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Human Antibody Effector Function

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I. Introduction

A molecular explanation of antibody effector function requires the description of multiple antibody molecules cross-linking an array of antigen molecules to multiple effector molecules. The antigen molecules are likely to be on a cell surface and the effector molecules are either large, as for complement, or also on a cell surface, as for Fc receptors. Therefore this is a complex problem.

As regard antibodies, we have crystal structures for Fab fragments, for Fab fragments complexed with a number of antigens (reviewed in Davies *et al.*, 1990), and for Fc from IgG (Deisenhofer, 1981; Sutton and Phillips, 1983). We also have the low-resolution structures of two mutant whole IgG molecules (Silverton *et al.*, 1977; Sarma and Laudin, 1982; Rajan *et al.*, 1983). These mutant molecules lack the hinge region and generally show poor effector activity (Burton, 1985). They crystallize and show a complete diffraction pattern probably because the loss of the hinge has reduced their flexibility. Native IgG molecules are flexible and do not give diffraction from the Fc part of the molecule (Huber *et al.*, 1976; Ely *et al.*,1978). Therefore, our best picture of the whole IgG molecule is probably built up by combining the crystal data on the fragments with techniques such as electron microscopy (EM) and solution studies giving information on whole antibody conformation and flexibility. For the other antibody classes we have less data and generally have to make some fairly broad extrapolations from IgG in order to have a working model. A small number of studies provide us with some ideas as regards the arrangement of antibodies in arrays such as might be found triggering effector systems.

No effector molecule has been crystallized. However, the gross shape of the complement C1q as a bunch of tulips is well known, and the dimensions of some of the other complement components interacting with antibody are emerging. Many of the Fc receptors have now been cloned and sequenced and shown to belong to the immunoglobulin supergene family. Coupled with recent data on the sites on anti-

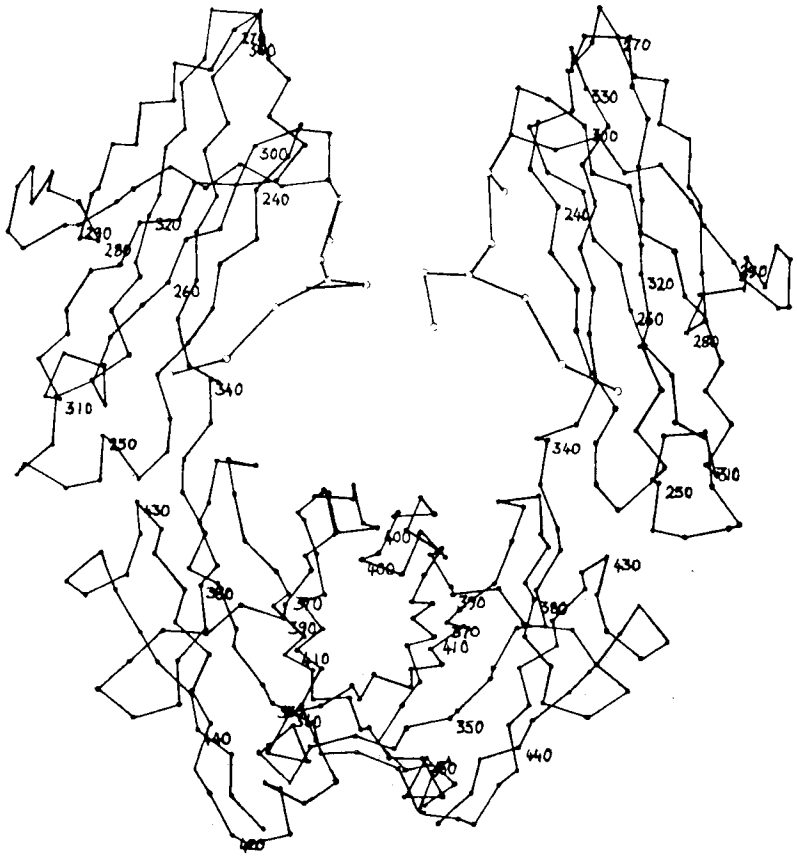


FIG. 1. The structure of Fc. The structure was solved for the Fc fragment from pooled human IgG (Deisenhofer, 1981); ●, α-carbon positions; ○, approximate centers of carbohydrate hexose units.

body molecules interacting with effector molecules, one can therefore place some useful constraints on how the three molecules, i.e., antigen, antibody, and effector, could be arranged in space relative to one another. Such arrangements then need to be placed into the context of arrays, and here we are largely at the stage of suggesting models for experimental investigation.

In this review we shall consider effector functions in turn, concentrating first on IgG, for which molecular information is most detailed,

FIG. 2. The structure of IgG. This picture has been generated by taking the known structures of a human $F(ab')_2$ fragment and a human Fc (Marquart *et al.*, 1980; Deisenhofer, 1981) and constructing the hinge of human IgG_1. The heavy chains are shown in dark blue and yellow, the light chains in green, and the C_H2 carbohydrate chains in light blue. The proposed C1q-binding site discussed in the text (Glu 318, Lys 320, and Lys 322) is shown in red on one of the C_H2 domains. The proposed Fc receptor ($Fc_\gamma RI$) binding region (Leu 234–Gly 237) is shown in white on both heavy chains. We thank Drs. Peter Artymiuk and Geoff Ford for permission to use this picture generated in collaborative work.

