ankylosing spondylitis. There is evidence that serum IgA concentrations, which are often high in untreated

disease, correlate with disease activity[18]. Serial measurements of serum IgA concentrations might, therefore, be used to assess the efficacy of therapy.

DISEASES ASSOCIATED WITH ABNORMALITIES OF IgE

Allergic diseases

The role of IgE antibodies in the immunopathogenesis of allergic diseases is well understood, and the demonstration of specific IgE antibodies by various methods including skin testing, provocation tests and *in vitro* methods such as the radioallergosorbent test (RAST) is a routine part of the investigation of those diseases. In contrast, the measurement of serum IgE concentrations is of diagnostic value in only a limited number of diseases. Although the serum IgE concentration is high in many patients with an allergic disease, in others it is normal despite the presence of specific IgE antibodies in tissues or serum. Presumably this is because most IgE antibodies are bound to mast cells in the mucosa of secretory surfaces and other connective tissues. Concentrations of serum IgE are much lower than those of the other immunoglobulin isotypes but with the use of sensitive immunoassays such low concentrations are easily measurable. A high serum IgE concentration may be found in all types of allergic disease but is found most frequently, and may reach the highest levels, in atopic eczema, particularly when associated with another allergic disease. Serum IgE concentrations may also be elevated in allergic asthma but the magnitude of the increase is less pronounced than in atopic eczema. Abnormalities of serum IgE are less common in allergic rhinitis. Indeed, in some studies as many as two thirds of patients with allergic rhinitis have had a normal serum IgE concentration, although most had a high normal concentration. Seasonal fluctuations in serum IgE occur in those individuals allergic to pollens and may account for normal findings in some patients. The highest serum IgE concentrations occur in patients with more than one allergic disease, and tend to be higher in seasonal rather than perennial allergies. Most cases of urticaria are not due to an allergy and, therefore, serum IgE concentrations are usually normal. Serum IgE is variable in food allergy and of little diagnostic value.

In most instances the diagnosis of an allergic disease requires detailed history taking and skin testing with the use of the serum RAST or provocation tests in selected cases only. In some instances the history of an allergic disease may not be clear-cut and, so as to identify those patients in whom further allergy investigations would be of value, the measurement of serum IgE concentrations can be useful. Examples are the diagnosis of allergic asthma in a child presenting with wheezy-bronchitis or nocturnal cough and also the differentiation of extrinsic from intrinsic asthma. However, it cannot be overstated that a normal serum IgE concentration does not exclude an allergic disease, particularly allergic rhinitis. A high serum IgE concentration in early childhood may be a predictor of the subsequent development of allergic disease.

Hyposensitization therapy results in an initial increase in serum IgE

followed by a decrease. Such changes, however, have not been found to be useful in monitoring the effectiveness of therapy.

Parasitic infestations

The serum IgE concentration is high, often very high, in patients with parasitic infestations including ascariasis, toxocariasis, capillariasis, schistosomiasis, ankylostomiasis and echinococcosis. IgE antibodies are an important part of the immune response against such parasites[19]. A part of the serum IgE is specific antibody against the parasite but most of it is not. That antibody which is not reactive with parasite antigens is produced because of polyclonal activation of IgE producing B-cells by the parasite or its products. Where parasitic infestation is a possibility in a patient with non-specific symptoms, the demonstration of a high serum IgE concentration may be a valuable indicator of the diagnosis. Furthermore, a reduction of serum IgE can sometimes be useful in gauging the success of therapy.

Serum IgE in immunodeficiency syndromes

Some patients with selective IgA deficiency or ataxia-telangiectasia also have IgE deficiency, but this is not clinically important. A high serum IgE concentration, on the other hand, is a more common and clinically significant finding in some immunodeficiency syndromes. In the Wiskott-Aldrich syndrome there is a relationship with the eczema which complicates that disease, and in some patients with selective IgA deficiency there is a relationship with allergic diseases.

High serum IgE concentrations are of particular significance in the immunodeficiency syndrome which is characterized by a high serum IgE concentration, eczema, recurrent staphylococcal abscesses and defects of neutrophil function, mainly impaired neutrophil chemotaxis. This syndrome has a variable clinical expression and includes conditions previously described as Job's syndrome and the hyper-IgE syndrome. It would appear that a variety of underlying immune defects can present with a similar syndrome[20]. Sometimes there is a family history of the same condition. Additional immunological defects can include reduced *in vitro* lymphocyte proliferation, other defects of neutrophil function and deficient systemic antibody responses. Clinical evidence of an allergic disease and positive prick skin tests are usually a feature, and it has been suggested that histamine released from mast cells as a consequence of allergen exposure causes a defect of neutrophil chemotaxis, and possibly the other immune defects as well. Some cases have improved with H_1 or H_2 histamine receptor blocker therapy. Measurement of serum IgE concentrations should, therefore, be included in the investigations of patients with recurrent staphylococcal abscesses, particularly if there is associated eczema or hayfever, because this might result in the demonstration of an immunodeficiency syndrome which can be treated with drug therapy.

DISEASES ASSOCIATED WITH ABNORMALITIES OF IgD

A polyclonal increase of serum IgD has been noted in a small number of diseases but there is, at present, no indication for routinely measuring serum IgD concentrations.

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9
Monoclonal Immunoglobulins

M. HAENEY

CONCEPT OF POLYCLONALITY AND MONOCLONALITY

Every immunoglobulin is the product of a single family or clone of plasma cells. Since almost all immunogenic substances have multiple antigenic sites, many different clones are involved in the specific antibody response, but each clone will synthesize and secrete a unique heavy and light chain variable region.

The proliferation of a clone of B-lymphocytes or plasma cells leads to an increased production of immunoglobulin molecules of identical class, subclass, V_H and V_L regions. Such monoclonal immunoglobulins have an identical amino acid sequence and electrophoretic mobility (Figure 9.1): they are termed paraproteins or 'M' components.

Paraproteins are secreted by many tumours of B-lymphocyte or plasma cell origin, but may also result from any aberrant immune response that gives rise to disproportionate proliferation of a clone of cells normally engaged in immunoglobulin production. Under special conditions, a single clone or restricted number of clones may respond to antigen-driven proliferation, producing a monoclonal or oligoclonal response. Restricted heterogeneity is most commonly due to the limited ability of the host to make a broad immune response. This is best illustrated following bone marrow transplantation: initially, only a few B-cell clones are able to respond to antigen and this is reflected in the presence of multiple paraproteins. As the graft proliferates, however, more and more donor clones respond and the paraproteins gradually evolve into a polyclonal response. Restricted responses are often seen in autoimmune disorders, such as systemic lupus erythematosus or chronic active hepatitis, where only a few clones are able to react with self-antigens.

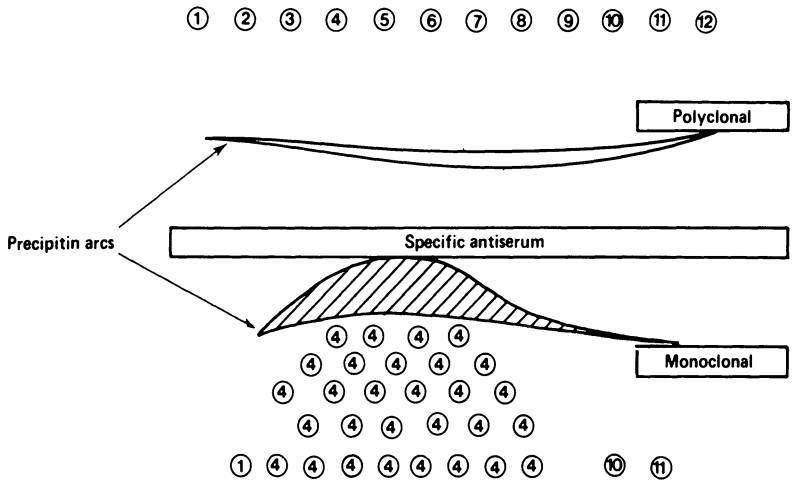

Figure 9.1 Schematic representation of monoclonal immunoglobulin detection. A normal serum immunoglobulin class is polyclonal, that is, composed of many different antibody specificities, here represented by clones (1)–(12). The malignant proliferation of a single clone of cells, e.g. (4), produces antibody of a single specificity with a distinct electrophoretic mobility. The monoclonal protein is detected by its characteristic precipitin arc on immunoelectrophoresis using antisera specific for different heavy and light chains

Monoclonal products of B-lymphocyte tumours

Most of the monoclonal immunoglobulins synthesized by B-lymphocyte tumours are structurally normal and some show antibody activity, most frequently directed towards auto-antigens or bacterial antigens.

Some malignant tumours produce abnormal immunoglobulin fragments (Table 9.1). The best recognized fragments are monoclonal free immunoglobulin light chains, or Bence-Jones protein. All plasma cells produce a slight excess of free light chains so the quantity of Bence-Jones protein detected is of importance. In practice, however, malignant cells have a more severe imbalance in heavy and light chain synthesis than do normal B-cells and produce larger quantities of Bence-Jones protein.

Some malignant tumours secrete Bence-Jones protein in the absence of complete immunoglobulin molecules. These neoplasms tend to grow faster

Table 9.1 Monoclonal products of B-lymphocyte and plasma cell tumours

Complete immunoglobulin molecules
Complete immunoglobulins and monoclonal free light chains (Bence-Jones protein)
Immunoglobulin fragments only
Free complete light chains (Bence-Jones protein)
Free incomplete heavy chains (heavy chain disease)
Half-molecules

and carry a poorer prognosis than tumours which produce intact immunoglobulins. Other tumours may secrete structurally abnormal light chain fragments or even fail to secrete at all: the highly malignant nature of these tumours has led to the concept[1] that the immunochemical dedifferentiation of the immunoglobulin product parallels the functional dedifferentiation of the malignant cell. Besides free light chains, B-lymphocyte tumours may produce free but structurally abnormal heavy chains. Four types of heavy chain disease (HCD) have been described: κ-HCD is the most common, γ-, μ- and δ-HCD are much rarer.

Clinical significance of monoclonal proteins

Many studies have been carried out to determine the frequency of paraproteins in healthy populations. The incidence in the general population is clearly age-related (Table 9.2): paraproteins have been found in about 1.5% of people aged over 50 years, and in approximately 3% of those older than 70. However, the incidence of overt myeloma in the general population is low and studies in Sweden show that there are about 5000 paraproteins per million of the population but only 50–60 cases of myeloma[2].

Table 9.2 Incidence of monoclonal proteins in healthy individuals

Group	*Percentage**
Blood donors (<60 years old)	0.1–0.3
Apparently healthy population	
>50 years old	0.9–2.1
>60 years old	1.4–3.2
>70 years old	3.1
>90 years old	6.0–19.0

*Percentages reported in various series

The paraprotein picture is different in hospital in-patients (Figure 9.2): three-quarters have, or will develop, malignant B-cell tumours, multiple myeloma being by far the commonest cause, accounting for three out of every five cases of paraproteinaemia. Only a quarter of patients have no detectable malignancy, although many have chronic disease.

DETECTION OF MONOCLONAL IMMUNOGLOBULINS IN BODY FLUIDS

Two important steps in the investigation of patients with suspected paraproteins involve the detection and characterization of paraproteins.

Detection of serum paraproteins

Paraproteins in serum samples are recognized by high resolution

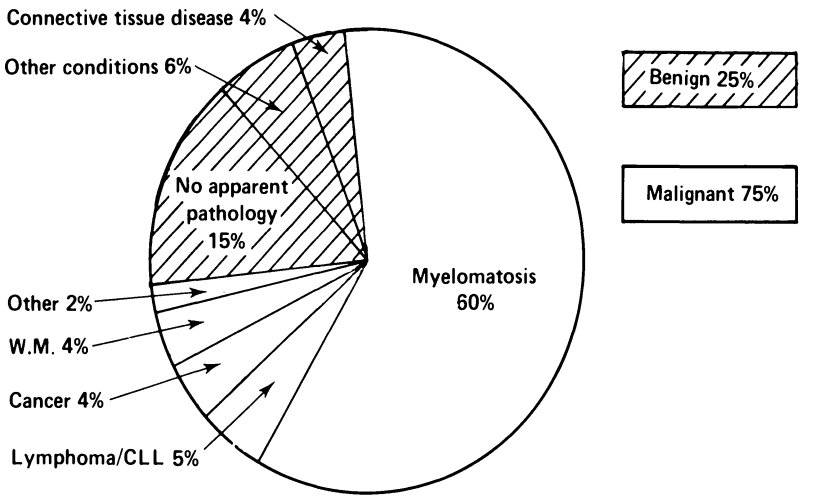

Figure 9.2 Final diagnosis in hospital patients with serum paraproteins of any immunoglobulin class. CLL = chronic lymphatic leukaemia; WM = Waldenström's macroglobulinaemia

electrophoresis on support media such as cellulose acetate or agarose, followed by staining of the protein bands. Fresh normal serum readily separates into pre-albumin, albumin and five globulin bands (Figures 9.3): $\alpha 1$, which is formed mainly by α_1-antitrypsin; $\alpha 2$, formed mainly by haptoglobin and α_2-macroglobulin; β_1 and β_2, due largely to transferrin and the third component of complement, respectively; and γ, which contains the immunoglobulins.

Paraproteins are identified by visual examination of the electrophoretic strip (Figure 9.3). At best, paraproteins appear as dense, well-defined bands, sometimes set against a pale background caused by low levels of other immunoglobulins. Although they are most commonly seen in the γ region, bands may appear in the β or even the α regions.

