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of alternative RNA processing remain to be established; aberrant splicing giving non-functional proteins does occur, although one cannot rule out that the RNA itself or the resulting peptides have some functional role.

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Immunoglobulin flexibility in complement activation

Arnold Feinstein, Neil Richardson* and Michael J. Taussig

Next to antigen-binding, the interaction between antibody and complement is among the most familiar and well-studied of all immunological events. Now that many of the molecular details are known, antibody-mediated complement activation provides a fascinating example of the subtle relationship between protein structure and function. In this discussion, Arnold Feinstein and his colleagues are concerned with the initial step in the classical pathway of complement activation, namely the activation of the first complement component (C1) after it has bound to a site on the Fc portion of an antibody molecule, and in particular with the problem of how the site is made available in different antibody classes.

The two antibody classes which activate the classical pathway, IgG and IgM, are represented in Figs 1–3 as space filling models, based on the results of chemical studies, electron microscopy and X-ray crystallography[1–3]. The arrangement of paired, homologous domains[4,5] is clearly seen. The N-terminal variable (V) domains of a heavy (H) and light (L) chain pair to form the antigen-combining site; in a different manner, the constant domain of the light chain (C_L) and the first constant domain of the heavy chain ($C\gamma1$) also form a close association. Heavy and light chains are linked by a disulphide bridge, the position of which depends on subclass; in the case of the human IgG_1 molecule (Fig. 1a) the H–L bridge connects the C-terminal of the light chain to that of the $C\gamma1$ domain. In IgG, there are two further heavy chain domains, $C\gamma2$ and $C\gamma3$, which comprise the Fc portion of the molecule. The $C\gamma2$ domains are the only ones not to pair by protein–protein interaction; instead, the space between them is bridged by oligosaccharide. The $C\gamma2$ domains are of particular importance in the present context as they carry the C1 binding sites[5–7]. The Fab and Fc regions of IgG are linked by a proline-rich stretch of the heavy chain which joins $C\gamma1$ to $C\gamma2$ and also contains the inter-heavy chain disulphide bridges. This is known as the hinge region, a term originally suggested by electron micrographs of antigen–IgG complexes, which showed that the angle between the Fab arms is variable and that the gross shape of IgG may be anything from an acute Y to an open T[8,9]. The length and flexibility of the hinge are dependent on IgG subclass and species; as we shall discuss below, these characteristics of the hinge seem to be determining factors in the initiation of complement activation.

IgM differs from IgG in several respects, most obviously in its pentameric structure (i.e. being composed of 5 μ_2L_2 IgM monomer units)[10]. IgM has never been crystallized, but a three-dimensional model, based on chemical studies and electron microscopy[11,12], has been proposed[2,3,13] (Fig. 1b–d). In this model, the free molecule is planar, with 5 pairs of Fab arms protruding from a central pentameric $(Fc)_5$ disc stabilized by disulphide bonds (Fig. 1b). For each $F(ab')_2$ unit (a pair of Fab arms linked through $C\mu2$ domains), both arms are shown lying flat in the plane of the disc, and in many electron micrographs this is indeed their appearance[11]. However, in some electron micrographs of free IgM, the radiating $F(ab')_2$ units do not appear bifurcated, which is interpreted to mean that the Fab arms are superimposed. We therefore propose a modification of the original model of Fig. 1b in which each $F(ab')_2$ unit is rotated through 90° about its two-fold axis of rotation; the plane in which each pair of arms lies is then at right angles to the disc, while the axis of symmetry of each $F(ab')_2$ remains in the plane of the disc in both models. Electron micrographs of side views of IgM cross linking flagella[12,14] support this modified model, which may well be the dominant conformation of the free molecule.