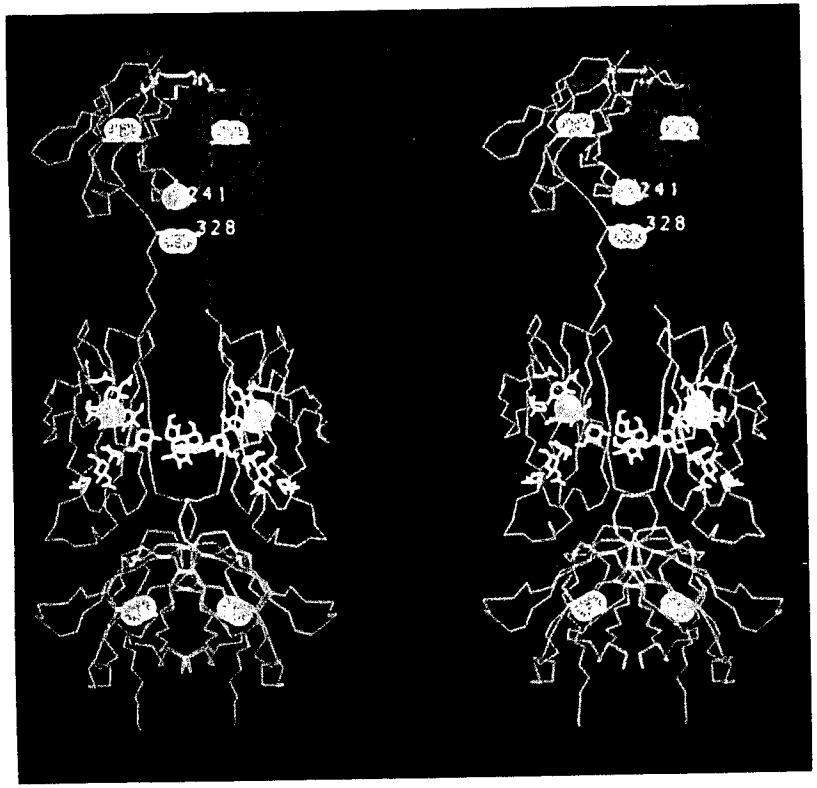


FIG. 3. The structure of IgE. A stereodrawing of the α-carbon trace of the revised model of Fc_ϵ from Helm *et al.* (1991) based on sequence homology with Fc of IgG (Deisenhofer, 1981) and taking into account the parallel nature of the inter-ϵ chain disulfide bonds. The $C_\epsilon2$ domains (red) are at the top, the $C_\epsilon3$ (green) are in the middle, and the $C_\epsilon4$ (magenta) are at the bottom. Carbohydrate side chains (yellow) as found in IgG_1 (Deisenhofer, 1981) are drawn attached to Asn 394 between the $C_\epsilon3$ domains. The intradomain and interchain cysteines (Cys 241 and Cys 328) are indicated by large circles (yellow). The white segment on the left ϵ-chain highlights the location of an $Fc_\epsilon RI$-binding peptide (residues 301–367).

and then on the other antibody classes where relevant. We shall attempt to build from unit interactions, e.g., one C1q head binding to one Fc site, to the more physiological situation, e.g., complement activation at an array of antigen-bound antibody molecules. There will be many instances wherein it will not be possible to be so tidy. Because there is a growing interest in the ability to engineer or design antibodies for specific effector function, we shall seek to highlight to what extent this is currently possible and describe some of the potential problems.

II. Antibody Structure: Considerations for Effector Function

A. Structure of Fc

The crystal structures of human (Deisenhofer, 1981) and rabbit Fc (Sutton and Phillips, 1983) from IgG have been determined to intermediate resolution and analyzed in detail in terms of potential interacting sites elsewhere (Burton, 1985). Nevertheless, the Fc is so central to effector function that it is worth summarizing a few central points.

The structure of human Fc is shown as an α-C trace in Fig. 1 and space-filled in Fig. 2 (compare also the Fc from IgE in Fig. 3). The two $C_\gamma3$ domains form a classical immunoglobulin domain pairing. There are extensive lateral van der Waals contacts between the domains as well as several hydrogen bonds between polar side chains and a pair of salt bridges. This close interaction results in approximately 1000 $Å^2$ of surface from each domain being removed from solvent contact. Each $C_\gamma3$ domain is linked by a loosely folded segment (Ser 337 to Gln 442) to the $C_\gamma2$ domain.

The $C_\gamma2$ domains are not paired in the usual fashion and indeed the polypeptide chains have no contact with one another until the hinge region. The hydrophobic face of the domain normally involved in pairing is partially covered by a branched N-linked carbohydrate moiety attached to Asn 297, which helps to stabilize the domain. Longitudinal interactions between residues in $C_\gamma2$ (residues 247–253 and 310–314) and in $C_\gamma3$ (376–379 and 428–433) further serve to stabilize both domains. About 500 and 750 $Å^2$ of surface area, respectively, are removed from solvent in these interactions.

The carbohydrate chains of the $C_\gamma2$ domain are not a single oligosaccharide moiety but consist of a set of about 20 structures based on a mannosyl chitobiose core, which can be represented as shown in Fig. 4. The possible role of carbohydrate in effector function is often investigated using aglycosylated IgG prepared by growing hybridomas in

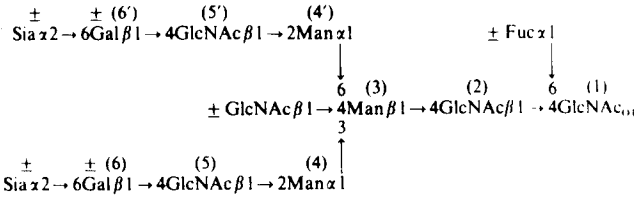


FIG. 4. The C_H2 domain N-linked carbohydrate of IgG. As shown, four types of mannosyl chitobiose cores are found (± bisecting *N*-acetylglucosamine/± fucose) and other chain variants include the presence or absence of galactose and sialic acid.

tunicamycin or by engineering Asn 297, the carbohydrate attachment residue, to another amino acid. The question then arises as to what effect loss of carbohydrate has on Fc structure. Proton nuclear magnetic resonance (NMR) (Matsuda *et al.*, 1990) comparing native and recombinant Fc indicates that the overall structures are quite similar; there is no evidence for example of inward "collapse" of the two $C_\gamma2$ domains. However, some differences are monitored, most notably by a reporter group (His 268) on a loop in spatial proximity to Asn 297. Small differences are also sensed by His 433 and His 435 ($C_\gamma3$ domain) at the junction of the $C_\gamma2$ and $C_\gamma3$ domains and remote from the glycosylation site. One caveat here is that the NMR studies refer to Fc fragments and the presence of Fab arms could affect an aglycosylated Fc.

Finally, the protein in Fig. 1 begins at residue 238 whereas Fc is generated by papain cleavage N-terminal to Cys 226. The intervening residues are not defined in electron density maps of human Fc presumably because of flexibility. This region is probably better considered as part of the hinge (the "lower hinge"; see below) even though it is coded for in the $C_\gamma2$ exon.

B. Conformation and Flexibility of IgG: The Hinge

Both immunoglobulin flexibility and the conformation of antibodies have been recently reviewed (Nezlin, 1990; Burton, 1990a,b; Schumaker *et al.*, 1991). Again only major points will be summarized. Flexibility of antibodies is classically equated with Fab arm flexibility (Y to T shape changes), which should allow "variable reach" for the antibody and therefore bivalent recognition of differently spaced antigens. However, the IgG molecule has available a number of modes of flexibility as illustrated in Fig. 5. It would seem very likely, although it is not formally demonstrated at this stage, that this flexibility would be

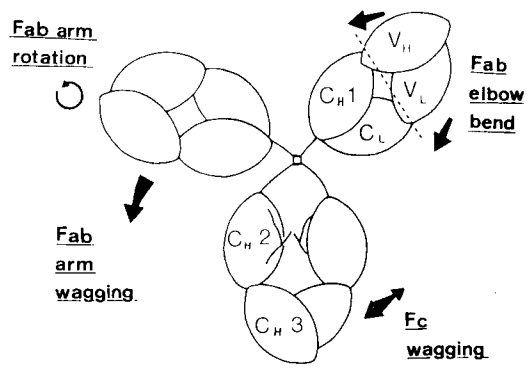


FIG. 5. Flexibility in the IgG molecule. The modes of flexibility have been most convincingly demonstrated by electron microscopy, as reviewed by Burton (1990b).

used in the allotted task of antibody to cross-link antigen and effector in a variety of situations.