Multiple bands may represent the products of biclonal tumours (see below) or, more commonly, Bence-Jones protein together with intact immunoglobulin paraprotein, or polymerized paraproteins, particularly those of IgA class. Sometimes, multiple banding is seen in a benign oligoclonal response to antigenic challenge.

Bands of low intensity which migrate close to, or within normal electrophoretic zones can be easily missed. On the other hand, 'false' bands which are not paraproteins may be misinterpreted as such by the inexperienced. The most common pitfalls are: (a) *haemoglobin haptoglobin complexes* in a haemolysed sample, migrating in theβ position; (2) *fibrinogen* in plasma or an incompletely clotted sample, causing a band in the mid γ zone; (3) *aggregated IgG* in aged serum or large amounts of *immune complexes*, usually in patients with circulating rheumatoid factor, producing a γ band; (4) *hyperlipidaemia* or (5) genetic variants of *transferrin* or *bisalbuminaemia*.

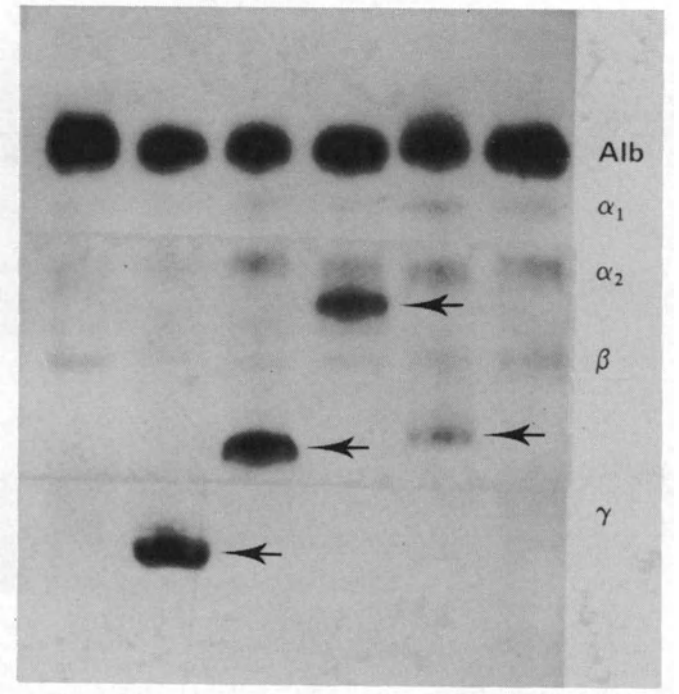

Figure 9.3 Cellulose acetate electrophoresis of six sera showing the separation into albumin, α1, α2, β and γ globulin bands. Immunoglobulins form the broad γ band. Of the six sera, the outer two are normal while the inner four are from patients with myeloma. Monoclonal proteins are seen as discrete bands (arrowed), usually in the γ region (IgG, IgM and IgD paraproteins), although they may occur in the β or more rarely the α regions (IgA paraproteins and free monoclonal light chains)

Detection of Bence-Jones protein in urine

The diagnosis of myeloma or amyloidosis may be overlooked if inappropriate tests for Bence–Jones proteinuria are employed. Not all proteinuria is albuminuria, and its investigation demands that the components be measured and identified by 24 hour urine protein excretion and immunochemical testing.

The classical heat precipitation test for Bence–Jones protein is insensitive and obsolete, while chemical tests such as Bradshaw's ring test are non-specific and lack sensitivity. The most reliable method is electrophoresis of concentrated urine followed by immunofixation or immunoelectrophoresis.

Characterization of paraproteins

Specific identification of heavy and light chains can be made by immuno-electrophoresis or immunofixation. Immunoelectrophoresis has, for many years, been the method of choice: monoclonal proteins give localized

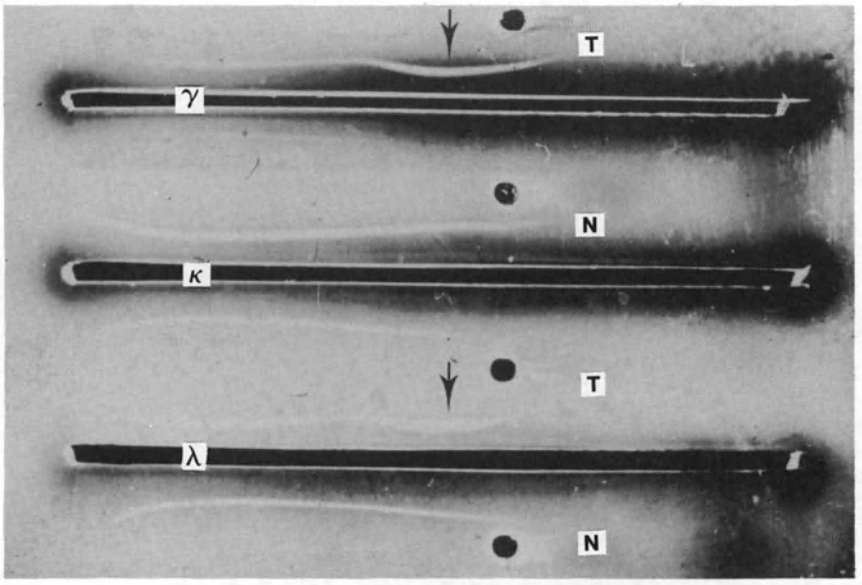

Figure 9.4 Immunoelectrophoresis in agar of test (T) and normal (N) serum. The anode is to the right. After electrophoresis, the troughs were filled with antisera to γ heavy chain, κ light chain or λ light chain. A monoclonal IgG (type λ) is seen as a localized, thickened bowing of the relevant precipitin arcs (arrowed)

thickening and bowing of the appropriate immunoglobulin precipitin lines (Figure 9.4). Interpretation does, however, require experience and the technique may be insensitive in typing faint immunoglobulin bands.

Immunofixation is gradually superceding immunoelectrophoresis because it has the advantages of sensitivity and good resolution, and the position of the fixed band can be compared with that seen on the original electrophoretic strip. Immunofixation is said to be ten times more sensitive than electrophoresis alone for the detection of Bence–Jones protein.

Quantitation of paraproteins

Serial measurement of paraproteins is essential for monitoring therapy of malignant B-cell tumours (see below). Accurate quantitation of absolute levels is, however, difficult because it is not possible to relate the concentration of a monoclonal immunoglobulin to a polyclonal reference serum. Routine immunochemical methods such as radial immunodiffusion or nephelometry are unreliable. Paraproteins are most satisfactorily measured by scanning densitometry of electrophoretic strips.

'BENIGN' MONOCLONAL GAMMOPATHY (MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE)

Benign monoclonal gammopathy can be defined as the presence of a monoclonal protein in a person showing no other manifestations of malignant disease: approximately 20–25% of all hospital patients with paraproteins (Figure 9.2) and the vast majority of asymptomatic people with paraproteins have benign monoclonal gammopathy by this definition.

The term 'benign' monoclonal gammopathy is, in many ways, inadequate because one cannot tell which paraproteins will remain benign and which will eventually be associated with myeloma or other malignant tumours of B-cells. For this reason, the term 'monoclonal gammopathy of undetermined significance' is preferable[3].

'Benign' paraproteins can be transient or persistent. Transient paraproteins represent antigen-stimulated clones which are still under the control of normal regulatory mechanisms. They are well recognized in B-cell depleted patients with hypogammaglobulinaemia where they are the products of the only clones able to respond. Transient paraproteins are also seen in circumstances where restricted antigenicity ensures that proliferation occurs in one or very few clones: these include infection by organisms such as mycoplasma or cytomegalovirus, and autoimmune disorders, for instance rheumatoid arthritis, coeliac disease and chronic active hepatitis.

Persistent benign paraproteinaemias probably represent responses to antigenic challenge by clones which have partly lost the normal feedback inhibition controlling clonal expansion. Chronic antigenic stimulation seems to be a triggering factor, and benign paraproteins are well recognized in chronic inflammatory disorders such as rheumatoid arthritis and osteomyelitis.

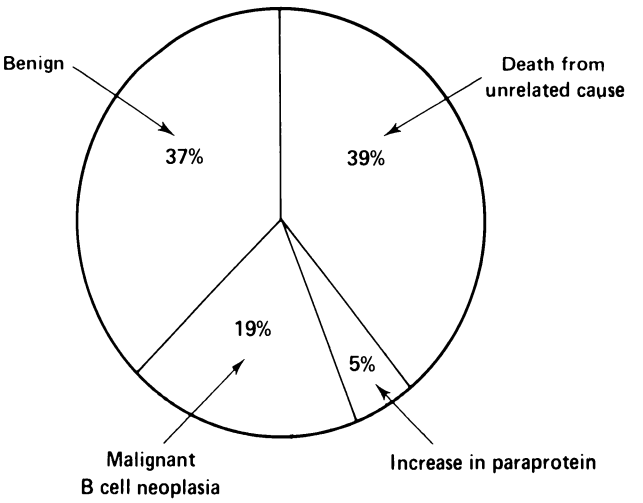

Figure 9.5 Outcome of 'benign' monoclonal gammopathy after 10 year follow-up. (**Data from** Ref. 3)

In clinical practice, the dilemma lies in deciding whether a small amount of paraprotein in an asymptomatic subject indicates an early but malignant B-cell tumour or a truly 'benign' monoclonal gammopathy.

Kyle[3] has recently reviewed the course of nearly 250 patients with 'benign' monoclonal gammopathy followed up for at least 10 years (Figure 9.5). In only about one-third had the level of the paraprotein remained stable without evidence of a malignant B-cell tumour; that is, truly benign monoclonal gammopathy. In 5% the paraprotein level had increased by over 50% and in some Bence-Jones proteinuria was also present. Forty-six of the patients (19%) had developed overt multiple myeloma (32 cases), Waldenströms macroglobulinaemia (6), amyloidosis (6) or a malignant lymphoproliferative process (2). The remaining 39% of patients died from unrelated causes.

The median interval from recognition of the monoclonal protein to the diagnosis of multiple myeloma was over 8 years, but ranged from 2 to 20 years. The median interval was nearly 10 years for Waldenströms macroglobulinaemia and almost 8 years for amyloidosis.

Although there are no firm criteria for establishing from the outset whether a paraprotein is benign or malignant, certain laboratory findings are of predictive value (Table 9.3).

Table 9.3 Findings suggestive of malignant paraproteinaemia

1.	Significant amounts of Bence-Jones proteinuria
2.	Suppression of normal immunoglobulin synthesis
3.	Marked elevation of serum paraprotein level
4.	Rising paraprotein level with time

The presence of immunoglobulin fragments, particularly Bence-Jones protein, is a strong indicator of malignancy. While small amounts of Bence-Jones protein can sometimes be secreted by benign clones, significant (i.e. greater than 0.3 g per 24 h) Bence-Jones proteinuria is an ominous diagnostic feature. Apparently benign cases of Bence-Jones proteinuria[3] must be followed indefinitely because the risk of serious disease is so great.

Immunosuppression is a strong pointer in favour of malignancy. Immune suppression usually affects IgM synthesis first, followed by IgA or IgG, and reflects synthetic failure rather than hypercatabolism.

Because benign lesions rarely produce high serum concentrations of paraproteins, the paraprotein level can be of differential value, provided the different halflives of the immunoglobulin classes are taken into account. Thus, benign paraproteins rarely exceed 30g/l for IgG (halflife: up to 23 days), or 10g/l for IgA (halflife: 6 days) and IgM (halflife: 5 days), although some exceptions exist.

The most reliable criterion of a benign paraprotein is the demonstration that its level is stable with time. A patient with a high and increasing serum paraprotein level, suppression of normal immunoglobulin synthesis and significant Bence-Jones proteinuria is likely to develop the full clinical picture of a malignant B-cell tumour within a relatively short time. Indefinite review

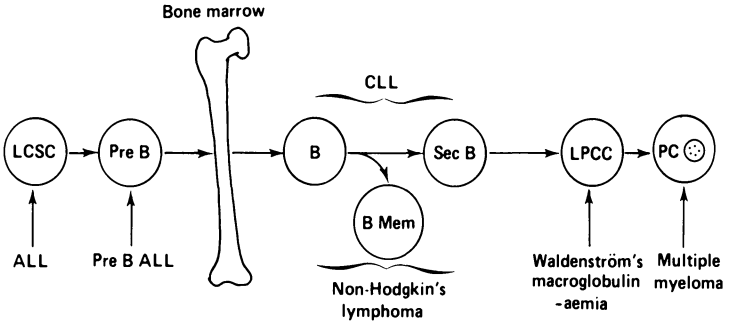

Figure 9.6 B-lymphocyte maturation steps with their malignant counterparts. LCSC = lymphocyte committed stem cell; Pre-B = pre-B lymphocyte; Sec B = secreting B-cell; LPCC = lymphoplasmacytoid cell; PC = plasma cell. ALL = acute lymphoblastic leukaemia; CLL = chronic lymphatic leukaemia

of all patients with paraproteinaemia at regular (3–6 monthly) intervals is therefore essential. In patients with 'benign' monoclonal gammopathy, treatment with cytotoxic drugs is contraindictated.