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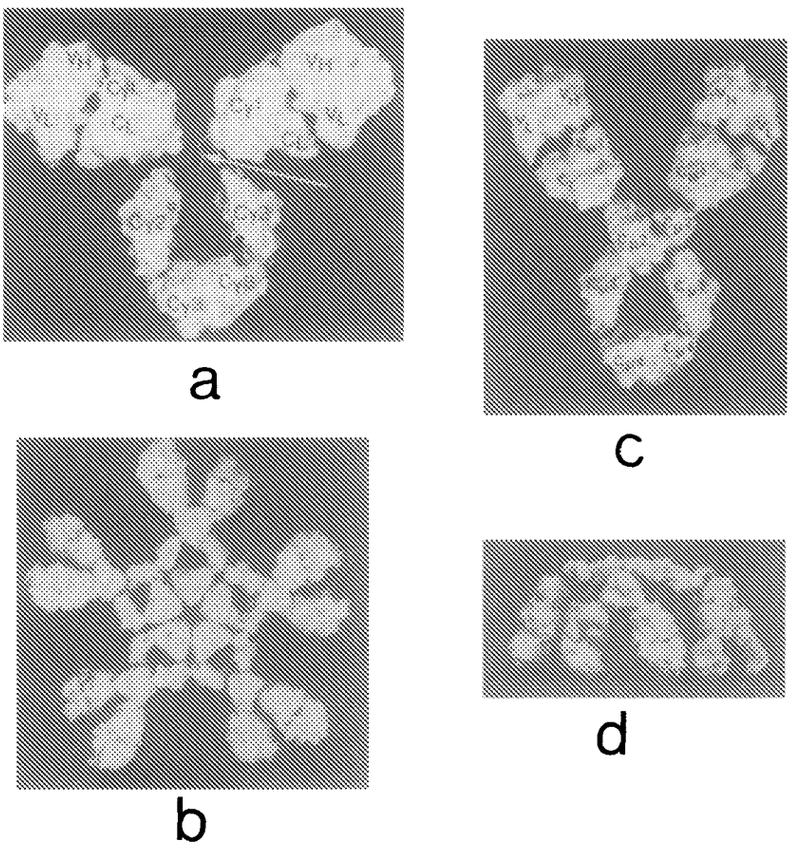

Fig. 1. *Plaster models of human immunoglobulins.*

a Human IgG1. o = site of attachment of oligosaccharide. b IgM pentamer in planar conformation, i.e. as free in solution. The plane of each pair of Fab arms may in reality be at right angles to the disc rather than in the plane of the disc as shown (see text). c IgM μ_2L_2 monomer. The Cμ2 domains take the place of the hinge of IgG. d Form of the IgM model representing the molecule bound to polymeric antigen and equivalent to the 'staple' of electron micrographs.

The main difference between a μ_2L_2 IgM monomer and IgG is the presence of an additional domain (Cμ2) in the IgM heavy chain, taking the place of the hinge region of IgG as the link between Fab and Fc (Fig. 1c). The effect of the paired Cμ2 domains in IgM is to immobilize the angle between the two Fab arms, so that each $F(ab')_2$ functions as a relatively rigid unit. On making a multipoint attachment to an antigen, a distortional conformational change of the IgM molecule occurs, leading to the staple-like appearance seen in electron micrographs[11,12,14]. This change alters the relative orientation of the domains of $(Fc)_5$ and is particularly associated with complement activation[15]. A model of the molecular conformation corresponding to the staple is shown in Fig. 1d and in Fig. 3, which appears in three dimensions when seen through the red–green viewer provided. The IgE molecule – the class responsible for atopic allergies such as hay fever and asthma – closely resembles a μ_2L_2 IgM monomer in possessing the additional domain (Cε2) instead of a hinge, leading to a similar rigidity and low Fab–Fab flexibility[3,16]. When B-cell receptor IgM molecules, or IgE bound to mast cells, are cross-linked by antigen, the rigidity of these Igs could be a crucial factor in transmitting signals to the respective cells.