The potentially most interesting flexibility is that associated with the hinge. Indeed the hinge is the region of greatest difference between isotypes of IgG, and because the isotypes also show great differences in effector function activity, there has developed a notion that activity and hinge flexibility are intimately linked. In simplified terms, one tends to associate good effector activity with flexible isotypes and poor activity with less flexible isotypes. We shall try to evaluate this association during the course of this review.

The hinge region can be conveniently divided into three parts (Fig. 6). The middle or core hinge contains a variable number of cysteines forming interheavy chain disulfide bonds and is connected to the folded Fc by the lower hinge and to the Fab arms by the upper hinge. NMR studies of human IgG_1 and isolated hinge peptides (Endo and Arata, 1985; Ito and Arata, 1985) indicate that the core hinge (Cys 226 to Cys 229) has a conformation little affected by the presence or absence of Fab and Fc and quite similar to that found in crystals of human IgG_1 Kol protein. The lower hinge (at least the part comprising Pro 230 to Leu 234) is suggested to be flexible and to have an extended conformation independent of the presence of the Fab arms but critically dependent on the intact core. The upper hinge (Lys 222 to Thr 225) is suggested to be the most flexible part, adopting the helical structure found in Kol crystals in the presence of the Fab arms. Nanosecond fluorescent depolarization experiments have been used to show a cor-

Antibody	Hinge		
	Upper	Middle	Lower
Mouse IgG3	EPRIPKPSTPPGSS	C	PPGNILGGP
Rat IgG2c	EPRRPKPRPPTDI	CSC	DDNLGRP
Human IgG3	ELKTPLGDTTHT	CPRCP(r.u.)$_3$	APELLGGP
Mouse IgG2b	EPSGPISTINP	CPPCKECHKC	PAPNLEGGP
G.pig IgG2	EPIRTPZBPBP	CTCPKC	PPPENLGGP
Human IgG1	EPKSCDKTHT	CPPC	PAPELLGGP
Rat IgG2b	ERRNGGIGHK	CPTCPTCHKC	PVPELLGGP
Mouse IgG2a	EPRGPTIKP	CCPPKC	PAPNLLGGP
Rabbit IgG	APSTCSKPM	C	PPPELLGGP
Rat IgG1	VPRNCGGD	CKPCIC	TGSEVSS
Human IgG4	ESKYGPP	CPPC	PAPEFLGGP
Rat IgG2a	VPRECNP	CGC	TGSEVSS
Mouse IgG1	VPRDCG	CKPCIC	TVPEVS
G.pig IgG1	QSWGHT	CPPCIPC	GAPZLLGGP
Human IgG2	ERK	CCVECPPC	PAPPVAGP
	<---------- Genetic hinge ---------->		

FIG. 6. Comparison of hinge sequences of IgG. In human IgG_1 the hinge exon corresponds to residues Gly 216 to Pro 230 (Eu numbering) (Kabat *et al.*, 1987). Cys 220 forms a disulfide bridge to the light chain, and so residues 216–220 belong structurally to the Fab rather than to the hinge. This also applies to mouse IgG_1, rabbit IgG, rat IgG_1, and rat IgG_{2a}. The designation of these residues in the other isotypes is less clear. For the sake of consistency we refer to the upper hinge as the sequence between position 220 and the first interheavy disulfide cysteine. The middle or core hinge contains a variable number of cysteines involved in interheavy chain disulfide linkages. The repeat unit (r.u.) in human IgG_3 corresponds to the sequence EPKSCDTPPPCPRCP. Crystallography indicates that Pro 238 in human IgG_1 is the first residue forming part of the folded C_H2 domain so that residues Ala 231 to Gly 237 (and equivalent residues in other isotypes) are assigned to the lower hinge. Alignments are after Burton (1985, 1987) and Feinstein *et al.* (1986).

relation between the upper hinge length and segmental flexibility, believed to be Fab arm motion (Oi *et al.*, 1984; Dangl *et al.*, 1988). In addition, protein engineering studies (Schneider *et al.*, 1988) have suggested that specific interactions between the hinge and the $C_\gamma1$ domain can affect this segmental flexibility. Molecular mechanics methods have been applied to model the hinge–$C_\gamma1$ domain interface in human IgG_4 and used to suggest that the interface may have a

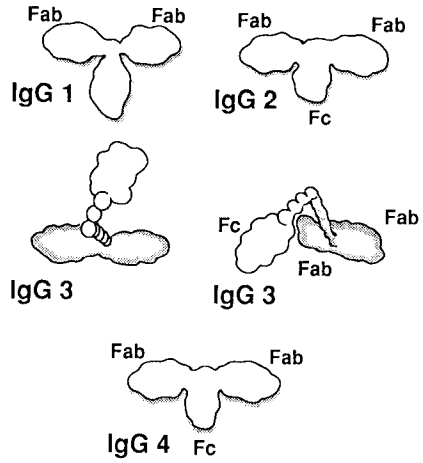


FIG. 7. Schematic representation of the conformations of the human IgG subclasses. These average solution conformations are deduced on the basis of small-angle X-ray scattering and sedimentation data (K. G. Davis and D. R. Burton, unpublished). Two views of IgG_3 are shown, the bent shape being very similar to that seen in the electron micrograph (Ryazantsev *et al.*, 1989). A tripod-like bent shape has been described from electron micrographs of human IgG_1 (Ryazantsev *et al.*, 1990). IgG_2 and IgG_4 show the closest approach of Fab and Fc in the average conformations above.

different conformation in IgG_2 than in the other human IgG subclasses (Snow and Amzel, 1988). Models for the solution conformations of the human IgG subclasses are shown in Fig. 7.

C. Other Antibody Classes

No crystal structures are available either for whole immunoglobulins or for Fc fragments other than those for IgG. Therefore, models for the other classes have been suggested on the basis of sequence homology with IgG together with EM and solution studies. These are presented in Fig. 8.