MALIGNANT PARAPROTEINS

A spectrum of malignant tumours of B-lymphocytes can give rise to paraproteinaemia. These tumours can be usefully thought of as representing cells frozen at different stages in the normal differentiation pathway of B-cells (Figure 9.6). Most forms of non-Hodgkins lymphoma and chronic lymphatic leukaemia, for instance, are malignant equivalents of the secreting or resting B-cell. Such cells express surface immunoglobulin but have little capacity for immunoglobulin secretion other than small amounts of IgM. In general, the more plasmacytoid the tumour, the more likely it is to produce a paraprotein and the more likely this is to be IgG or IgA rather than IgM.

Multiple myeloma

Myeloma is a malignant neoplasm of plasma cells and is almost invariably associated with paraproteinaemia or Bence-Jones proteinuria. Myeloma accounts for about 1% of all malignancies and 10% of haematological tumours. It is diagnosed with increasing frequency because of a growing awareness of its various presentations and improvements in immunochemical analysis. The incidence of the disease in Caucasians is about 1 per 50000.

Diagnostic criteria

The Medical Research Council's criteria for the diagnosis of myelomatosis are:

(1) The demonstration of a monoclonal immunoglobulin in serum or urine.

(2) The demonstration of neoplastic plasma cells in the bone marrow.
(3) The radiological demonstration of bone destruction.

At least two of these criteria are required to make the diagnosis.

Clinical features

Myeloma is usually a disease of the elderly; the mean age at presentation is approximately 60 years and it is uncommon below the age of 40 years. Both sexes are affected equally.

Presenting symptoms in patients with myelomatosis are related to either the extent of proliferation of the tumour mass or the nature or quantity of the paraprotein secreted. The appearance of a monoclonal protein may precede other manifestations by many years; thus, in about 10% of subjects, a monoclonal protein is found by chance on routine screening of serum samples or during investigation of an apparently unrelated disorder.

During the early phase, patients may be asymptomatic or complain only of non-specific symptoms such as lethargy, malaise and weight loss. Progressive doubling of the tumour mass eventually results in classical symptoms of established myeloma.

Skeletal manifestations

Tumour deposits cause bone pain, particularly in the ribs, spine and pelvis. Pathological fractures are common. Osteolysis is probably due to the production of osteoclast activating factor by plasma cells, leading to increased bone reabsorption. The consequent calcium release causes hypercalcaemia in about 35–45% of patients.

Hypercalcaemia

Hypercalcaemia and hypercalciuria give rise to feelings of nausea, anorexia, polydipsia and polyuria.

Renal impairment

Over half of all patients with myeloma develop renal insufficiency, and this is the second most common cause of death after infection. Aetiological factors include hypercalcaemia, hyperuricaemia, tubular precipitation of paraprotein, infection, Fanconi's syndrome, hyperviscosity, amyloidosis, glomerulosclerosis and drug nephrotoxicity[4].

Renal failure is strongly associated with excessive excretion of free light chains in the urine, an event which is toxic to proximal renal tubular cells. The evidence[5] suggests that some Bence-Jones proteins are more nephrotoxic than others, particularly those with a high isoelectric point. Renal failure is commonly precipitated by some intercurrent event such as infection or dehydration. Renal amyloidosis occurs in 5–15% of myeloma patients and causes glomerular lesions as well as interstitial damage. The prognosis is poor (see below).

Bone marrow infiltration

Plasma cell infiltration of the bone marrow causes a normochromic, normocytic anaemia and thrombocytopaenia. Suppression of normal immunoglobulin synthesis leads to recurrent pyogenic and viral infections and is a major cause of death.

Vascular features

Features of the hyperviscosity syndrome or cryoglobulinaemia may be clinically important in myelomatosis, although they are more usual in macroglobulinaemia.

Rare presentations

Acute spinal cord compression is an uncommon presenting feature and requires emergency decompression. Amyloidosis secondary to myelomatosis can cause a carpal tunnel syndrome or peripheral neuropathy. Occasionally, patients present with skin changes such as pyoderma gangrenosum, erythema annulare, pruritus, xanthomata or yellow discoloration. Other unusual presentations are related to the antibody activity possessed by the paraprotein (see below).

Diagnostic tests

Monoclonal immunoglobulins

Electrophoresis of *both* serum and concentrated fresh urine is essential and will show a paraprotein in almost every case of myelomatosis. Fewer than 1% of patients lack detectable paraprotein; that is, have non-secreting myeloma, and in most of these immunofluorescent examination of bone marrow will show intracellular immunoglobulin which is not secreted. Non-secretory myeloma is clinically indistinguishable from the secretory form and carries a similar prognosis[6].

The heavy and light chain components of the paraprotein can be typed by immunoelectrophoresis or immunofixation: IgG, IgA or free monoclonal light chains (Bence–Jones myeloma) account for the majority of cases (Table 9.4). About 17–25% of patients have Bence–Jones protein only with no serum paraprotein. Such cases have a poorer prognosis because diagnosis may be delayed, the tumour seems to be more invasive and faster-growing, it occurs in a younger age group, and is more likely than other types of paraprotein to be complicated by amyloidosis and renal impairment.

IgD accounts for only 1% of myeloma proteins but 80–90% of these are of λ light chain type. Rarely, two distinct serum paraproteins (biclonal myeloma) can be identified in the same patient. IgM and IgE paraproteins associated with myeloma are rare[6].

Measurement of serum immunoglobulin concentrations is required for two reasons: first, to assess the degree of suppression of non-myeloma

Table 9.4 Paraprotein type in myelomatosis

Type	*Percentage*
IgG	50–60
IgA	20–25
Light chains only	17–25
IgD	1–2
Biclonal	1
IgM	Rare
IgE	Rare

immunoglobulin synthesis and second, to measure the paraprotein level. Unfortunately, routine immunochemical methods may give misleading values, especially for IgM paraproteins. Scanning densitometric analysis of stained electrophoretic strips gives more reliable results for following the growth of the tumour.

Neoplastic plasma cells

The bone marrow shows increased numbers of abnormal plasma cells, including bi- or trinucleated cells with unevenly sized nuclei in over 90% of cases. The cytoplasm of such cells is basophilic and may contain acidophilic inclusions (Russell bodies) or crystals which can sometimes be shown to consist of immunoglobulin.

The number of plasma cells present is, in general, a poor guide to the

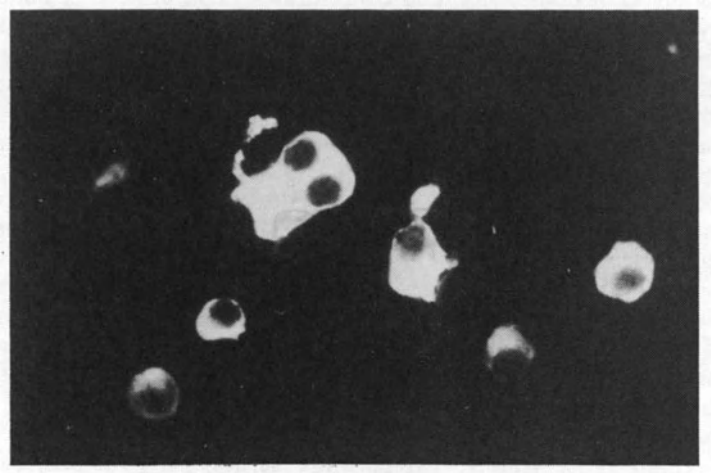

Figure 9.7 Direct immunofluorescent examination of bone marrow from a patient with IgA myeloma. The marrow was incubated with a fluorescein labelled antiserum specific for α heavy chains. Dense cytoplasmic staining is seen; many of the cells also show unevenly-sized nuclei or binucleated forms

progress of the disease. Although typical cases of myelomatosis show bone marrow plasmacytosis in excess of 20% (normal <5%), this figure can be reached in some reactive conditions and is not therefore diagnostic. Marrow involvement tends to be patchy in the early phases of the disease: a marrow specimen may then show no increase in plasma cells or only minor plasmacytosis. Direct immunofluorescent or immunoperoxidase examination of bone marrow specimens using labelled specific antisera to heavy and light chains will confirm the monoclonal nature of the condition and help to distinguish reactive from malignant plasmacytosis (Figure 9.7).

Bone destruction

Osteolytic lesions can be found in the skull in over half of all patients (Figure 9.8). The combination of osteolytic lesions and a normal serum alkaline phosphatase level is highly suggestive of myelomatosis. Generalized osteoporosis is also common but of little diagnostic value. Many patients also show compression fractures of vertebrae.

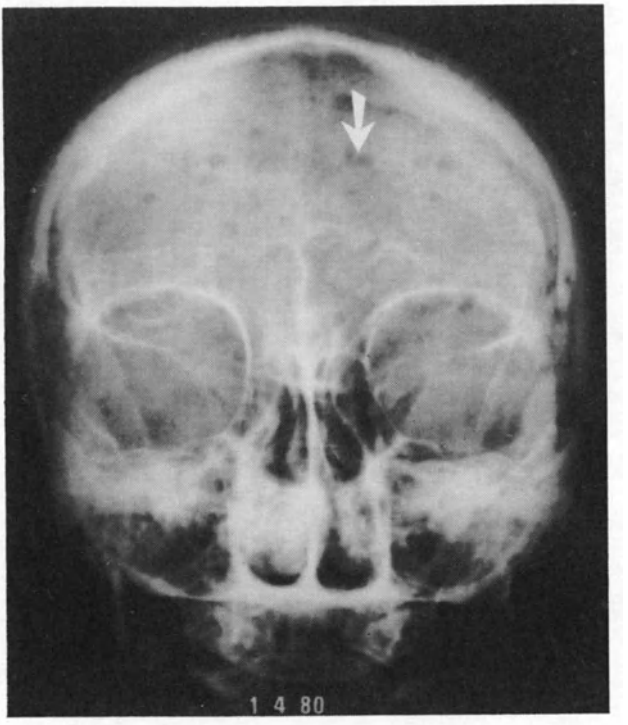

Figure 9.8 Multiple osteolytic lesions (arrowed) in the skull of a 67 year old woman with Bence-Jones myeloma

Table 9.5 Myeloma staging of Durie and Salmon and its relationship to survival

	Stage I *Low cell mass*	*Stage II* *Intermediate cell mass*	*Stage III* *High cell mass*
Haemoglobin (g/dl)	>10.0		<8.5
Serum calcium (mmol/l) (mg/dl)	<3.0 <12.0	Intermediate values	>3.0 >12.0
Radiology	Normal, or one lytic lesion		Lytic lesions +++
Monoclonal protein (g/l)	IgG<50 IgA<30 Urinary Bence-Jones protein <4g/day		IgG>70 IgA>50 Urinary Bence-Jones protein >12g/day
Myeloma cell mass ($\times 10^{12}/m^2$)	<0.6		>1.2
Median survival (months)	39	27	17

Myeloma staging and prognosis

During the clinical phase of myelomatosis, the mass of malignant plasma cells grows exponentially and there is a good relationship between the paraprotein level and the estimated tumour mass, a fact of considerable importance if the paraprotein level is to be used to monitor therapy.

At the lower limit of paraprotein detection of about 2g/l for IgG, the cell mass is 2×10^{10} cells or about 20g of tumour. Symptoms of myeloma begin when the paraprotein level is about 40g/l, equivalent to a tumour burden of approximately 5×10^{11} cells. Death occurs when the tumour mass reaches 5×10^{12} cells or 3kg.

Using these concepts, a series of independent studies have found that estimates of the myeloma cell mass at presentation correlate with survival to a clinically useful degree. Durie and Salmon[7] found that certain independent factors correlated well with the calculated tumour mass, and proposed a clinical staging system which divides patients into three groups based on the degree of anaemia and hypercalcaemia, the extent of osteolytic lesions and the serum concentration of the monoclonal protein (Table 9.5). Therapeutic response rates were similar in all three groups but survival was markedly better in patients with stage I disease (low tumour mass) than in those with stage III disease (high cell mass). These findings (Table 9.5) tend to validate the staging method and the use of the paraprotein level to monitor response.

Table 9.6 Factors correlating with poor survival in myelomatosis

Raised blood urea	>13mmol/l (>80mg/dl)
Low serum albumin	<30g/l
Low haemoglobin	<7.5g/dl
Heavy Bence-Jones proteinuria	

Several clinical features are of prognostic significance (Table 9.6). Impaired renal function is found in approximately half of patients at presentation and carries a poor prognosis.

Principles of management

Detailed discussion of treatment is outside the scope of this chapter but has been recently and extensively reviewed[8,9].

Before the introduction of melphalan and cyclophosphamide, survival of patients with myeloma was always less than a year, and usually less than 6 months. Since the first flush of success following the use of alkylating agents, however, progress in treating multiple myeloma has been disappointingly slow.

There are a number of difficulties in assessing the effectiveness of treatment of myeloma. First, some studies have included cases of indolent or smouldering myeloma. Second, various types of myeloma have significantly

different outlooks: patients with IgG (κ) myeloma, for instance, survive much longer (median 50 months) than those with λ Bence-Jones myeloma (median 22 months). Response to treatment and survival must, therefore, be evaluated separately for each type of myeloma. Third, there are problems in comparing response rates in different studies. Since truly 'complete' remissions are never achieved in myeloma, results compare various degrees of 'partial' remission. Some groups use $>50\%$ tumour regression as a 'response', but others use $>75\%$ as the cut-off point.