Complement activation

The initial event in the activation of the complement cascade by the classical pathway is the binding of C1 to sites on the Fc portion of IgG or on the $(Fc)_5$ disc of IgM[17,18]. C1 is a macromolecular assembly of three glycoproteins, C1q, C1r and C1s. Binding to Ig occurs through C1q, a large molecule (mol. wt about 460 000) which in electron micrographs resembles a 'bunch of tulips', with six globular heads each connected by a collagen-like strand to a central stalk (Fig. 4)[17]. Components C1r and C1s combine as a tetramer, $C1r_2$–$C1s_2$, a linear structure of eight globular domains which associates relatively loosely with C1q to form the C1 assembly. C1r and C1s are proenzymes which are converted into active serine proteases following firm binding of Clq heads to Ig. In practice, 'firm' implies at least bivalent binding (Fig. 4). Binding of C1q to free IgG molecules in solution is monovalent; it is demonstrable but weak (K values in the range $1–5\times10^4M^{-1}$), and the dissociation will occur before activation of $C1r_2$–$C1s_2$[18,19]. However, when C1q binds two (or more) adjacent IgG molecules, the binding constants are increased by at least three orders of magnitude ($5\times10^7M^{-1}$ for bivalent binding, $1\times10^{10}M^{-1}$ for trivalent)[19] and the bound time of the complex is then sufficient for activation of the $C1r_2$–$C1s_2$ proenzymes. Thus C1 activation by IgG only occurs when the latter is aggregated, usually through formation of an antigen–antibody complex (Fig. 4). The possibility that conformational changes in the Fc region of IgG are required for C1 activation is now thought very unlikely[2,5,20]. (The situation with IgM is rather different, and will be dealt with separately below.)

C1q-binding sites are present on the Cγ2 domains of IgG, though their precise location is still a matter of argument. Charged amino acid residues are involved,

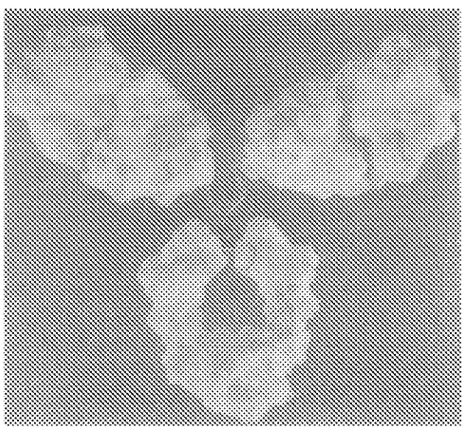

Fig. 2. *Plaster model of human IgG1.*
Based on the data of Refs 39 and 40. The hinge region is shown here as extended polypeptide chains 6 nm in length which connect at their N-terminal ends to Fab units and at their C-terminal ends to Cγ2 domains (see text for details of actual arrangement).

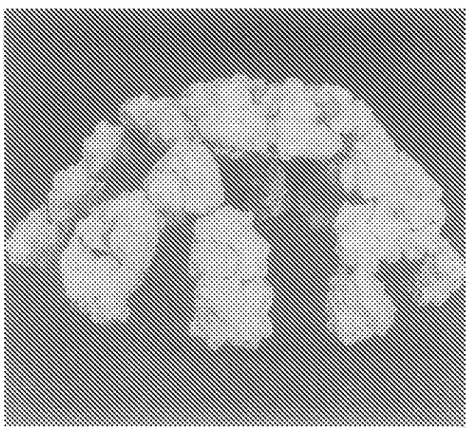

Fig. 3. *Plaster model of IgM in the conformation adopted after making multiple attachment to a particulate antigen.*
The span of the IgM molecule shown here represents approximately 25 nm.

either in the 285–292 or the 317–340 regions of Cγ2[21–23]; both these areas are exposed on the outer surface of the domain. A complementary array of charges on the C1q head would allow binding to occur by 'surface matching'. An important aspect of C1q binding – and hence of complement activation – is the variable efficiency of the different IgG subclasses and of the IgGs of different species[1,3,20,24]. In man, for example, there are four IgG subclasses and the order of their reactivity with complement is IgG3>IgG1>IgG2>IgG4; binding of C1 by native myeloma IgG is some 40 times greater for IgG3 than IgG2, while binding of C1 by IgG4 may not be demonstrable at all[25,26]. This variation seems not to be due to the affinity of Fc binding sites for C1q, since the isolated Fc fragment of the IgG4 subclass is as effective in C1 fixation as that of IgG_1[27]; moreover, the primary structures of the Cγ2 domains of these two subclasses are more than 90% homologous, and such variations as there are do not involve the probable C1q-binding sites. It seems, rather, that the inability of certain IgG subclasses to fix complement well occurs for steric reasons, namely interference by the Fab arms in the approach of C1q to the Cγ2 sites, and that the likelihood of such interference occurring is dependent on the properties of the IgG hinge region.