III. Complement Activation by Antibodies

The complement cascade can be usefully divided into two phases (Howard and Hughes-Jones, 1988): (1) the deposition of C3 on the antigenic cell surface, thereby facilitating phagocytosis—in the classical pathway this requires the sequential activation of C1 and C42;

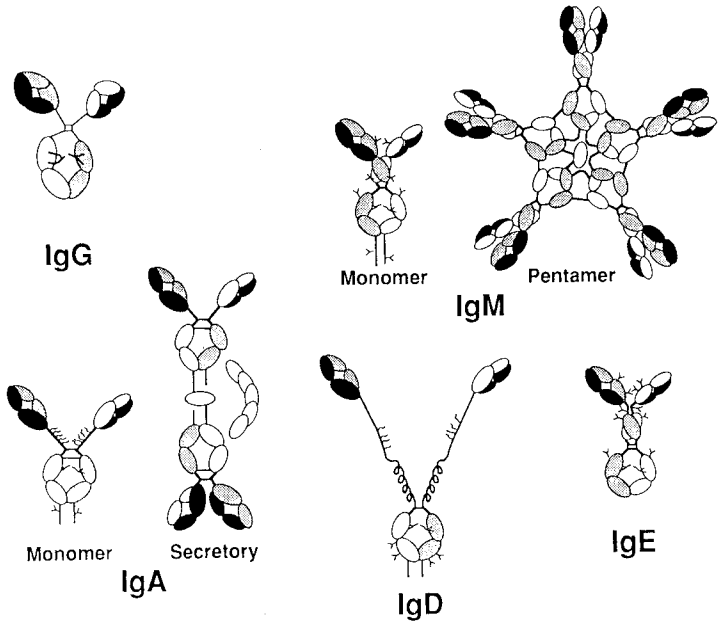


FIG. 8. Schematic representations of the structures of the five antibody classes. Heavy chains are shown blank and stippled, light chains are dark. The N-linked carbohydrate structures are represented as Y shapes and O-linked carbohydrates are shown as wavy lines. The representation for IgG can be compared directly with Fig. 2. The other structures are suggested on the basis of sequence comparison with IgG, extrapolation from known features of IgG structure, and physical studies (Burton, 1987, 1990b). IgM monomer has a pair of $C_\mu 2$ domains replacing the hinge, unpaired $C_\mu 3$ domains, and C-terminal tailpieces. The pentamer incorporates a molecule of J chain, which may adopt an Ig domain structure. Monomeric serum IgA_1 shown has an extended hinge with 10 O-linked carbohydrate chains, an unusual positioning of the $C_H 2$ domain carbohydrates that makes the unpaired configuration of $C_\alpha 2$ domains speculative, and C-terminal tailpieces. IgA_2 has a much shorter hinge, with the light chains generally disulfide linked to one another rather than to the heavy chain. Secretory IgA is a dimer in which monomers are disulfide linked via J chain and the arrangement stabilized by secretory component, a structure of five Ig-like domains. IgD has a very extended hinge divided into a region rich in O-linked carbohydrate and a highly charged region, possibly in helical conformation, and short tailpieces. IgE closely resembles IgM monomer.

(2) the formation of the membrane attack complex resulting in cell lysis—this requires the activation of C5 and attachment of C6–9.

Antibody is involved in the first phase. Attachment of C1 to antibody in an appropriate arrangement, typically spatially associated by reaction with antigen, leads to C1 activation. C1, a giant complex of the C1q

hexamer and the $C1r_2\,C1s_2$ tetramer, binds via C1q to antibody, resulting in C1r then C1s activation. Activated C1s ($\overline{C1s}$) then cleaves C4 to reveal a labile thioester bond, which can now interact covalently with the cell surface or the proximal antibody molecules. Activated and anchored C4 then accepts a C2 molecule, which is also cleaved by $\overline{C1s}$. The $\overline{C42}$ complex is now able to cleave C3, again to reveal a labile thioester bond, which rapidly becomes covalently attached to groups in the local area.

In molecular terms we should like to understand the binding of C1q to single antibody molecules, the binding of C1q to antibody arrays, the changes leading to C1 activation, and the arrangement of the molecules involved in C3 deposition.

A. The Interaction of C1q and Antibody

1. Complement C1q

C1q is a hexavalent molecule with a molecular weight of approximately 460,000 and a structure likened to a bouquet of tulips in which six collagenous base and arm regions (stalks) are connected to six globular head regions (Reid and Porter, 1981; Reid, 1983; Cooper, 1985; Kilchherr *et al.*, 1985; Schumaker *et al.*, 1987) (Figs 10–14). The basic structure is conserved, for example, between man and bullfrog (Alexander and Steiner, 1980), and there is great propensity for C1q and IgG from different species to interact (Burton, 1985). The ability to generate isolated C1q heads by controlled collagenase digestion allows IgG binding to be localized to the heads. EM studies indicate considerable flexibility in the C1q molecule (variable base–arm angle), which could have importance in recognizing antibody arrays, but this flexibility is reduced in the physiological ligand C1 (Poon *et al.*, 1983; Schumaker *et al.*, 1987).

2. Thermodynamics of the Interaction

One of the early problems in this field was why complement was triggered by antigen-complexed antibody but not by free antibody, as occurs, for example, in high concentration in serum. It was suggested that the binding of antigen might transmit conformational changes to the Fc region, which would enhance C1q binding and lead to activation. The thermodynamics of the interaction give no support for such a notion for complement triggering by IgG.

Isolated C1q heads bind to normal pooled human IgG in aggregated form with a binding constant of the order $(1–5) \times 10^4\ M^{-1}$(Hughes-Jones and Gardner, 1979). This value is also observed for the binding of C1q to monomeric pooled human IgG (Sledge and Bing, 1973;

Hughes-Jones, 1977; Müller-Eberhard, 1975). Only when both of the interacting partners are capable of multivalent interaction does the affinity increase to the order of $10^8\ M^{-1}$ (Burton, 1985). Detailed modeling studies show that these differences in affinity, founded on multivalent versus monovalent interaction, are sufficient to explain the binding of C1q to antibody associated on a cell surface even in the presence of physiological monomeric IgG (Dower and Segal, 1981).

Other arguments favoring the associative versus the allosteric model of IgG complement triggering have been discussed elsewhere (Metzger, 1978; Burton, 1985). Perhaps one of the greatest problems for an allosteric model is provided by the complement-activating ability of the human IgG_3 molecule, which has a hinge length of the order of 15 nm including 21 proline residues and 11 interheavy chain disulfide bonds. It is not easy to conceive of the transmission of a common conformational change through this structure on the occupation of the antibody combining site by diverse antigen.

The binding of the human IgG subclasses in monomeric form to human C1q has been investigated by ultracentrifuge studies (Schumaker *et al.*, 1976). Assuming two IgG sites per C1q head, which gives the best fit to the experimental data, the rank order IgG_3 ($K_a = 2.9 \times 10^4\ M^{-1}$) > IgG_1 (1.2×10^4) > IgG_2 (0.6×10^4) > IgG_4 (0.4×10^4) was found. There was considerable error in the last value. The current consensus implies that there is no appreciable affinity of IgG_4 in associated form for C1q (Garred *et al.*, 1989; Horgan *et al.*, 1990; Tan *et al.*, 1990).