Induction of remission

Intermittent and continuous treatment regimes have been used; the main advantage of intermittent oral therapy is that severe marrow suppression is less common. Melphalan and cyclophosphamide are equally effective in inducing remission but both are toxic, particularly in the presence of renal failure. About 50–75% of patients will show a partial remission[8]. However, most patients subsequently relapse; the median duration of remission is 9 months, with median survival from starting treatment of about 24 months. Because of the shortcomings of melphalan or cyclophosphamide plus or minus prednisone, various combinations of chemotherapeutic agents have been tried, with controversial results[8,9]. Although there have been claims that combinations of agents such as vincristine, doxorubicin (Adriamycin), carmustine and others produce results superior to those achieved with melphalan and prednisone, this difference has not been proved conclusively. Furthermore, a combination of intravenous agents is less convenient, more expensive, produces more side-effects, and reduces the therapeutic options for future treatment.

Maintenance of remission

Most myeloma patients who achieve remission have less than 90% tumour regression and only about 10% have as much as 99% regression. Thus, even with a good remission the burden of residual tumour cells exceeds 10^{10}. The question of how long to continue chemotherapy is controversial. Stopping treatment after 1–2 years usually results in relapse, which may not respond to retreatment. However, continued chemotherapy may lead to refractory anaemia, a greater incidence of infections, or acute leukaemia (see below). Indeed, in several studies, patients maintained beyond 12 months on various cytotoxic drugs had no significant lengthening of survival compared with those given no maintenance therapy. Patients must, however, be closely monitored to detect relapse.

Management of relapse

When relapse occurs (Figure 9.9), the growth rate or doubling time of the tumour will be faster than before treatment in over 40% of cases (growth rate escape). This accelerated tumour growth is most commonly reflected in the

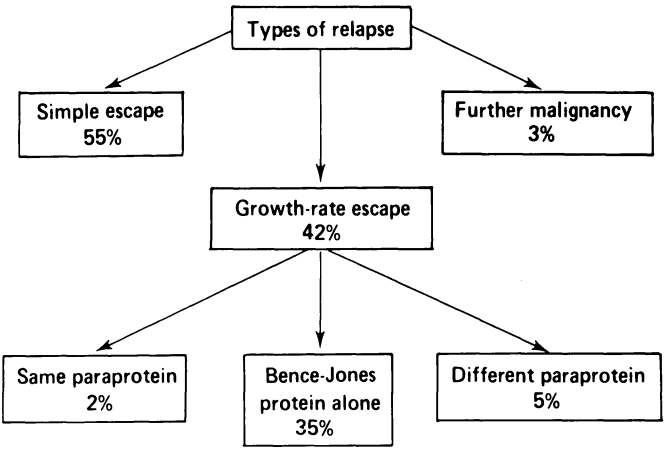

Figure 9.9 Modes of relapse in treated cases of myelomatosis. (Data from Ref. 10)

production of Bence–Jones protein alone; occasionally there is an increased rate of synthesis of either the presenting paraprotein or another monoclonal immunoglobulin. In just over half the patients, the serum paraprotein level and by implication the myeloma cell mass grows at a rate identical to that before treatment (simple escape). Sometimes, escape from the therapeutic control is due to the development of lymphoma or leukaemia. The incidence of leukaemia among myeloma patients is 30–230 times greater than expected[11] and appears to be linked to the dose of alkylating agents received by the patients. Presumably these drugs induce chromosomal damage and gene mutations or activate latent oncogenic viruses. Representatives of almost every type of acute leukaemia have been described but usually the leukaemic cells are myeloblastic or myelomonoblastic.

Only 10% of relapses respond to those first-line drugs used to induce remission. The mechanism by which selective resistance to alkylating agents occurs is not known. In suitable patients, anti-tumour agents such as vincristine, doxorubicin (Adriamycin) and carmustine (bischloronitrosurea; Bicnu) may induce a further remission in 30–70% of cases[9].

Despite the use of these various cytotoxic regimes, however, patients with multiple myeloma have a median survival of only 2–3 years[8]. Only about 2% of patients survive for more than 10 years and these tend to be younger individuals who do not have hypercalaemia at presentation, have better renal function, and show a good response to chemotherapy[12].

General care

Myeloma affects many organs and while cytotoxic therapy is the mainstay of treatment, management of complications (Table 9.7) must not be overlooked. In particular, the importance of emergency treatment of hypercalcaemia[13] and spinal cord compression[14] must be emphasized.

Table 9.7 Management of complications of myelomatosis

Hypercalcaemia
Rehydration
Steroids
Phosphate infusion
Mithramycin
Infection
Appropriate antibiotics
Immunoglobulin replacement
Renal impairment
Prevent dehydration
Avoid fluid restriction (e.g. before intravenous pyelography)
Treat hypercalcaemia and hypercalciuria
Standard lines of treatment
Spinal cord compression
Laminectomy
Localized irradiation
Hyperviscosity
Plasmapheresis

Patients who present with renal failure (Table 9.8) commonly do so after a precipitating event such as infection or dehydration: a rare but well-recognized trigger is intravenous urography. This investigation is most likely to be requested in the patient with undiagnosed myeloma who presents with renal impairment of uncertain cause. In these circumstances, dehydration prior to urography can lead to renal failure. If intravenous urography is required, then the patient should not be dehydrated beforehand.

Table 9.8 Common causes* of renal failure in multiple myeloma

Acute renal failure (50%)	*Chronic renal failure* (50%)
Hypercalcaemia (50%)	Bence-Jones proteinuria (80–90%)
Volume depletion (20%)	Hypercalcaemia (40–60%)
Nephrotoxic antibiotics (20%)	Hyperuricaemia
Intravenous urography (1%)	Amyloidosis (10%)
Others	Pyelonephritis

*Data from Ref. 8

Prompt rehydration, antibiotics, management of hypercalcaemia and hyperuricaemia, plus short-term dialysis if necessary, will often restore renal function. In patients who have urinary light chains without renal failure, a continued high-fluid intake of 3 litres a day helps prevent a deterioration in renal function[15].

Localized plasmacytomas

Although multiple myeloma is the usual mode of presentation of malignant plasma cell tumours, some plasmacytomas remain solitary either in bone marrow (that is, solitary myeloma) or in soft tissues (that is, extramedullary plasmacytoma) (Figure 9.10). Such localized plasmacytomas differ from multiple myeloma in their clinical features and prognosis[16].

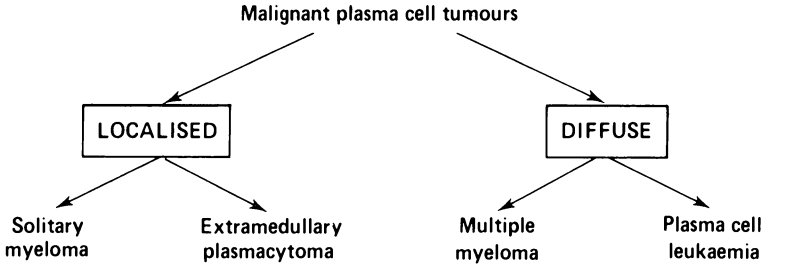

Figure 9.10 Classification of malignant plasma cell tumours

Patients with *solitary myeloma* typically present with skeletal pain relating to their tumour, and cord or nerve root involvement is common. Only 25% have a serum monoclonal component, usually in low concentration. About 60% of patients with solitary myeloma go on to develop myeloma within 10 years and a further 25% have either a local recurrence or a new lesion at a distant site. Dissemination is significantly more frequent if local treatment is inadequate.

Extramedullary plasmacytomas may affect a variety of tissues, but over 80% occur in the upper respiratory tract and oral cavity. Presenting symptoms depend on the site and extent of local disease: they include hoarseness, epistaxis and local pain. Only about one quarter of patients have a serum monoclonal protein and fewer than 4% have Bence-Jones protein. However, these figures are probably an underestimate because the diagnosis of extramedullary plasmacytoma is rarely suspected prior to surgery, and immunochemical studies are usually performed only after partial or complete removal of the tumour. Local recurrence of the tumour usually reflects inadequate local treatment with surgery and/or low dose radiation. Only about 6% of cases show local recurrence if higher doses of radiation are used from the outset.

Twenty per cent of extramedullary plasmacytomas arise at sites other than the head and neck, most frequently the lungs, lymph nodes, spleen and gastrointestinal tract. Dissemination is frequent.

The prognosis for patients with extramedullary plasmacytomas presenting in the upper respiratory tract and oral cavity is better than for patients with tumours in other sites and for those with solitary myeloma.

Plasma cell leukaemia

Plasma cell leukaemia is a rare form of plasma cell malignancy with an

extremely poor prognosis. The diagnosis is made when the proportion of plasma cells in the peripheral blood exceeds 20% of the white blood cell count, although in some series this criterion has been reduced to 10%.

The reported incidence amongst myeloma patients varies according to the diagnostic criteria but is usually less than 3–4%. Men are more likely to be affected than women, with a mean age of 50–60 years. The clinical picture is dominated by fatigue, weight loss and haemorrhage. Bone disease is less frequent than in myeloma but hepatomegaly, splenomegaly, lymphadenopathy, renal failure and hypercalcaemia are more common. In the majority of patients, the bone marrow is heavily infiltrated with abnormal plasma cells of varying maturity.

Waldenström's macroglobulinaemia

Waldenström's macroglobulinaemia (WM) is one of the commonest causes of serum paraproteins of IgM class (Figure 9.11). By current criteria, WM is best regarded as a slowly growing lymphoplasmacytoid lymphoma in which the malignant cells gradually infiltrate lymph nodes, spleen, liver and bone marrow.

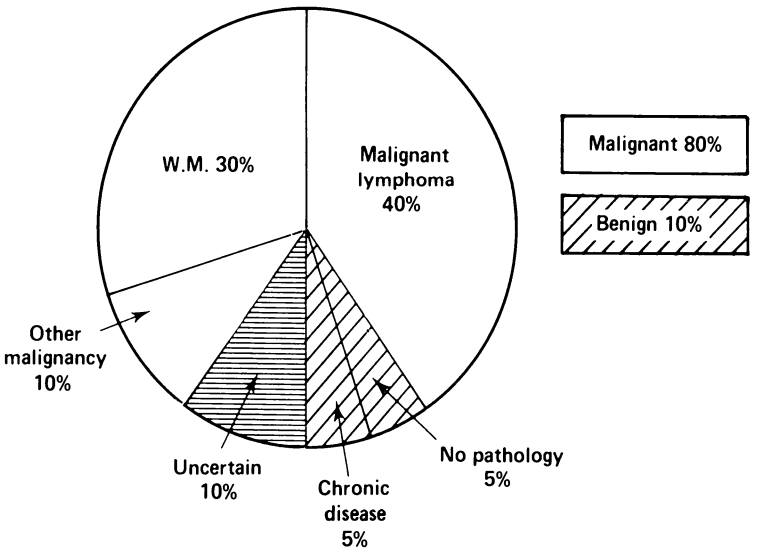

Figure 9.11 Final diagnosis in patients with serum paraproteins of IgM class. W.M. = Waldenström's macroglobulinaemia

Clinical features

Both males and females are affected, although males predominate. The usual age at diagnosis ranges from 50 to 75 years.

Table 9.9 Comparative clinical features of Waldenström's macroglobulinaemia and multiple myeloma

Clinical features	*Waldenström's macroglobulinaemia*	*Multiple myeloma*
Hyperviscosity syndrome	+++	+
Changes in visual acuity	+++	+
Changes in optic fundi	+++	+
Mucosal bleeding	+++	+
Hepatosplenomegaly	+++	+
Lymphadenopathy	+++	+
Anaemia	++	+++
Neuropathy	+	+
Leukopaenia	—	+
Thrombocytopaenia	—	+
Renal failure	—	++
Hypercalcaemia	—	++
Recurrent infection	—	++
Bone pain	—	+++
Lytic bone lesions	—	+++

Unlike multiple myeloma, symptoms of WM (Table 9.9) are usually directly attributable to the effect of the IgM paraprotein on serum viscosity rather than to the tumour itself.

There is a marked variation in the level of viscosity that produces symptoms in individual cases, but a relative serum viscosity of over 4.0 (normal 1.5–1.8) is usually symptomatic. Several factors contribute to the hyperviscosity syndrome (Table 9.10): high molecular weight paraprotein increases serum viscosity and reduces peripheral circulation; cryoglobulin precipitation causes further vascular sludging, ischaemia and necrosis, and adsorption of macroglobulin to the surface of platelets, red cells and phago-

Table 9.10 Paraprotein characteristics contributing to the hyperviscosity syndrome

High molecular weight
High serum concentration
Confinement to the intravascular pool
Polymer formation
Spatial configuration
Cryoglobulin formation
Interaction with other plasma proteins
Adsorption to surfaces of platelets, erythrocytes and phagocytes

cytes may contribute to a bleeding tendency, anaemia and polymorphonuclear dysfunction.

The clinical features of hyperviscosity can be vague and its onset insidious: weakness, fatigue, mucosal bleeding, and neurological manifestations such as headache, vertigo, ataxia, impaired vision or a deteriorating conscious level may be the dominant presentation. In some instances, patients present with congestive cardiac failure, hypertension, coma or epilepsy. On examination, the fundi may show characteristic venous engorgement and tortuosity ('string of sausages'), haemorrhages and papilloedema. Anaemia, lymphadenopathy and hepatosplenomegaly are common findings (Table 9.9) and reflect infiltration of the reticuloendothelial system by the malignant lymphoplasmacytoid cells. Peripheral neuropathy is often found and may be due to the antibody activity of the paraprotein directed against nervous tissue.