Hinge structure

The hinge stretches from the end of the Cγ1 domain to the start of Cγ2 (Table 1). In human IgG1, mouse IgG1 and rabbit IgG, the N-terminus of the hinge is marked by the cysteine residue which forms the interchain disulphide bridge with the light chain, while in other subclasses the limit is the C-terminal end of the Cγ1 domain fold[3]. The C-terminus of the hinge in practically all IgGs is the proline residue with which Cγ2 commences. The hinge is coded by a separate exon[28] and the extent of the 'genetic hinge' is somewhat longer

Table 1. Correlation of hinge sequences and complement activation among IgG of different species

	Upper										Middle												Lower										Complement fixation
	216								224														234										
Human IgG1	E	P	K	S	C	D	K	T	H	T	–	C	P	P	–	–	–	–	–	–	C	P	A	P	E	L	L	G	G	P		+
Human IgG2	E	R	K	–	–	–	–	–	–	–	–	C	C	V	E	C	P	P	–	–	C	P	A	P	P	V	A	G	–	P		–
Human IgG4	E	S	K	Y	G	–	–	–	–	P	P	C	P	P	–	–	–	–	–	–	C	P	A	P	E	F	L	G	G	P		–
Mouse IgG1	V	P	R	D	C	G	–	–	–	–	–	C	K	P	C	I	–	–	–	–	C	T	V	P	–	–	E	–	V	S		–
Mouse IgG2a	E	P	R	G	P	T	I	K	–	–	P	C	C	P	P	K	–	–	–	–	C	P	A	P	N	L	L	G	G	P		+
Mouse IgG2b	E	P	S	G	P	I	S	T	I	N	P	C	P	P	C	K	E	C	H	K	C	P	A	P	N	L	E	G	G	P		+
G. pig IgG1	Q	S	W	G	H	T	–	–	–	–	–	C	P	P	C	I	P	–	–	–	C	G	A	P	Z	L	L	G	G	P		–
G. pig IgG2	E	P	I	R	T	P	Z	B	P	B	P	C	T	C	P	K	–	–	–	–	C	P	P	P	E	N	L	G	G	P		+
Rabbit IgG	A	P	S	T	C	S	K	P	M	–	–	C	–	–	–	–	–	–	–	–	P	P	P	E	L	L	G	G	P			+

1st inter-H S-S

last inter-H S-S

Genetic hinge

Alignment of sequences after Burton (Ref. 5)

For technical reasons we are unable to reproduce this figure in colour. See the June issue of *Immunology Today* for full colour illustration.

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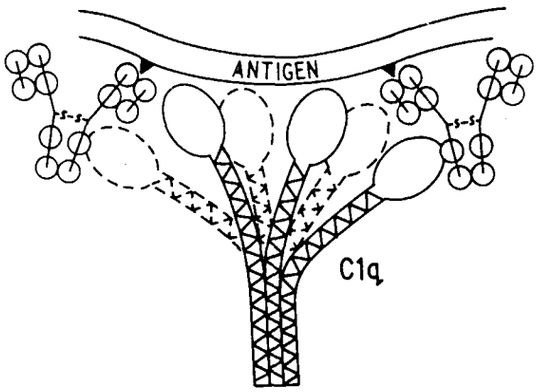

Fig. 4. *Model of the binding of C1q to an IgG–antigen complex. Two IgG molecules are shown bound to antigen, with a C1q molecule attached to the complex. (After Dwek, R.A. et al. (1984) Biochem. Soc. Symp. 49, 123.)*