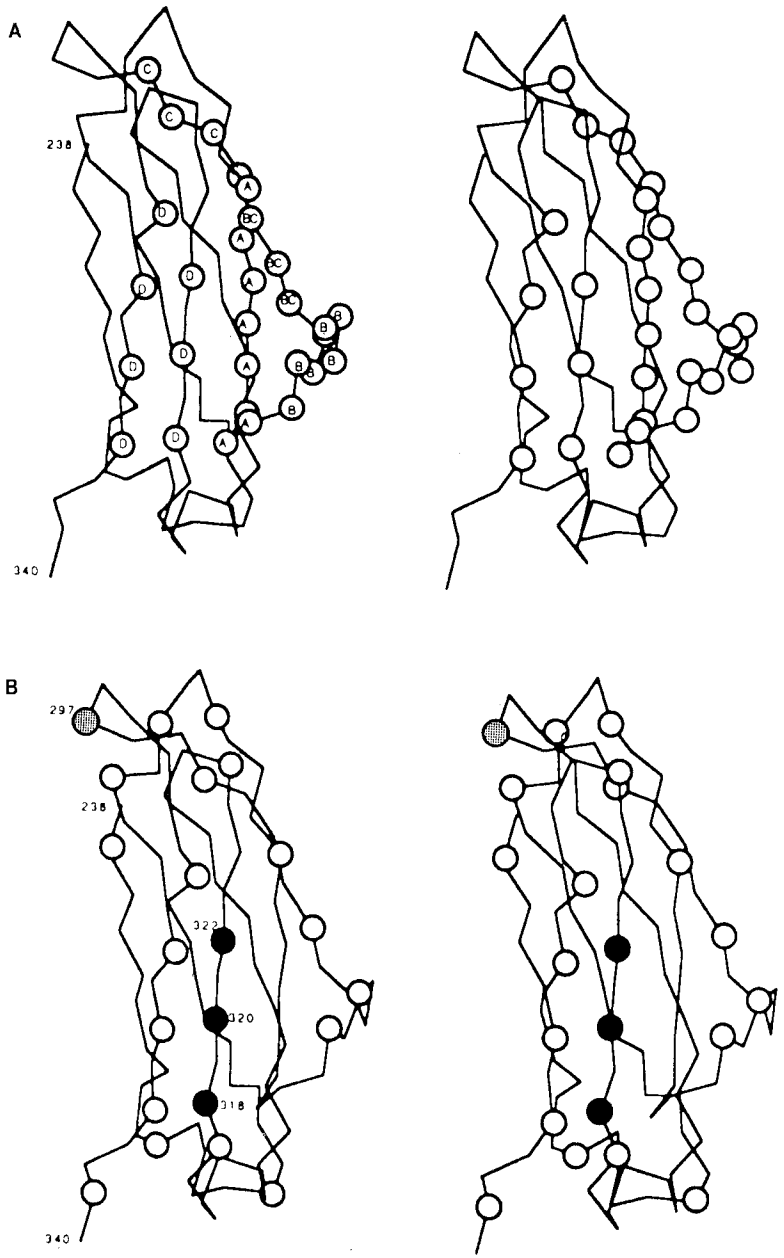
In contrast to IgG, IgM occurs in its native form in an associated state, primarily a pentamer (Fig. 8). Uncomplexed, the pentamer expresses a single C1q-binding site with an affinity estimated as $5 \times 10^5\ M^{-1}$ (Feinstein *et al.*, 1983) or as $10^4\ M^{-1}$ (Poon *et al.*, 1985). Complexed with antigen the affinity of the C1q–IgM interaction increases to of the order of $5 \times 10^7\ M^{-1}$. This increase arises in antibody excess (IgM binding multivalently to the same molecule expressing multiple epitopes) or in antigen excess (IgM cross-linking different antigen molecules). However, only the former case leads to C1 activation (Feinstein *et al.*, 1983). Therefore, it is suggested that the functionally important increase in affinity arises from the exposure of new C1q-binding sites on a single IgM molecule on complexation rather than the spatial association of monovalent pentamers. The ability of single, complexed IgM molecules to activate C1 supports this view (Borsos and Rapp, 1965a,b; Ishizaka *et al.*, 1968; Feinstein *et al.*, 1983). Similarly, on a cell surface it is found that complement activation only occurs when more than one antigen-binding site in the IgM molecule is occupied (Borsos *et al.*, 1981).

Interestingly Fc5, isolated by proteolytic removal of the $F(ab')_2$ units, shows the same binding affinity for C1q as native uncomplexed IgM. Hence the creation of new C1q sites cannot simply be the result of antigen binding somehow moving $F(ab')_2$ units to "unblock" sites on an unaltered Fc5. The implication is that some sort of conformational change in Fc5 must accompany functional IgM binding to antigen (see below).

3. *C1q–IgG Interaction at the Molecular Level*

A number of studies have indicated the importance of charged groups in this interaction (Hughes-Jones and Gardner, 1978; Burton, 1985). The most detailed (Burton *et al.*, 1980; Emanuel *et al.*, 1982) indicated that about 12 ions are released into solution on the binding of one molecule of C1q to an IgG immune aggregate. Because the structural information on Fc is relatively so good, many studies have approached the molecular details of the interaction by attempting to localize the site on Fc binding to C1q. A definitive delineation of the site would likely require cocrystallization of an Fc·C1q head complex but protein engineering of mouse IgG_{2b} (Duncan and Winter, 1988) gives indication that three charged residues, Glu 318, Lys 320, and Lys 322, constitute the essential binding motif. This motif is part of a site earlier proposed (Burton *et al.*, 1980). Figure 2 shows the localization of the motif and Fig. 9 shows the mutations that were made in the protein engineering approach.

For the purposes of design one would like to know whether the motif is the entire binding region, and, if so, any importance of the context of the motif. The ability of a peptide mimic of the motif to inhibit the activation of complement with an inhibition constant close to that observed for IgG (Duncan and Winter, 1988) would suggest that the motif is sufficient. Certainly many mutations were carried out in the proximity of the motif (Fig. 9) with no effect on C1q affinity. The involvement of three charged groups if interacting with three similar groups on C1q would imply the release of six ions per C1q head bound. The observation of 12 ions released would therefore be consistent with two heads bound per C1q molecule to an immune aggregate, which is in agreement with the thermodynamics. Other mutations in the motif implied that a positive charge is required at positions 322 (Arg can substitute for Lys) and probably a hydrogen bond at position 318 (Thr can substitute for Glu). Position 320 will accept either Arg or Gln with retention of C1q binding, but the latter mutation abrogates complement activation. This underscores the existence of requirements additional to C1q binding for effective complement activation. It should



also be noted here that experiments with IgG molecules with heterologous heavy chains have been used to suggest that only one heavy chain is essential for C1q binding (Clark *et al.*, 1989a).