In contrast to myeloma, lytic bone lesions and heavy Bence-Jones proteinuria are rare in WM; thus, bone pain, hypercalcaemia and severe renal failure tend to be uncommon.

Diagnostic tests

Immunological findings

The characteristic abnormality is an excessive production of pentameric monoclonal IgM, although this is not diagnostic in its own right. Rarely, the typical lymphoid involvement of WM occurs with an IgG or IgA paraprotein and may represent lymphoplasmacytoid cells which have differentiated past the heavy chain 'switch'.

Haematological findings

Rouleaux formation in peripheral blood smears is seen in nearly all patients, with relative peripheral lymphocytosis, moderate anaemia, abnormal liver function, cold agglutinins and cryoglobulins in varying proportions.

The bone marrow is almost invariably infiltrated by lymphoplasmacytoid cells. Lymph node biopsies also show a diffuse infiltration with round cells, but the basic node architecture is retained. Immunohistochemical studies of infiltrating cells frequently show early forms with membrane-bound IgM and IgD, both of which bear the same idiotypic specificities as the secreted paraprotein.

Treatment

Because major features of WM are due to hyperviscosity, plasmapheresis will provide rapid symptomatic relief; excess IgM is removed and the serum viscosity and plasma volume restored to normal. A plasma exchange of 2–4 l can reduce the IgM level to 10–20% of its pretreatment level, but a more gradual effect is achieved by 1 l exchanges daily for 5–10 days. Subsequently, maintenance plasmapheresis (1 l exchanges every 2–4 weeks) will help to keep a patient asymptomatic, provided the serum viscosity remains below 3.0.

Although the viscosity level at which symptoms and signs develop varies widely from patient to patient, this 'symptomatic threshold' tends to be relatively constant for each individual and has made the serial determination of serum viscosity an important part of management of patients with WM.

However, plasmapheresis does nothing to retard the basic cause of the condition. Chemotherapy is therefore indicated if plasmapheresis becomes necessary more frequently than every 2–4 weeks. Chlorambucil (Leukeran) is usually given in low doses on a daily basis with frequent monitoring of blood counts.

Prognosis

Waldenström's macroglobulinaemia has a variable course but carries a better prognosis than myelomatosis. Mean survival time from diagnosis is 4–5 years.

Non-Hodgkins lymphoma and chronic lymphocytic leukaemia

Both conditions are malignant tumours, usually of B-cell lineage. The non-Hodgkins lymphomas are the malignant counterparts of the secreting B-lymphocyte (Figure 9.6). Paraprotein production is relatively low and only about 10–20% of patients with lymphoma and 5–15% of those with chronic lymphatic leukaemia have paraproteins, most commonly of IgM class, although the incidence increases if more sensitive detection techniques are used[17].

In both disorders, treatment is that of the underlying condition with symptomatic relief of hyperviscosity where appropriate.

Heavy chain diseases

This rare group of disorders is characterized by the production of incomplete heavy chains *without* associated light chains. Most of the heavy chain fragments show internal deletions which seem to be due to structural mutations. Four types are recognized: α, γ, μ and δ chain diseases, α chain disease being the most prevalent.

Frank paraproteinaemia is often absent, so these diseases present problems of laboratory diagnosis. The diagnosis may be suspected on immunoelectrophoresis or immunofixation if, as is usually the case, the free heavy chains display a different electrophoretic mobility from the bulk of the immunoglobulins. The best method of confirming the presence of free heavy chains is by rocket immunoselection. In this technique, the sample is electrophoresed through a zone of agarose containing antisera to κ and λ light chains: all intact immunoglobulin molecules are precipitated. The free heavy chains, however, continue to migrate through the first zone but are precipitated in a second zone of agarose containing appropriate antisera to heavy chains.

Alpha heavy chain disease (α-HCD)

The essential abnormality is the production of abnormal α chains by

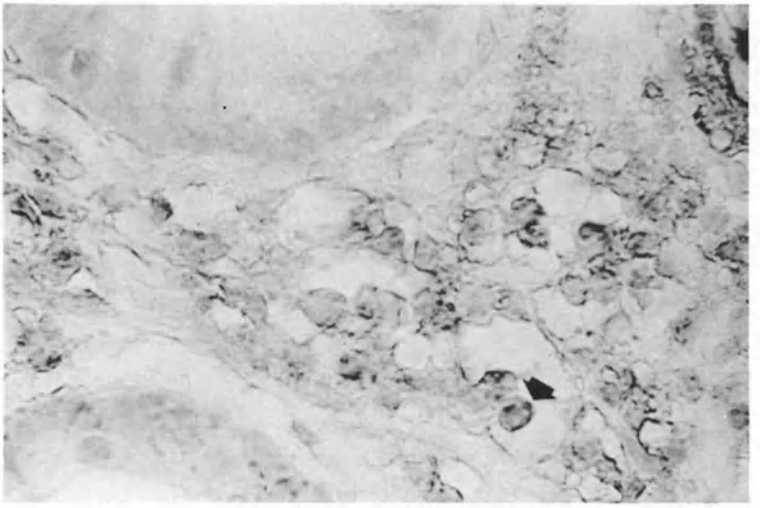

Figure 9.12 A small intestinal biopsy from a man with α-heavy chain disease. The tissue section has been incubated with a peroxidase-labelled antiserum specific for α heavy chains. Lymphoplasmacytic cells contiaining α chains without associated light chains are present (arrowed) in the lamina propria

lymphoplasmacytic cells infiltrating the proximal small intestinal mucosa (Figure 9.12) and mesenteric lymph nodes. The cells secrete dimers of α 1 chains which show partial or complete deletion of the variable (V_H) and first constant (C_{H1}) regions. The diagnosis depends on finding the characteristic α chain fragments in the serum, intestinal secretions or concentrated urine. A distinct paraprotein is often not seen on electrophoresis.

Alpha heavy chain disease, also called immunoproliferative small intestinal disease[18], seems to develop in two stages: an early pre-malignant infiltrative phase, followed by later progression to an immunoblastic lymphoma derived from the same clone of B-lymphocytes. Most cases have been described in patients from the Mediterranean area and Middle East, regions with low hygienic standards and a high rate of parasitic infestations.

The clinical picture is strikingly uniform: almost all patients have been between 10 and 30 years old and have presented with severe, chronic diarrhoea, malabsorption, weight loss, abdominal pains and clubbing.

No specific dietary agent or enteric pathogen has been clearly implicated in the pathogenesis of the condition but, in the early stage, complete remissions have been achieved by the use of oral antibiotics alone, implying that B-cell proliferation stops if the putative persistent antigenic stimulus can be eliminated.

Gamma heavy chain disease (γ-HCD)

Gamma heavy chain disease most commonly presents in elderly men. The clinical picture resembles malignant lymphoma, with recurrent febrile

episodes, painful lymphadenopathy, hepatosplenomegaly and palatal oedema. A clear-out paraprotein is usually lacking, the disease being characterized by production of monoclonal γ heavy chain fragments, usually of the $\gamma 3$ subclass.

Usually the illness worsens steadily, with intermittent remissions and relapses complicated by bacterial infections.

Mu heavy chain disease (μ-HCD)

Mu heavy chain disease seems to be a rare variant of chronic lymphatic leukaemia in which μ chain fragments are present in the serum as pentamers. In contrast to α- and γ-HCD, some patients have amyloidosis or pathological fractures; and most excrete Bence–Jones protein of κ type which is not structurally linked to the μ chains.

Delta heavy chain disease (δ-HCD)

The only known case was reported in an elderly man with a clinical picture similar to multiple myeloma: marked marrow plasmacytosis and osteolytic lesions were present, with free δ chains in the serum. Death occurred from renal failure.

COMPLICATIONS OF PARAPROTEINS

Cryoglobulinaemia

Cryoglobulins are immunoglobulins which form precipitates, gels or even crystals at temperatures below 37°C. Sometimes, other plasma proteins are also involved. The clinical features are caused either by increasing viscosity in peripheral capillaries with consequent tissue hypoxia or by complement activation by complexes of aggregated immunoglobulin. The severity of symptoms is mainly dependent on the temperature at which cryoprecipitation occurs rather than on the concentration of the cryoglobulin.

Testing for cryoglobulins

Since cryoglobulins which precipitate above 22°C are likely to be of clinical significance, blood should be collected in a pre-warmed syringe and taken directly to the laboratory, where it is allowed to clot at 37°C before centrifugation. The serum is kept in a calibrated tube at 4°C for up to 72 hours. Symptomatic cryoglobulins are readily detectable after 12–24 hours while those which precipitate only after 4–7 days at 4°C are unlikely to be clinically relevant.

Classification

Type I cryoglobulins (25%) are monoclonal proteins which lack recognizable

antibody specificity. The cryoglobulin is most commonly IgM; less frequently it is IgG and usually belongs to the IgG2 or IgG3 subclass. In most cases, there is underlying malignant disease, typically Waldenströms macroglobulinaemia or myeloma.

This type of cryoglobulin has an inherent tendency to cryoprecipitate. Symptoms result from hyperviscous sludging of cryoprecipates in cutaneous vessels, producing vascular purpura, cutaneous ulceration, peripheral gangrene and Raynaud's phenomenon.

Type II cryoglobulins (25%) are of mixed type, in which the monoclonal protein (usually IgM, occasionally IgG or rarely IgA) has specificity directed against the Fc portion of polyclonal IgG. Cryoprecipitation occurs when complexes of IgG–anti-IgG are formed. This type is associated with lymphoma, chronic lymphocytic leukaemia, macroglobulinaemia or myeloma. Patients often present with features of immune complex disease, namely diffuse vasculitis, arthritis and glomerulonephritis characterized by electron-dense tubular deposits[19].

Type III cryoglobulins (50%) are of mixed polyclonal type in which IgM rheumatoid-like factors react with IgG. The cryoglobulin concentration is usually less than 1g/l. Approximately one-third of cases are idiopathic, while the remainder are associated with SLE, polyarthritis nodosa, rheumatoid arthritis, Sjögren's syndrome and chronic infections such as shunt-nephritis or bacterial endocarditis.

Treatment

Treatment of cryoglobulinaemia is generally directed towards management of any recognized underlying disorder. Commonsense measures such as avoidance of cold environments are helpful. A combination of plasmapheresis and cytotoxic drugs can sometimes induce a remission of symptoms.

Hyperviscosity

A paraprotein which polymerizes or binds to other serum proteins to form high molecular weight complexes may cause a considerable increase in plasma viscosity and lead to the features of the hyperviscosity syndrome (see above).

The tendency to cause hyperviscosity is most common with IgM paraproteins, particularly when the serum concentration exceeds 30–40g/l; Waldenström's macroglobulinaemia therefore accounts for about 90% of cases. IgA paraproteins are the next commonest cause because of their tendency to polymerize. IgG paraproteins rarely produce hyperviscosity unless their serum concentrations are extremely high: of the subclasses of IgG, IgG3 polymerizes most readily.

The severity of the resulting decrease in tissue perfusion depends on the underlying state of the vascular bed, the shear rate, interactions with cells,

and other factors, and accounts for the marked individual variation in symptoms of hyperviscosity for similar paraprotein concentrations.

Two important points about the hyperviscosity syndrome are that, firstly, it can be diagnosed by clinical examination (see above) and, second, most of the symptoms and signs are readily reversible by prompt plasmapheresis.

Amyloidosis

In the various forms of amyloidosis, biochemically distinct amyloid proteins make up the β-pleated sheet fibrillary structure which confers on amyloid its characteristic staining properties and apple-green birefringence under polarized light[20].

One form, light chain associated amyloidosis, is nearly always associated with B-lymphocyte malignancies which produce excessive amounts of free light chains. Amyloid complicates about 5–15% of myeloma and 20% or more of cases of 'Bence-Jones' myeloma, but is also seen in other B-lymphocyte malignancies.

The main component of light chain associated amyloid fibrils is a protein called AL which consists of either whole intact light chains or fragments containing part or whole of the light chain variable regions (V_l). Where the circulating paraprotein has been characterized, the light chain is always identical to, or the precursor of the AL subunits.

About 15% of Bence-Jones proteins appear to be 'amyloidogenic'; that is, they have the property of polymerizing and forming fibrillary sheets which resemble amyloid after *in vitro* proteolytic cleavage. This property is more commonly seen with λ than with κ light chains.

Patients with 'Bence-Jones' myeloma who secrete 'amyloidogenic' light chains often present with features directly attributable to tissue deposition of amyloid, such as the carpal tunnel syndrome, macroglossia, congestive cardiac failure with or without conduction defects, arthralgia, peripheral or autonomic neuropathy, and gastrointestinal bleeding. In contrast, patients without evidence of amyloid but a similar degree of plasma cell proliferation and concentration of monoclonal light chains tend to present with features typical of myeloma.

Systemic amyloidosis is almost invariably fatal, renal failure and cardiac involvement being the most frequent causes of death[21]. In light chain associated amyloidosis, survival from the time of diagnosis in non-myeloma patients is about 12–15 months. In those with myeloma, the prognosis is even worse, averaging 4–9 months, a considerably shorter survival time than for non-amyloidotic cases of myeloma.

Paraproteins with antibody activity

Although the antibody specificity of most monoclonal proteins is unknown, there are many examples of paraproteins with demonstrable antibody activity: some are directed against bacterial or viral antigens but only a few are of any clinical importance.