at the N-terminal end than the 'functional hinge'. Table 1 shows that the primary amino acid sequence of the hinge region in IgG of different species and subclasses is variable in length and composition. One consistent feature, however, is the presence of cysteines which form the inter-heavy chain disulphide bridges; this part of the hinge is also rich in prolines in all cases. It is useful to divide the hinge into three sequential segments, namely upper, middle and lower, as conveniently illustrated in human IgG1. The upper (N-terminal) hinge segment runs from the end of the Cγ1 domain (Cys-220) to the first inter-heavy chain disulphide (Cys-226). In crystals of the Kol IgG1 myeloma, it is well-defined due to crystal packing interactions, and its structure has been deduced from X-ray crystallography[29] to be an open turn of solvent-accessible helix; however, the absence of internal interactions means that little inherent stability would be expected in solution[4]. The upper hinge is responsible for flexibility between the Fab arms as well as rotational flexibility of each individual Fab. In contrast, the middle hinge (Cys-226 to Pro-230) is a rigid polyproline double-helix stabilized by the interchain disulphide bridges; it thus provides an inflexible spacer element between Fab and Fc. The interchain bridges maintain the spatial disposition of the Cγ2 domains, which have no significant protein–protein interaction. The lower (C-terminal) hinge, from Pro-230 to the start of the first β-strand of the Cγ2 domain (Pro-238) is another flexible segment; it is disordered in Kol crystals[28] and thus no information on its structure is available from X-ray crystallography. It will be noticed that the hinge region of human IgG3 has been omitted from Table 1. This is because this subclass has a uniquely elongated hinge of about 70 amino acid residues, with 21 prolines and 11 cysteines, four times the length of the IgG1 hinge. A model of the IgG3 hinge shows it to be an inflexible polyproline double helix[29].

Hinge function

An important function of the hinge is to provide segmental flexibility within the IgG molecule. Thus, the upper hinge allows the angle between the Fab arms to vary (Fab–Fab flexibility), as clearly seen in electron micrographs[8,9], and provides Fab arms with freedom to rotate. The fact that the distance between the binding sites and their relative rotation are not rigidly fixed means that IgG can make simultaneous use of both antigen-binding sites in reacting with single particles carrying repeating epitopes, almost irrespective of how those epitopes are spatially arranged[2]. Were it not for this flexibility, bivalent binding of IgG to particulate antigens such as bacterial flagellum or tobacco mosaic virus would be impossible, because the polarity of their repeating epitopes precludes double attachment of a symmetrically rigid IgG molecule whose binding sites would be anti-parallel[2]. The advantage of double attachment to a single particle is of course the tremendous gain in binding strength which bivalency gives over the use of single binding sites (avidity over affinity)[30]. Hinge flexibility, especially in the lower (C-terminal) hinge region, also leads to variation in the relative positions of Fab arms and the Fc region (Fab–Fc flexibility). Because of the conformational disorder between Fab and Fc, X-ray diffraction patterns for crystals of whole IgG molecules fail to reveal the Fc region[29] and precise information on the relative positions of Fab and Fc in native IgG is lacking. In fact the only intact IgG molecules for which entire three-dimensional structures can be deduced from crystallography are those in which the hinge has been deleted[31].

Hinge-dependent Fab–Fab and Fab–Fc flexibility seem to be of particular importance in ensuring that the C1q binding sites on the Cγ2 domain are available for complement fixation. Feinstein and colleagues pointed out that a correlation could be made between the length of the upper hinge segment and the complement-activating ability of different IgG subclasses and species, and suggested that restriction in the upper hinge limits flexibility and leads to steric interference with C1q binding[1–3]. Subsequent results of IgG sequencing have maintained the correlation in the IgGs of man, mouse, guinea pig and rabbit, as shown in Table 1. A comparison between human IgG1, mouse IgG1 and rabbit IgG is particularly clear because of the delineation of the upper hinge segment by the disulphide bridges, as noted above. Human IgG1 and rabbit IgG, which are both efficient in complement activation, have six and five residues, respectively, in the upper hinge segment, whereas mouse IgG1, a poor activator, has only two (the N-terminal cysteine is included in the length). Mouse IgG1 thus has a very restricted hinge and its relative inflexibility has been confirmed by physical methods[16]. A similar comparison can be made within human IgG subclasses: IgG1 binds C1 some six times more effectively than IgG2, while C1 binding by IgG4 is undetectable[25,26], even though the purified Fc fragments of IgG1 and IgG4 show comparable activity[27]. Reference to Table 1 shows that the upper hinge is virtually absent in IgG2 and limited to three residues in IgG4, two of which are prolines. In both these classes, flexibility between Fab and Fc segments can be expected to be highly restricted, and this is also indicated by substituting the IgG4 upper hinge into the IgG1 model[2]. It is also noteworthy that the subclass of human IgG with the longest hinge of all, IgG3, is the most effective complement activator, and here one could argue that the length of the hinge renders steric interference between Fab and Fc even less likely than in IgG1, whatever the extent of Fab–Fab flexibility. Conversely, the hinge-deleted variants of human IgG1 (Dob and Lec) fail to bind C1 or activate the classical pathway[32] and models of these proteins derived from X-ray crystallography show a close approach between the Fab domains (C_L, Cγ1) and