Other studies are suggestive of some importance for the context of the motif. A substitution of Gln by Glu at position 324 in mouse IgG_{2a} adjacent to the motif abrogates C1q binding and complement activation (Nose *et al.*, 1988; Nose and Leanderson, 1989). The behavior of different IgG isotypes also suggests the importance of context. The human IgG subclasses all possess the motif and yet IgG_4 does not significantly bind C1q. The favored interpretation of this has been that the Fab arms of IgG_4 obstruct the C1q-binding site because of the "restricted" hinge of IgG_4 (Burton, 1985). The key experiments here are the description of Fc4 (Fc fragment of IgG_4) binding C1 with an affinity comparable to that for Fc1 and, in aggregated form, activating complement (Isenman *et al.*, 1975). The inability of hinge-deleted human IgG_1 mutants to bind C1 or activate complement seem to further provide examples of just such Fab arm obstruction (Klein *et al.*, 1981). New protein engineering studies (Tan *et al.*, 1990; Norderhaug *et al.*, 1991) provide a challenge to this interpretation of IgG_4 function. In particular, an IgG_4 molecule wherein the hinge exon of IgG_4 is replaced with those found in IgG_3 fails to bind C1q or activate complement. An IgG_3 molecule with the genetic hinge of IgG_4 still binds C1q effectively (Tan *et al.*, 1990). IgG_3 molecules with an IgG_4 hinge or an IgG_4 hinge and CH1 domain activate complement more effectively than wild type IgG_3 (Norderhaug *et al.*, 1991).

Therefore, it would appear that there is a structural lesion in Fc4 that leads to its inability to bind C1q. The data of Isenman *et al.* would require that this lesion is sensitive to the presence of the Fab arms. The

FIG. 9. Mutations made in a protein engineering strategy to localize the C1q-binding site on IgG (after Duncan and Winter, 1988). The diagram shows an α-carbon trace of the C_H2 domain from human Fc (Deisenhofer, 1981). (A) Groups of residues that had been proposed as C1q-binding sites. A, Residues 274–281 (Boackle *et al.*, 1979); B, residues 282–292 (Lukas *et al.*, 1981); C, residues 290–295 (Brunhouse and Cebra, 1979); D, residue 318, 320, 322, 331, 333, 335, and 337 (Burton *et al.*, 1980). (B) Residues in which exposed side chains were altered by site-directed mutagenesis in the homologous mouse IgG_{2b} antibody. Residues altered to Ala were S239, K248, I253, D265, S267, D270, Q274, E283, H285, Q290, E294, N297, K317, E318, K320, K322, K326, E333, T335, S337, and K340. P331 was mutated to Gly and E235 (not shown) was mutated to Leu. Open circles show mutants that were still lytic; closed circles show mutants that were nonlytic and are suggested to comprise the C1q-binding site, i.e., E318, K320, and K322. The carbohydrate attachment residue, N297, is shown shaded.

positions in the CH2 domain distinguishing IgG_4 from the other subclasses have been discussed previously (Burton, 1985; Jefferis, 1986). The sequence Ser 330–Ser 331 (Ala-Pro in the other subclasses, Fig. 1) in close proximity to the motif stands out but there are other differences, such as Phe 234 (Leu) and Gln 268 (His). The ability of an IgG_4/IgG_1 switch mutant (CH1 to residue 291 of CH2 from IgG_4; residue 292 to end of CH2 and CH3 domain from IgG_1) to activate complement implies that residues 292–340 in the CH2 domain contain the amino acids responsible for the inability of IgG_4 to activate (Tao *et al.*, 1991).

The case of human IgG_4 may have implications for the C1q-binding and complement-activating patterns of all the isotypes. Basically there have been two schools of thought. Both saw the C1q-binding site as being present on most isotypes but modulated by the Fab arms. The hinge, and in particular, the upper hinge, was seen as crucial. The first school tended to emphasise the necessity for flexibility in this upper hinge in binding C1q and pointed to a correlation between C1q binding and segmental flexibility (Oi *et al.*, 1984; Dangl *et al.*, 1988). The second school tended to associate "restricted" hinges, which could arise from shorter upper hinges, with structural accommodations placing Fab arms closer to Fc (Burton, 1985). It pointed to solution data indicating more compact conformations for isotypes such as human IgG_2 and IgG_4 with their inferior C1q-binding ability compared to the more extended IgG_1 and IgG_3 (Gregory *et al.*, 1987). The data of Tan *et al.* show that upper hinge flexibility per se (at least in the nanosecond time range) is not necessary for C1q binding. Thus, for example, two "rigid" mutant IgG_3 molecules are able to bind C1q as effectively as the "flexible" wild-type IgG_3 molecule. They also show that close approach of the Fab arms is probably not the reason for the failure of IgG_4 to bind C1q unless CH2 folded domain sequences were somehow controlling hinge conformation and therefore Fab arm disposition.

What is the origin of the poor C1q binding of isotypes such as mouse IgG_1 and guinea pig IgG_1 and the loss of C1q binding associated with hinge deletion (Klein *et al.*, 1981; Michaelsen *et al.*, 1990; Tan *et al.*, 1990)? Fab arm obstruction is an appealing explanation brought into question by the human IgG_4 results. Other unidentified structural lesions are a possibility. In particular, hinge deletion may subtly perturb conformation. Interestingly, for mouse IgG_1, replacement of the $C_\gamma2$ domain with that of mouse IgG_{2b} generates C1q binding and complement lysis at a level comparable to that for IgG_{2b} (Clackson and Winter, 1989). This again militates against the importance of the upper hinge (hinge exon) but leaves open the role of the lower hinge ($C_\gamma2$ domain exon). Mouse IgG_1 has a restricted hinge in terms not only of a short upper hinge but also of a short lower hinge where it lacks the characteristic Gly-Gly sequence. It should be noted, however, that introduction of the mouse IgG_{2b} lower hinge into IgG_1 does not generate a lytic antibody (T. Clackson, personal communication).

The exceptionally long hinge of IgG_3 is something of an enigma. Two groups have now reported that most of the hinge can be deleted without any detrimental effects on C1q binding or complement activation (Sandlie *et al.*, 1989; Michaelsen *et al.*, 1990; Tan *et al.*, 1990). In fact, an IgG_3 with a single hinge exon is more effective at C1q binding and a molecule with an extra exon is less effective.

Aglycosylation is a context to which C1q binding is sensitive to varying degrees. Aglycosylation of mouse IgG_{2a} or mouse IgG_{2b} produces only a small (roughly threefold) reduction in C1q binding affinity (Leatherbarrow *et al.*, 1985; Duncan and Winter, 1988). Again, a small reduction (twofold) has been reported for human Fc1 (Matsuda *et al.*, 1990). In contrast, complete abolition of C1q binding has been reported for aglycosylated human IgG_1 and a dramatic decrease for IgG_3 (Morrison *et al.*, 1989). Even for those cases where C1q binding is minimally affected, whole complement activation, where looked at, is abolished.