Primary cold haemagglutinin disease

Patients with cold haemagglutinin disease present with a chronic haemolytic anaemia and severe Raynaud's phenomenon on exposure to cold. The associated paraprotein is nearly always IgM (type κ); it may be a primary benign condition or arise secondary to a malignant B-cell tumour (for example, lymphoma). The paraprotein reacts with components of erythrocyte membranes, usually the I antigen.

Rheumatoid factor

Paraproteins may have antibody specificity for IgG, that is, rheumatoid factor activity; they are usually IgM but monoclonal IgG and IgA rheumatoid factors can occur.

Anti-nervous tissue activity

IgM and occasionally IgG paraproteins with activity against nervous tissue are well described. The patients characteristically present with a chronic sensorimotor neuropathy of insidious onset, and tremor and ataxia are common. Nerve conduction studies show severe reduction of motor and sensory nerve conduction velocities. Nerve biopsies demonstrate a demyelinating neuropathy with deposition of the monoclonal protein on the myelin sheaths of surviving myelinated nerve fibres, and in one study, the IgM paraprotein was shown to react with a carbohydrate determinant present on human myelin-associated glycoprotein[22].

Anti-axonal or glial activity has been recorded in over 60% of individuals with IgM paraproteins, although only about 6% of patients who present with cryptogenic peripheral neuropathy have paraproteins exhibiting nervous tissue activity[23].

Other specificities

Rare examples of paraproteins with activity directed against dextran, actin, factor VIII, fibrin, transferrin, α_2-macroglobulin, phosphorylcholine or lipoprotein have been described.

Papular mucinosis (lichen myxoedematosus)

This rare skin condition is characteristically associated with deposition of mucinous material in the dermis and a slow-migrating IgG serum paraprotein almost exclusively of λ light chain type. Other evidence of myeloma is usually absent and the condition typically has a prolonged benign course.

These paraproteins do not share the same idiotype, suggesting that they are not antibodies directed against the same antigen determinant.

Acquired C1 inhibitor deficiency

Cases of angio-oedema and acquired deficiency of C1 inhibitor have been

described, most frequently in association with a circulating IgM monoclonal protein which presumably initiates classical pathway complement activation and consumption of the C1 inhibitor[24].

Acknowledgements

I am indebted to Mrs Eileen Walker for typing the manuscript.

Figures 1, 2, 7 and 11 were redrawn or reproduced from Haeney, M. (1985). *An Introduction to Clinical Immunology* (Butterworths - Update Publications) with the kind permission of the Editor of *Hospital Update.*

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10
Immunoglobulins in CSF

G. KEIR AND E. J. THOMPSON

ORIGIN OF CEREBROSPINAL FLUID PROTEINS

In normal cerebrospinal fluid (CSF) the majority of the proteins, including the immunoglobulins, are derived from the blood plasma which gain access to the ventricular and subarachnoid space by a filtration-type process across various blood–CSF barriers. Factors which influence the eventual CSF concentration of a protein derived from blood plasma include:

(1) The plasma concentration of the protein; assuming the blood–CSF barriers are intact then CSF concentration is proportional to the plasma concentration.
(2) The size of the protein molecule; for those proteins having a globular shape the ability of the protein to cross the combined blood–CSF barriers is inversely related to its diameter, which in turn is related to its molecular weight.
(3) The functional status of the combined blood–CSF barriers; damage to these barriers results in increased transudation of all plasma proteins into the CSF, thus increasing their absolute concentration.

SYNTHESIS OF IMMUNOGLOBULINS WITHIN THE CENTRAL NERVOUS SYSTEM

Local synthesis of immunoglobulins within the central nervous system (CNS) can be defined as the presence in CSF of an immunoglobulin at a concentration greater than would be expected for a given serum immunoglobulin concentration and blood–CSF barrier state.

ELECTROPHORESIS OF CSF

In addition to the quantitative alterations seen in local immunoglobulin

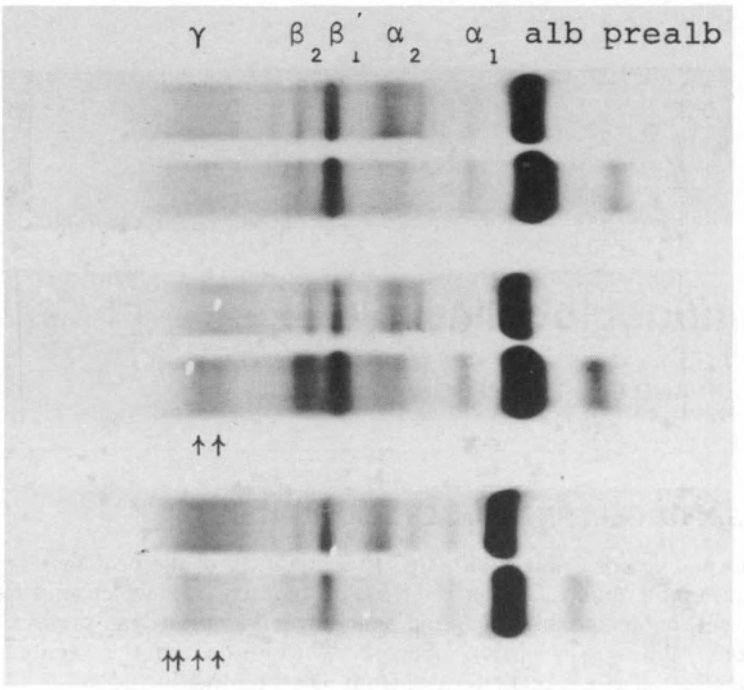

Figure 10.1 Agarose electrophoresis of serum and CSF proteins stained with a general protein stain. The CSF (lower track of each pair) was concentrated 200-fold prior to electrophoresis. Bands (arrows) can be seen in the gammaglobulin regions of those samples from patients with MS (lower two pairs of tracks). Other, non-gamma proteins may also be seen to be present in normal CSF in amounts excessive to that found in serum, e.g. pre-albumin

synthesis, there are well documented qualitative changes observed when CSF is electrophoresed. CSF electrophoresis is performed as for serum (see Chapter 9), with some adaptations (see Figure 10.1).

A variant of the electrophoresis technique is that of isoelectric focusing. Separation by this technique requires a stabilized pH gradient generated within a gelatinous support, such as agarose or polyacrylamide gel. Proteins migrate through the pH gradient under the influence of an applied electric field until the pH of the gradient matches their isoelectric point (pI). When this is achieved the proteins will have no net charge and their migration will cease. A heterogeneous mixture of proteins will, therefore, separate according to the pI values of the component proteins. This approach offers much higher resolution than conventional electrophoresis.

OLIGOCLONAL BAND PATTERNS

When there is local synthesis of immunoglobulins, particularly immunoglobulin G (IgG), within the CNS, electrophoresis of the CSF shows bands

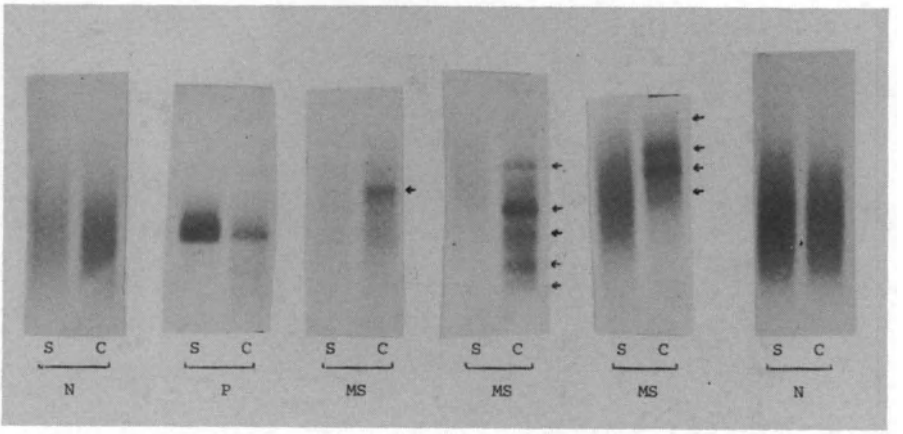

Figure 10.2 Immunofixation of serum (S) and CSF (C) IgG Fc after electrophoresis on agarose. Samples are from normal (N) individuals and patients with multiple sclerosis (MS) and paraproteinaemia (P). Note the presence in the cases of MS of oligoclonal bands (arrows), which are present in CSF but absent from the corresponding serum. In the case of IgG paraproteinaemia the abnormal band is present in both fluids. The use of immunofixation increases the specificity of identification of oligoclonal bands by using specific antiserum to IgG for their identification

superimposed upon the otherwise diffuse gammaglobulin zone. There are usually between one and five such bands visible by electrophoretic methods using agar, agarose, cellulose acetate or polyacrylamide as a support (Figures 10.1 and 10.2), whilst isoelectric focusing can show 30 or more bands (Figure 10.3). Such patterns have been termed *oligoclonal* (small number of clones). Each band probably represents the product of a discrete clone of secretory lymphocytes within the central nervous system. Although this has never been experimentally verified the concept is a useful one. Since IgG constitutes over 90% of the total gammaglobulins in CSF it can be assumed, unless otherwise indicated, that the term oligoclonal refers to bands of IgG.

The observation of oligoclonal bands is a consequence of the normally low level of gammaglobulin seen in CSF. The normal CSF gammaglobulin level, as IgG, is less than 40 mg/l, compared to that found in serum of up to 16 g/l. This represents a difference in concentration of some 400-fold. Assuming an intrathecal concentration of locally synthesized IgG of 10 mg/l, this would constitute 25% of the total CSF IgG and would easily be observed as oligoclonal bands. Synthesis of up to 200 mg/l of IgG is possible in multiple sclerosis. The synthesis and release of an equivalent amount of clonal IgG into the blood vascular system would lead to the bands constituting only 0.05–0.1% of the total gammaglobulins. Such bands would be totally hidden by the polyclonal background gammaglobulins. Clonal bands are usually only seen in the plasma in extreme conditions, e.g. in multiple myeloma and some viral diseases. Calculations of this type also raise one further point about oligoclonal bands. Quantitative measurement of CSF IgG can allow the concentration within the CSF to double before it lies outside the upper

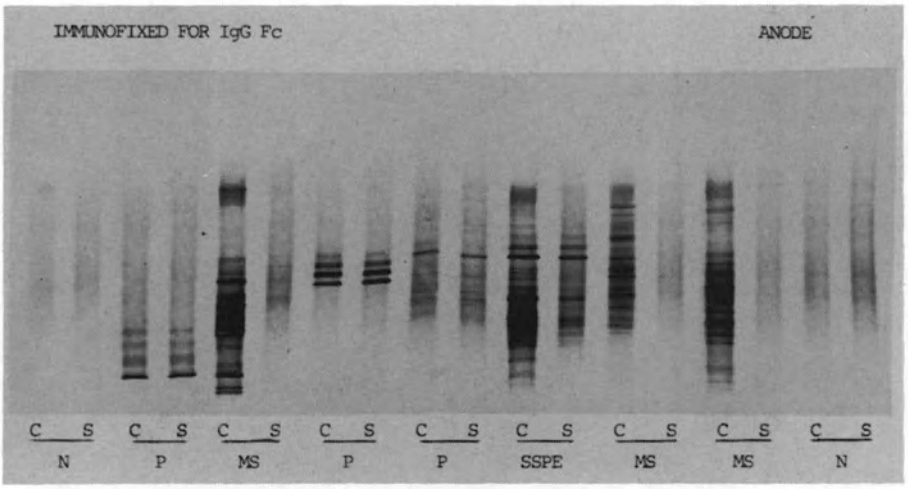

Figure 10.3 Immunofixation of serum (S) and CSF (C) IgG Fc after isoelectric focusing on agarose. Samples are from normal (N) individuals and patients with multiple sclerosis (MS); paraproteinaemia (P) and subacute sclerosing panencephalitis (SSPE). Multiple bands are seen in CSF only, or in both CSF and serum, depending upon the primary pathology. Compare the increased resolution of this technique with that of electrophoresis shown in Figure 10.2

limit of normal. Qualitative oligoclonal band studies however, will clearly demonstrate local synthesis when the quantitative immunoglobulin level is within the normal range. For this reason oligoclonal band studies are the method of choice in the detection of local synthesis (Figure 10.4).

Conditions associated with local synthesis of gammaglobulins

Oligoclonal bands may occur in three groups of CNS diseases:

(1) The demyelinating disorders.
(2) In chronic central nervous system infections by viruses, bacteria, amoebae and helminths.
(3) In association with a miscellaneous group associated with altered immune responsiveness and, including such conditions as Behçet's disease, neurosarcoidosis, CNS lupus and the uveo-meningitides.

The incidence of detection of oligoclonal bands in various disorders is given in Table 10.1.

Demonstration of oligoclonal bands

Oligoclonal band patterns may be observed following the electrophoresis of CSF and sometimes serum. They may also be seen in other body fluids, for example in cases of MS the aqueous humour may exhibit oligoclonal bands.