Cγ2, such that the binding of a globular head of C1q is likely to be obstructed[31,32]. These examples support the view that it is the role of the hinge to ensure that access of C1q to its Cγ2 binding site is unhindered[33], and that the variability of hinge length and flexibility among IgG is resonsible for the differences in their complement fixing activity.

Flexibility and complement activation

A recent study has elegantly confirmed the predictions concerning upper hinge length, segmental flexibility and complement activation, using a series of monoclonal mouse antibodies all of which shared the same (anti-dansyl) combining site[16]. Starting from a hybridoma line producing IgG1 anti-dansyl antibodies, the fluorescence-activated cell sorter was used to isolate cells which had switched to production of different isotypes, namely IgG2b, IgG2a and IgE; thus all these molecules possess identical V regions, but have the isotype-specific heavy chains, including the respective hinge regions. The flexibility of these Igs in the nanosecond time range was estimated from emission anisotropy curves of the bound dansyl chromophore, using nanosecond fluorescence polarization spectroscopy. IgG2b emerged as the most flexible isotype and IgG1 and IgE as the least, with IgG2a intermediate. Reduction of interheavy chain disulphide bridges increased flexibility for IgG1, but had no effect on IgG2a and IgG2b, where the Fab units already possessed a high degree of rotational freedom. When the complement fixing ability of the anti-dansyls was compared, IgG2b was the most effective, with IgG2a a very close second, but IgG1 was highly inefficient in comparison, both in terms of the amount of complement fixed and in the amount of antigen (dansylated BSA) required to trigger complement activation. IgE was inactive in complement fixation. That the differences in complement activation between the IgG subclasses reflect the extent to which each binds C1q has been confirmed by direct assay of the binding of radiolabelled C1q to monoclonal anti-DNP antibodies (from three different hybridomas, rather than variants of a single clone) where the order of reactivity was IgG2a>IgG2b≫IgG1[34]. The greater efficiency of IgG2 subclasses over IgG1 is also seen in haemolysis[35]. Our interpretation of these findings and the correlations in Table 1 is that in complement-fixing IgG, the flexibility conferred by the upper hinge helps to avoid steric interference between Fab arms and the binding sites for C1q on the Cγ2 domain.

The role of molecular flexibility in complement activation is very different in the case of IgM. The C1q binding sites are located on $(Fc)_5$, either on the Cμ3 or Cμ4 domains[15]. Since it is well established that single IgM molecules bound to antigen can fix complement[15,36,37], these sites must be unavailable when IgM is free in solution. It has been shown that, in its native planar conformation, IgM expresses only a single C1q-binding site, with a binding constant of $5\times10^5\ M^{-1}$, leading to a bound half life for C1q of only a few seconds[15]. We have already noted that there are no hinge regions in IgM corresponding to those of IgG, but in their place Fab and Fc are linked by the paired, disulphide-bridged Cμ2 domains (Fig. 1c); the flexibility associated with the hinge of IgG is therefore absent. Instead, binding to polymeric or latticed antigen stabilizes dislocation of the $F(ab')_2$ units out of their original plane, leading to the distorted form of the molecules seen in Fig. 1d and in Fig. 3 which appears in three-dimensions when seen through the red–green viewer provided. That this is the configuration of IgM which is the best activator of complement is supported, among other observations, by studies of IgM–dextran complexes[3,15]. C1 is only activated when such complexes are made in antibody excess where, as demonstrated by electron microscopy, most of the IgM is in the 'staple' form. Antigen excess, on the other hand, favours cross-linking of antigenic sites on separate dextran particles and IgM thus remains in its native planar state; C1 activation is then negligible. It seems that in the configuration corresponding to the staple, at least one further C1q binding site becomes available on $(Fc)_5$ as a result of the molecular distortion attendant on the movement of the Fab arms[15]. Bivalent attachment of C1q is then possible, with increase of K to $5\times10^7\ M^{-1}$, allowing $C1r_2$–$C1s_2$ to activate[15,18]. In contrast with the noncomplement-fixing IgG subclasses, such as human IgG4, or hinge-deleted IgG molecules, it does not appear that C1q sites on native IgM are simply rendered unavailable due to steric hindrance by the Fab arms. Thus, free $(Fc)_5$ discs prepared by trypsin or papain digestion of IgM fail to activate C1, indicating that the appearance of additional sites on $(Fc)_5$ is dependent on a conformational change[15].