Recombinant hybrid molecules in which Fc is linked to other proteins afford an opportunity to look at the effect of context on effector function. Two groups have reported on recombinant CD4–immunoglobulin molecules (Capon *et al.*, 1989; Traunecker *et al.*, 1989; Byrn *et al.*, 1990). Replacing the V_H domain of human $\gamma1$ chain by the first two or all four of the CD4 cytoplasmic domains gives molecules expressed as dimers in a eukaryotic cell line in the absence of light chains. Both molecules bind gp120 and FcRI, but neither binds C1q. However, a molecule in which both V_H and $C_\gamma1$ domains of mouse IgG_{2a} are replaced by the first two domains of CD4 does bind C1q. It would seem that the presence of the $C_\gamma1$ domain, perhaps unable to pair with the C_L domain, is detrimental to C1q binding. In this vein, attempts to graft C1q-binding sites into different molecules will undoubtedly be revealing as regards to site requirements.

Considering the interaction site on C1q, there are charged regions on each of three chains in the heads, e.g., the sequence Glu 198–X–Asp 200–Lys 202 on the A chain, which might interact with the charged motif on IgG (Reid *et al.*, 1982).

4. *C1q–IgM Interaction at the Molecular Level*

As for IgG, it appears that charged groups are important in the interaction of IgM and C1q (Hughes-Jones and Gardner, 1978; Poon *et al.*, 1985). It is estimated that 12 ions are released into solution on the binding of C1q to uncomplexed IgM in solution (V. N. Schumaker, personal communication) and 8–9 ions are released on the binding to IgM interacting with a cell surface. Given the differences in binding affinities described earlier, the C1q-binding site on IgM is not expected to be identical to that of IgG. Indeed, the IgG motif is not found on IgM.

The C1q-binding site(s) are located on the Fc pentamer (Fig. 8) but the precise domain ($C_\mu3$ or $C_\mu4$) has been debated. An interesting mutant IgM molecule with a single amino acid change (Pro → Ser 436) in the $C_\mu3$ domain has been isolated and shown to have decreased affinity for C1q (Wright *et al.*, 1988). This has been taken as evidence to support the $C_\mu3$ domain as binding C1q. It is noteworthy that the residue analogous to Pro 436 in IgG is Pro 331 in the $C_\gamma2$ domain, which is on the edge of the proposed IgG-binding site. Further, a Pro → Ser mutation at this position is prominent in the non-C1q-binding IgG_4 isotype. The effect of the mutation in IgM is complex in that it renders half of the mutant molecules incapable of binding C1q and the other half capable of binding but with lowered affinity. A model based on an equilibrium between different IgM conformations has been proposed to explain these observations (Wright *et al.*, 1988). Another mutant (Asn → Gln 402, comparable position Asn 297 in IgG, the $C_\gamma2$ glycosylation site), which does not glycosylate, shows decreased complement-activating ability compared to wild-type IgM (Muroaka and Shulman, 1989).

The binding of IgM to antigen has been studied by electron microscopy and a model has been suggested for how extra C1q-binding sites would become available on antigen complexation (Beale and Feinstein, 1976; Feinstein and Richardson, 1981; Feinstein *et al.*, 1983, 1986). Briefly, pentameric uncomplexed IgM generally appears crudely as a star-shaped molecule with $F(ab')_2$ unites emerging in various orientations from a planar Fc5 unit. On the binding of specific IgM to *Salmonella paratyphi* flagella, new staple-like IgM molecules are observed in which the $F(ab')_2$ units are all dislocated out of the plane of the Fc5 disk and are bound to the flagella. Similarly, in an antidextran IgM/dextran system, the ability to activate C1 correlates with the appearance of IgM bound to single molecules of dextran in the staple form. Therefore, it is suggested that some distortion introduced into the IgM molecule in the star-to-staple transition reveals extra C1q sites and triggers complement activation.

However, as commented above, removal of the $F(ab')_2$ units does not reveal extra sites, implying the star-to-staple transition generates some change in Fc5 conformation. Again, the problems of transmitting changes through the domain structure of immunoglobulins have been used to argue against an allosteric mechanism. Instead it has been suggested (Feinstein *et al.*, 1986) that pivoting about the inter-$C_\mu3$ bridges and readjustment of the spatial relationship of neighboring $C_\mu4$ dimers might reveal extra C1q sites. Certainly protein engineering experiments indicate the importance of the inter-$C_\mu3$ bridges involving Cys 414, because the pentameric IgM Ser 414 mutant is unable to activate complement.

Perhaps one of the most intriguing developments in this area recently has been the demonstration in a number of laboratories of the propensity of IgM to form hexamers, particularly in the absence of J chains (Cattaneo and Neuberger, 1987; Davis *et al.*, 1988; Randall *et al.*, 1990). The hexamer is found to activate whole complement 10- to 20-fold more efficiently than the pentamer (Davis and Shulman, 1989; Randall *et al.*, 1990). Because J chains are not necessary for either assembly or secretion of polymeric IgM from B cells, it has even been suggested that their function may be to regulate the lytic efficiency of IgM by controlling the pentamer : hexamer ratio (Randall *et al.*, 1990). The existence of both C1q and IgM as hexamers is striking and at least suggestive of some involvement of symmetry in the triggering process.

5. *C1q-Associated IgG at the Molecular Level*

The most detailed description available of associated IgG comes from two-dimensional crystallization studies of a monolayer of a dinitrophenol (dnp)–lipid binding a mouse monoclonal anti-dnp IgG_1 antibody (Reidler *et al.*, 1986). The antibodies form hexagonal arrays in which the Fcs are dislocated out of the plane of the Fabs to generate an angle of about 80°. The monomers interact with one another via both Fc and Fab regions. We have constructed graphics representations based on this idea (Burton *et al.*, 1989; Burton, 1990a) when association of Fc regions was made most readily though interaction of the large hydrophobic patch at the CH2/CH3 domain interface. In fact, although the precise amino acids in this region are not conserved between IgGs of different species and subclasses, there is a strong conservation of character as an extensive exposed hydrophobic patch. The patch is the recognition site for staphylococcal protein A, and the common reactiv-

ity of protein A with IgG is comprehensible in terms of its conservation (Burton, 1985).

Two major features of the two-dimensional crystal studies are that the IgG molecule is viewed as dislocated and in Fc–Fc interaction. Independently, from studies on the interaction of IgG and Fc receptor, we suggested that IgG might be dislocated and in Fc–Fc interaction on an antigenic surface (Burton, 1986). We highlighted a possible link between IgG and IgM in their mode of binding C1q and complement activation (Fig. 10). Thus, IgM is normally in an associated state but must dislocate to bind C1q effectively. IgG is normally monomeric but on binding antigen it would form a defined polymer in a dislocated conformation, which would trigger complement. The complement-activating molecular species in the two cases is very similar according to this model. It is made more plausible by the description of hexavalent IgM, which, in a dislocated conformation, would be expected to closely resemble the two-dimensional crystal view of hexameric IgG. Interestingly, hexagonal symmetry was also described some time ago from EM studies of Fc crystals (Pinteric *et al.*, 1971).