Parallel studies on CSF and serum may show:

(1) Bands in serum, but absent from CSF. An example of this would be in

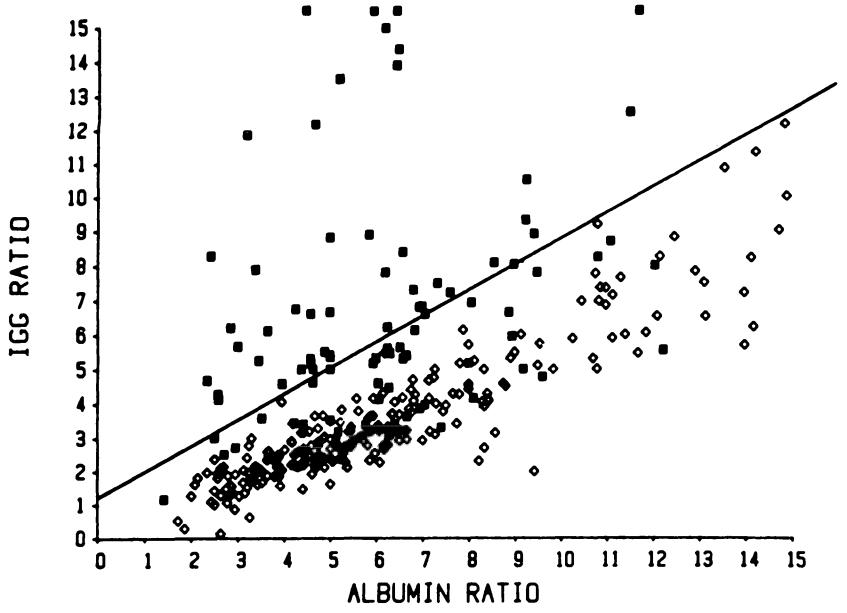

Figure 10.4 A comparison of the sensitivity between isoelectric focusing and quantitative determination of CSF IgG in the detection of local synthesis. Open diamonds (◇) indicate individuals showing no oligoclonal bands by isoelectric focusing on CSF, whilst the solid squares (■) indicate patients in which the CSF does have oligoclonal bands. The graph axes show the (CSF/serum concentration × 1000) for the relevant protein. Higher values of albumin ratio imply increasing damage to the blood–CSF barriers. Points lying above the line indicate a CSF IgG concentration which is greater than that expected for a given barrier state. The line was drawn by eye to maximize the sensitivity of the quantitative assays but prevent false positives by isoelectric focusing. Note, however, the large proportion of oligoclonal band positive samples which lie below the line and which represent false negative findings based upon quantitative assays

19S IgM paraproteinaemia. The high molecular weight of the IgM molecule prohibits its migration across intact blood–CSF barriers with the consequence that the paraprotein is normally undetectable in CSF when its serum concentration is low.

(2) The same bands present in both serum and CSF. In general all immunoglobulins of a given class cross the blood–CSF barriers with equal ease. This means that if a band is seen in the γ region of serum, the CSF should show a band of the same mobility and intensity relative to the background. Such a situation is found in most paraproteinaemias.

(3) Bands present in both fluids but these bands differ in either relative intensity, or distribution, or both. This implies there is a difference in immunoglobulin production within the CNS compared to that in extra CNS tissues. An example is subacute sclerosing panencephalitis (SSPE) in which serum oligoclonal bands are seen in two thirds of cases, but these need not correspond exactly to those observed in the CSF.

Table 10.1 Conditions associated with oligoclonal bands in CSF or both CSF and serum

Conditions	*Incidence of oligoclonal bands*	*Comments*
Demyelinating diseases		
Clinically definite MS	>95%	low incidence of serum bands
Optic neuritis	uncertain (about 50%)	
Chronic central nervous system infections		
Viral:		
measles	true incidence	serum bands
mumps	not known but	common if
rubella	probably quite	test timing
varicella zoster	low because timing	optimal
herpes	of test too early	
'slow' virus:		
papovavirus	uncertain	
SSPE	100%	serum bands in 2/3 cases
Bacterial:		
neurosyphilis	high	
tuberculous meningitis	uncertain	
Amoeboid:		
CNS malaria	uncertain	
Helminth:		
cysticercosis	uncertain	
Unknown:		
Guillain-Barré syndrome	variable	oligoclonal bands may be eclipsed by high CSF total protein
chronic, relapsing meningitis	uncertain	
uveo-meningitis	uncertain	
Inborn errors		
adrenoleukodystrophy	high	no serum studies done
Autoimmune disorders with neurological complications		
sarcoid	all uncertain	
Behçet's	but probably	
SLE	in the region of 50%	
Other neurological diseases		
stroke	low	
CNS tumours	low	oligoclonal bands in these conditions may signify infectious complications
paraneoplastic syndromes	low	
ataxia telangiectasia	uncertain (about 50%)	

(4) Bands present in CSF but absent from serum. This is the situation in locally restricted synthesis of immunoglobulins within the CNS, as is characteristically observed in multiple sclerosis (MS). Faint bands are occasionally seen in the serum in MS, but never of the intensity observed in

CNS infections. This is probably because the concentration gradient for the oligoclonal band immunoglobulins in MS is from the thecal into the vascular space, whilst in infections the gradient for some of the bands is from plasma to CSF.

Two points must be emphasized. First, the observation of oligoclonal bands in CSF is not a test which is specific for diagnosing multiple sclerosis. The presence of oligoclonal bands is a sign of pathology; further studies are required to identify the cause. Secondly, parallel studies on both serum and CSF are essential. The importance of this can be shown by studies in cases of adrenoleukodystrophy showing oligoclonal CSF IgG bands by immuno-electrophoresis, an unexpected observation since the primary lesion is probably an inborn error of metabolism. Parallel serum studies have not been carried out, however, so it is impossible to say whether the oligoclonal response is restricted to the CNS, or represents a much more generalized phenomenon. Quantitative studies have supported the notion of local synthesis in this condition.

Oligoclonal bands in the presence of a raised CSF total protein

The influence of the blood–CSF barriers must always be taken into consideration when looking for oligoclonal bands. In the presence of overt barrier damage, locally synthesized oligoclonal bands may be completely eclipsed by transudated polyclonal gammaglobulins from the serum. Such obscuring effects may occur in cases of bacterial and viral meningitis and in the Landry–Guillain–Barré syndrome during the phase of the disease in which the CSF total protein is raised. In multiple sclerosis the CSF total protein concentration is not usually increased, and values greater than 800 mg/l make a diagnosis of MS doubtful.

QUANTITATIVE ASSAYS OF CSF IgG

Apart from the detection of the oligoclonal nature of the antibody response within the CNS there is a need for a reliable method for quantitating the immunoglobulins in CSF. Quantitative measurements are necessary for longitudinal studies of antibody responses within the CNS.

There are many approaches used in the reporting of quantitative measurements. One popular method is to report the immunoglobulin as a percentage of either the CSF total protein or the CSF albumin. This fails to adequately compensate for blood–CSF barrier function. Further, each of the individual immunoglobulin classes constitutes a greater proportion of the serum total protein than of the CSF total protein. For example, IgG constitutes 20% of the serum total protein but only 5% of the CSF total protein, whilst the corresponding values for IgA and IgM are even more disparate. When the blood–CSF barriers are damaged the CSF total protein increases, but the increase in IgG is proportionately greater. Thus as the CSF total protein increases the more likely it is that the immunoglobulin as a percentage of the total protein will be abnormally high. In addition, it is

impossible to determine whether an increase in CSF immunoglobulin reported in this fashion results from an increase in the plasma concentration of the immunoglobulin, which can vary by up to a factor of five.

Measurement of the CSF/serum concentration ratio for a protein corrects the CSF value for the serum level. Further, by measuring two proteins in the CSF and serum, for example IgG and albumin, the CSF IgG level can be corrected for barrier function, as any damage to the barrier would result in transfer of albumin as well as IgG into the CSF. An increase in CSF/serum IgG ratio in the presence of an increased CSF/serum albumin ratio indicates that the rise in CSF IgG is secondary to barrier damage. An increased IgG ratio with a normal albumin ratio implies local synthesis. This approach leads to an increased discrimination between causes of increased immunoglobulin levels.

Two methods are commonly used for reporting the protein ratios. The first is to combine the two ratios, by dividing the IgG ratio by the albumin ratio to obtain the IgG quotient which is a dimensionless figure. Secondly, the two ratios may be plotted graphically, with IgG ratio on the y-axis and albumin ratio on the x-axis. This latter method is shown in Figure 10.4, which is also a comparison between the sensitivity of electrophortic studies with quantitation in the detection of local synthesis.

More sophisticated mathematical manipulations of quantitative data have been suggested but they have not been shown to better the discriminative value of the quotient.

ORIGINS OF LOCALLY SYNTHESIZED CSF IMMUNOGLOBULINS

Radioactive tracer studies have shown that in normal individuals it is likely that all of the CSF gammaglobulins are derived from plasma.

The demonstration of locally synthesized oligoclonal bands within the CSF demonstrates a fundamental quality of immune responses within the brain. Such immunoglobulins can only have their origins in stimulated B-lymphocytes and plasma cells within the cerebral parenchyma and/or the CSF space. Indeed it has been demonstrated in MS that there is a migration of B-lymphocytes from the peripheral blood into the cerebral parenchyma, in particular into the perivenular space of the cerebral blood vessels. Such sequestered lymphocytes are then able to secrete their humoral products directly into the brain extra cellular fluid. It has been estimated that there are some 10^8 plasma cells present in the brain of an MS patient, with a total synthetic capacity for IgG of up to 100 mg/day. Normal brains contain few or no plasma cells. In multiple sclerosis, immunoglobulin-containing cells are closely confined to areas of demyelination, and older plaques have significantly fewer immunoglobulin-containing cells than new plaques. In SSPE, however, the immunoglobulin-containing cells are found to be widely disseminated throughout the parenchyma, paralleling the distribution of the measles virus which is also widely dispersed within the oligodendrocytes of the brain and occasionally the spinal cord.

Plasma cells are found within the CSF in MS, and CSF lymphocytes have

been demonstrated to synthesize IgG and IgA *in vitro*. Since few, if any plasma cells are normally present in the peripheral blood it is unlikely that the CSF plasma cells would have arisen by crossing the meningeal capillaries. They must be capable of moving from the perivenular space through the parenchyma to the CSF space. Presumably the widespread destruction of the parenchymal tissue seen in MS simplifies this journey. Also, most of the early plaques seen in MS occur in the periventricular areas which are close to the CSF reservoirs.

In contrast to MS the CSF lymphocyte count in SSPE is usually normal. In herpes encephalitis CSF lymphocytes have been shown to synthesize antibody with anti-herpes virus activity.

Free immunoglobulin light chains in the CSF

In cases of MS in which oligoclonal IgG is observed there is a high incidence of free light chains in the CSF. Free light chains are a well recognized phenomenon in oncology where the presence of excessive amounts of *monoclonal* free light chains in the urine (Bence–Jones' protein) is associated with malignant tumours of the B-cell series, typically multiple myeloma and lymphomas (see Chapter 9). The free light chains observed in MS are not the same, however, for they are *oligoclonal* free light chains, characterized by multiple bands occurring outside the gammaglobulin region. Each band has either κ or λ chain identity, but no corresponding heavy chain reactivity. They are most easily observed by immunoblotting after polyacrylamide gel electrophoresis.

Clinically the presence of oligoclonal free light chain bands in MS appears to be related to a recent exacerbation of the disease. This implies that free light chain production is associated with recruitment or differentiation of B-lymphocytes into active plasma cells. Thus it can be hypothesized that as the plaque ages, so the intensity of the response to the antigens fades and the excessive free light chain production is controlled, with consequent fading of the free light chain bands. The evolution of a new plaque results in a fresh cycle of antibody response and the appearance of new bands of immunoglobulins and free light chains.

Free light chains have also been observed in the CSF of cases of SSPE and progressive rubella virus panencephalitis. In some instances such molecules are paired to form dimers.

Brain tissue immunoglobulins

When the isoelectric focusing distribution of the oligoclonal immunoglobulin bands of different MS plaques is studied, some bands are found in more than one plaque, whilst others are specific to only a single plaque. This shows that, although the stimulating antigen may be the same for all cases, the clones synthesizing the antibodies arise from different parental cells, which themselves occupy spatially localized areas within the brain. In SSPE, by contrast, oligoclonal bands from different plaques are found to be similar in spectral distribution suggesting that antibody production in SSPE is much more generalized, i.e. less spatially restricted, when compared to that in MS.

During the clinical evolution of MS it has been observed that the distribution of the oligoclonal bands observed on polyacrylamide gel electrophoresis can change, although others find no change in the band pattern when using agarose electrophoresis. Quantitative studies show variations in the CSF IgG concentration with time. In SSPE the oligoclonal band pattern is known to change during the course of the disease.

SUBCLASS STUDIES OF CSF IMMUNOGLOBULINS

Several early studies into IgG subclasses in MS showed that the predominant response was restricted to the IgG1 subclass. Later studies, however, have shown that if the analytical approach is sufficiently sensitive, the oligoclonal bands can be found to have components from all four of the IgG subclasses. Any given band may be composed entirely of one given subclass, or more than one subclass may exist within any single band. This probably reflects the inability of the analytical method to separate two or more distinct co-migrating antibody molecules, rather than any peculiarities about the immunoglobulins themselves. The incidence of bands of a given subclass is similar to the incidence of that subclass in the normal sera. Both quantitative and qualitative studies have shown that subclass studies are of limited diagnostic value.

IDIOTYPE STUDIES ON CSF IMMUNOGLOBULINS

Individual antigenic determinants on antibody molecules (idiotypes) offer one means of identification of unique immunoglobulins within a given class. Most commonly, idiotype determinants are formed within the region of the immunoglobulin molecule which includes the antigen binding site.