For IgM, the continuity of domain interaction from Fab to Fc, in the absence of flexible hinge regions, is probably the important factor in inducing the appearance of C1q binding sites on $(Fc)_5$ and enabling complement activation to take place. We would like to propose the means by which this occurs. In the native IgM molecule, neighbouring μ_2L_2 subunits are linked by two disulphide bridges. One occurs between neighbouring Cμ3 domains, while the other is close to the C-terminals of the μ chains[2,3,38]. The latter bridge does not couple Cμ4 domains together directly, but occurs near the ends of the additional octadecapeptide 'tail pieces' which are continuations of the Cμ4 domains. We suggest that in adopting the staple form, there is simultaneously a degree of flexion between the Cμ2 and Cμ3 domains and pivoting about the inter-Cμ3 bridges; in addition, the tail pieces would act as flexible spacers and allow a change in the spatial relationship of the Cμ4 domain pairs of neighbouring subunits to occur at the same time. By this process of conformational change, C1q sites are revealed. In short, the staple form can be thought of as approximating to a ring of IgG molecules, the C1q sites of which were hidden in the native planar conformation of IgM by the close approximation of subunits.

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Computer models of the human immunoglobulins

Shape and segmental flexibility

Richard Pumphrey

At present there is interest in the design and deployment of engineered biosensor molecules. Antibodies are the most versatile of the naturally occurring biosensors and it is important to understand their mechanical properties and the ways in which they can interact with their natural ligands. Two dimensional representations are clearly inadequate, and three dimensional representations are too complicated to manipulate except as numerical abstractions in computers. Recent improvements in computer graphics allow these coordinate matrices to be seen and more easily comprehended, and interactive programs permit the modification and reassembly of molecular fragments. The models which result have distinct advantages both over those of lower resolution[1], and those showing every atom, which are limited to the few fragments[2–5] or mutant molecules[6] for which the X-ray crystallographic coordinates are known.

In this review Richard Pumphrey describes the shape and flexibility of immunoglobulin molecules in relation to the three dimensional structure.

Computer generated models of the immunoglobulin classes and the T-cell antigen receptor are shown on the centre pages of this issue. The starting point for the computer graphics were sets of X-ray diffraction coordinates taken from the Brookhaven Data Base. The peptide sequences were taken from Kabat *et al.*[7]. A new homology table of all the human immunoglobulin domains was prepared using the coordinates of the paradigm domains and secondary structure prediction[8]. Each domain of each immunoglobulin was processed separately to generate new coordinates corresponding to the amino acid sequence: the starting coordinates were taken from the domain closest in beta-pleated sheet sequence and the bends then modified where necessary to accommodate the variations in sequence, taking the predicted secondary structure into account. These processed domains were then glycosylated and reassembled into complete molecules to give structures consistent with available structural data from biochemical (Refs 7, 9–13 and references therein), physical[14,15] and electron microscopic techniques[16,17]. I have also used new electron micrographs of human IgE and IgG subclasses in the assembly of these models.

A 5 angstrom (Å) diameter cylinder drawn between adjacent alpha-carbon atoms has been used as a simplified representation of the peptide chain. This emphasizes the 'hard' external surface of the molecules and has many advantages. The smaller amino acids lie within, or nearly within, the cylinder, and the larger side chains on the exterior are mostly polar, and will not have a rigidly

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For technical reasons we are unable to include the centre page diagram in this edition. See the June issue of *Immunology Today* for the centre pages.