When antibodies are formed against these idiotype determinants on molecules isolated from CSF of MS patients it is found that the idiotype is restricted to that band and is not detected on other bands observed in the same patients' CSF, or from the CSF of the majority of other patients. It is occasionally found that other heterologous MS CSF (i.e. CSF from other MS patients) show idiotypes with cross-reactivity.

The CSF to serum ratios of idiotypes is consistent with substantial local synthesis of the idiotypic immunoglobulin within the CNS, although immunoglobulins with the same idiotype as those of the CSF may be found in the serum. This implies either that an initial production of immunoglobulins of that idiotype occurs outside the nervous system, or that the immunoglobulins are moving from the CSF to the blood vascular space.

Longitudinal studies have shown that the absolute concentration of an immunoglobulin of a particular idiotype within the CSF fluctuates. Unfortunately these studies neglected to take into consideration the influence of the functional status of the blood–CSF barrier, so the true significance of these observations is unknown.

When anti-idiotype serum, raised against the predominant CSF oligoclonal

band, was used to study the IgG eluted from different plaques it was found that the concentration of that idiotype was similar in all of them. This suggests that, in some instances at least, the IgG is disseminated throughout the brain, perhaps reflecting a similar dissemination of clonal antibody-producing cells.

Idiotype studies in SSPE have shown similar results and have confirmed that some of the oligoclonal bands are locally synthesized in this condition. Anti-idiotype serum raised against the bands from the CSF of cases of SSPE shows little cross-reactivity with heterologous SSPE or CSF from MS patients.

ANTIGENIC SPECIFICITIES OF OLIGOCLONAL GAMMAGLOBULINS

It would be a very powerful diagnostic aid to be able to determine the antigen specificity of the oligoclonal band response. In the case of the infective CNS disorders it has been amply demonstrated that when oligoclonal antibody responses exist, over 90% of the total acitivity of the oligoclonal bands is directed against the invading organism. This is confirmed by the observation that the oligoclonal bands can be absorbed out by using the appropriate antigens. Such studies which integrate the presence of oligoclonal antibody bands with particular antigen specificity offer more clinically useful information than simple antibody titres in CSF. It has been reported, however, that isoelectric focusing does not identify all of the oligoclonal bands in SSPE as having anti-measles activity. Although measles antibody activity of oligoclonal bands has diagnostic significance in SSPE, its role in pathogenesis is not defined. T-cell-mediated immunological mechanisms are much more important in combating measles virus infection.

Approximately 60% of MS patients have raised anti-measles antibody titres in the CSF, out of proportion to their serum levels. This observation has been the subject of much debate regarding a possible relationship between measles virus and MS. This association is now seen to represent a non-specific response with the anti-measles activity thought to be the end result of a much wider stimulation of the humoral immune system. This culminates in the activation of memory cells whose specificity is against measles virus, this being an anamnestic feature of normal immune systems. This theory may be supported by the observation that measles antibody activity in the CSF increases in acute mumps meningitis. When viral immunoblots are done on patients with MS, many patients show activity but this activity only represents a minor proportion of the total oligolconal response. Unfortunately it has not yet been possible to identify the antigen specificity for the predominant oligoclonal bands in MS. Trivial antibody activity against a variety of other viral and bacterial antigens has been demonstrated in MS. The types and number of locally synthesized antibodies against viruses and bacterial antigens in MS show no correlation with either disease duration or severity.

The pattern of antiviral activity seen in cases of optic neuritis with oligoclonal bands is similar to that found in MS, supporting the view that these may represent different facets of the same disease.

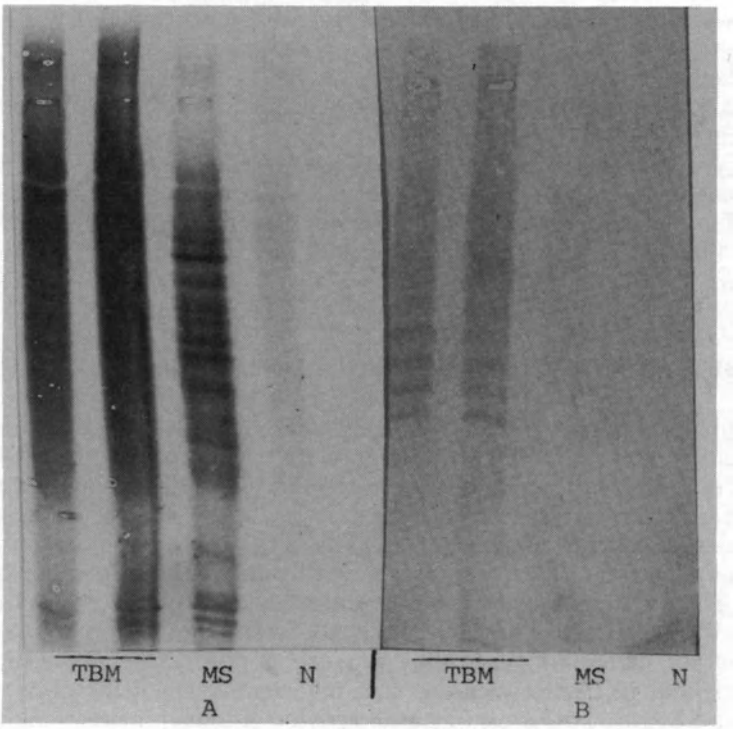

Figure 10.5 Antigen-specific oligoclonal bands in a case of tuberculous meningitis (TBM). The tracks of group A represent the oligoclonal IgG distribution after isoelectric focusing. In group B, nitrocellulose membrane was impregnated with a cultured sonicate of *Mycobacterium avium-intracellulare* and placed in contact with the isoelectric focusing gel. Antibodies reacting with the bacillus were transferred into the paper and detected using an enzyme-conjugated anti-human IgG. TBM tracks are analysed in duplicate. Note the lack of reaction by the MS and normal (N) controls. Also note how this method can detect oligoclonal bands which are not displayed by normal methods. The use of *M. avium-intracellulare* is a safer alternative to other pathogenic tubercle bacilli

In cases of cerebrovascular disease with oligoclonal bands the antibodies in the bands do not appear to be directed against structural brain constituents. Such subjects do have local synthesis against one or more viral antigens so this may represent either an anamnestic response or infectious complications to the vascular disease arising from the compromised blood–brain barrier.

An example of an antigen-blot for tubercle bacillus in a case of tuberculous meningitis is shown in Figure 10.5.

INVESTIGATIONS OF IMMUNOGLOBULINS OTHER THAN IgG

IgM

The normal CSF IgM concentration is less than 0.5 mg/l. Increased levels of IgM have been found in many conditions including MS and CNS infections. When quantitating CSF IgM it must be appreciated that the serum/CSF ratio for IgM is in the region of 1600, compared to 400 for IgG. This means that CSF IgM levels are much more likely to give misleading values in the presence of barrier damage than IgG. This is reflected in the observation that the normal CSF IgM ratio is distributed in a log-normal fashion.

The very low values for CSF IgM make oligoclonal band studies difficult, but not impossible. Several groups of workers have reported oligoclonal IgM in the CSF in MS and CNS infections, but the true incidence of this observation is subject to debate.

Theoretically the study of IgM offers two advantages over the measurement of IgG:

(1) IgM antibodies predate those of other antibody classes in the ontogeny of the immune response, offering the potential of earlier detection of an abnormality of antibody responses.

(2) The presence of IgM antibodies implies recent recruitment of B-cells into the immune response. Continued synthesis of IgM usually means that the provocative agent is still present. This can be of value in cases of chronic CNS infection. For example, in neurosyphilis successful treatment of the treponemal infection leads to the disappearance of oligoclonal IgM followed by a gradual loss over 2–3 years of the oligoclonal IgG pattern. Conversely, a failure to eradicate the IgM response reflects a failure of therapy.

IgA

Quantitative studies have shown CSF IgA to be elevated in cases of MS. Lymphocytes isolated from CSF in MS and herpes encephalitis have been shown to synthesize IgA *in vitro*. IgA-containing cells have been demonstrated by immunohistochemistry in the brains of MS patients. Oligoclonal bands comprising IgA have not been identified.

When IgA is synthesized within the CNS it is the dimeric form which is preferentially produced. Thus the ratio of dimeric to monomeric IgA, or the expressing of the dimer as a fraction of the total IgA may be the best method for determining local synthesis of IgA.

IgD

This has been reportedly absent from normal CSF, although it is present in autologous serum. Increased CSF levels have been found in MS. Cerebrospinal fluid IgD levels are, however, very sensitive to changes in barrier function, so further work needs to be done to determine the value of such measurements.

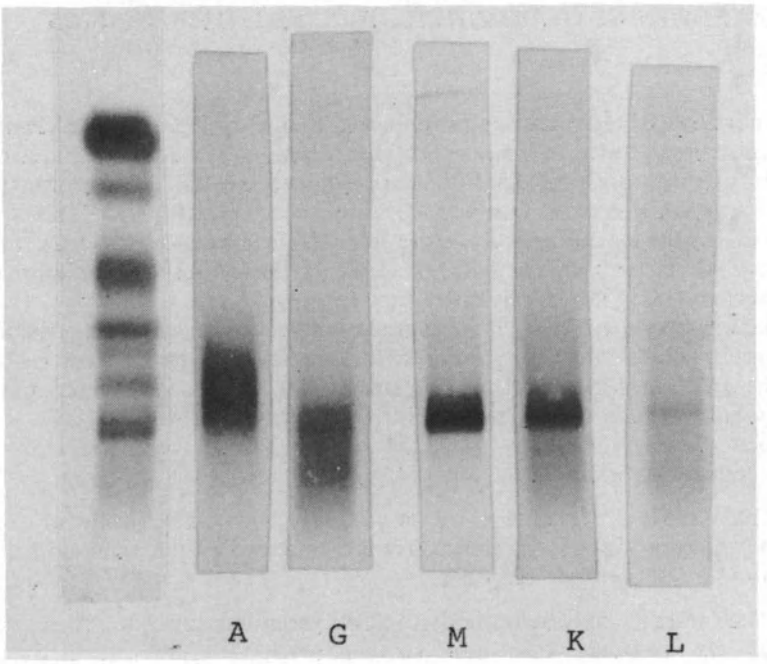

Figure 10.6 Paraproteinaemia associated with a peripheral neuropathy. Compare the gamma region of this serum with that of the normal serum in Figure 10.1. The monoclonal gammaglobulin is shown by immunofixation to be of type IgM (κ light chain). There is a small amount of non-specific reaction with G (IgG Fc) and L (λ light chain) antisera, possibly due to the paraprotein forming small amounts of non-specific complexes with normal immunoglobulins

MONOCLONAL IMMUNOGLOBULINS IN THE CSF

It is not uncommon to find monoclonal immunoglobulins (paraproteins, M-proteins) or their fragments in the CSF. Usually these simply represent transudates of the corresponding plasma protein. Rarely, it is possible to find that these proteins are being secreted by soft-tissue plasmacytomas present within the brain or spinal cord, or even myelomas present in the marrow of the skull and with osteolytic lesions leading directly into the cranial cavity. Under such circumstances it is possible to determine if the paraprotein is being secreted directly into the CSF by determining the relative concentration of the abnormal protein to an internal reference protein. For this purpose transferrin is used as a suitable marker. Transferrin is present in serum at a concentration which is not subject to wild variation by pathophysiological factors. If the paraprotein to transferrin ratio is higher in the CSF than in the corresponding serum then the paraprotein is probably being secreted preferentially into the CSF. This implies the source is likely to be on the thecal side of the dura mater or, alternatively, the paraprotein is leaking directly through the dura.

Neurological disturbances are well documented in paraproteinaemic states, particularly malignant states such as multiple myeloma and Waldenström's macroglobulinaemia. The most common neurological symptoms are peripheral neuropathies, excluding those resulting from the physical aspects of myeloma, e.g. collapsed verterbrae and radiculopathies. Such neuropathies may be of sensory, motor or combined type. In patients with malignant paraproteinaemia, such as myeloma, peripheral neuropathy occurs in 3–5% of cases (Figure 10.6). However, this can be increased to 40–60% when neurophysiological investigation of subclinical peripheral neuropathy is carried out. Often the neuropathy may precede the clinical diagnosis of myeloma by many months, particularly in cases of sclerotic myeloma in which half of the cases have a peripheral neuropathy.

Benign IgM paraproteins are sometimes found in patients presenting with neuropathies. The paraprotein concentration may be low and levels of polyclonal immunoglobulins may be normal. Immunofluorescence studies have shown that biopsied peripheral nerves of some patients demonstrate IgM bound to the nerve sheath. Ultrastructural studies of biopsied nerves have shown a characteristic increase in the intraperiod spacing of the myelin sheath, particularly in the outermost lamellae, a feature which has not been reported in any other human disease. There may also be high-titre IgM binding to heterologous myelin, possibly to the myelin-associated glycoprotein (MAG) component of peripheral and central myelin.

When paraproteins are found in the CSF there is generally an increase in CSF total protein which may be due to the direct effect of the paraprotein, or more likely, the result of blood–CSF barrier dysfunction resulting from complications of malignancy, such as vertebral collapse or cerebral ischaemia.

In general it is good practice to investigate any case of unexplained neuropathy for paraproteins to exclude these as a cause.

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