Experimental evidence on associated IgG conformation in less artificial situations than an EM grid is sparse. There is evidence of Fc–Fc interactions in the formation of immune precipitates (Moller, 1979; Rodwell *et al.*, 1980; Easterbrook-Smith *et al.*, 1988). Fc–Fc interactions, reflected in a cooperative binding, have also been observed for the binding of mouse IgG_3 to a surface antigen (Greenspan *et al.*, 1987). However, we have observed no cooperative binding of human anti-rhesus D IgG_1 or IgG_3 to red cells or of human anti-NIP (3-iodo-4-hydroxy-5-nitrophenylacetate) IgG_1 or IgG_3 to NIP-coated lymphoid cells (Woof *et al.*, 1992).

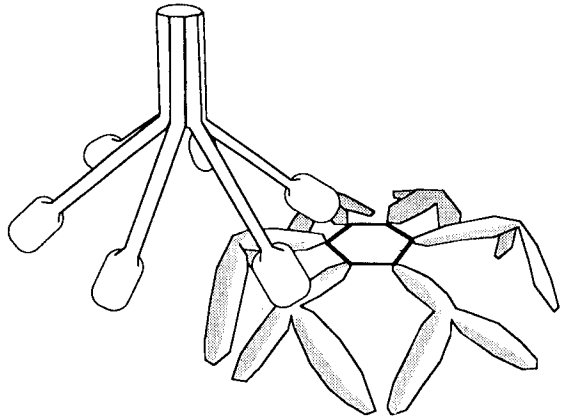


FIG. 10. Schematic representation of C1q binding to an array of IgG molecules. The IgG molecules are arranged in a hexameric array as suggested by two-dimensional crystallization studies (Reidler *et al.*, 1986). The Fcs are dislocated (Burton, 1986) and roughly at right angles to the plane of the corresponding Fabs. For clarity, the IgG molecules are shown schematically in a ribbon format: the two-dimensional crystallization studies indicate lateral Fc–Fc and V domain interactions. The C1q molecule, roughly to scale, is shown interacting with two IgG molecules. Distortions of C1q would be required to recognize adjacent IgG molecules.

Howard and Hughes-Jones (1988) have focused on synergistic lysis of red cells to propose a model for the interaction of C1q and IgG on an antigenic surface. Briefly, as first reported by Elliot *et al.* (1978), antibodies bound together to a single antigen molecule on a cell surface have unusually strong lytic activity. For example, single monoclonal IgGs are often poor agents of complement lysis, but the addition of a second monoclonal IgG to a different epitope on the same antigen can cause a dramatic increase in lysis to a level much greater than the sum of that seen with the individual antibodies (Howard *et al.*, 1979; Bindon *et al.*, 1987). Howard and Hughes-Jones reported that in a synergistic situation of two rat monoclonal IgG_{2b} antibodies binding to MHC class I antigen on a red cell surface, the stoichiometry of C1q binding is two C1q molecules bound per monoclonal antibody pair. The "autonomous" model of Fig. 11, in which two C1q molecules are bound bivalently to opposite faces of the same pair of IgG molecules, themselves bound to the same antigen molecule, was proposed to explain this data. Additional support for the model was derived from the ability of pairs of functionally monovalent antibody (one Fab arm inactivated by association with a nonfunctional light chain) to show a full synergistic lysis effect. This excludes cross-linking of different antigen molecules as an important factor in triggering red cell lysis in this case.

The autonomous model is clearly of some considerable interest and one would like data from other systems to examine its validity. A study (Bindon *et al.*, 1987) of C1q binding to synergistic rat IgG_{2b} monoclonal antibodies binding to human leukocyte common antigen is consistent with an antibody:C1q stoichiometry of about 3:1, but the experimental errors here could be quite large (G. Hale, private communication). An earlier study (Hughes-Jones *et al.*, 1983) of synergistic rat IgG binding to MHC class I antigen on red cells found that the total number of C1q-binding sites was approximately the same whether the synergistic pair was composed of two IgG_{2b} molecules or an IgG_{2b} with an IgG_{2a} molecule (which by itself did not significantly bind C1q). The

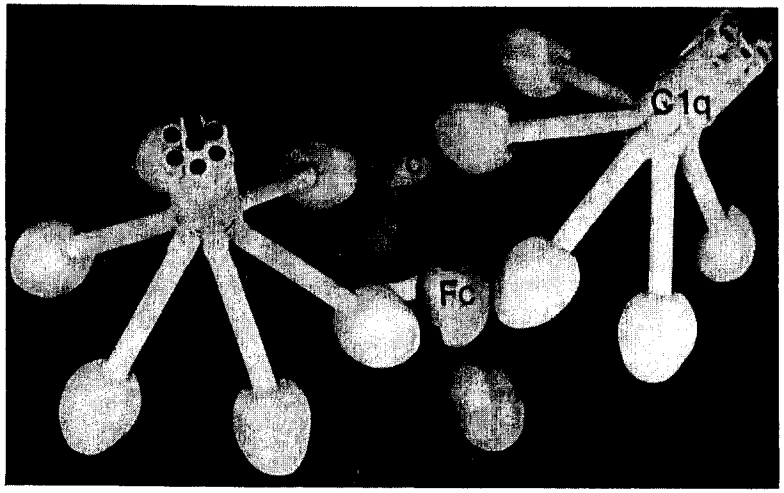


FIG. 11. The autonomous model of complement activation (Howard and Hughes-Jones, 1988). The scale model shows the binding of two C1q molecules to two adjacent Fc pieces. The two IgG molecules are bound to two different epitopes on the same antigen molecule.

binding constant was decreased in the latter case. Furthermore, an $F(ab')_2$ fragment of the IgG_{2a} could not establish a full synergistic effect. These data imply some role for the Fc part of the IgG_{2a} molecule in C1q binding and are perhaps indicative of stabilization of an antibody array rather than autonomous binding.

The human IgG subclass pattern seen in the binding of C1q to associated IgG roughly mirrors that seen for monomeric IgG. More C1q becomes bound to IgG_3 associated on hapten-coated red cells or on immobilized hapten–bovine serum albumin than to similarly associated IgG_1 (Brüggemann *et al.*, 1987; Bindon *et al.*, 1988a; Garred *et al.*, 1989). However, the extent of IgG_3 superiority depends on epitope density and complement concentration. The difference appears to reside in the number of C1q sites made available in the two cases, the binding constants being very similar. More sites are made available by the $IgG_{3m(g)}$ than the $IgG_{3m(b)}$ allotype. There appear to be some very subtle antibody conformational or geometric requirements for the generation of a site capable of binding C1q multivalently. Another study of two IgG_1 antibodies against different epitopes on the same synthetic branched polypeptide showed that, with equal amounts of antibody

bound to the polymer, one antibody bound severalfold more C1q than the other. No difference in sequence in the hinge or $C_\gamma2$ domains between the two antibodies was found (Horgan *et al.*, 1990). This study again implies preferred antibody conformations or arrangements for productive C1q binding.

Relatively significant C1q binding to associated IgG_2 is observed at higher epitope densities and complement concentrations whereas at lower ones such binding virtually disappears (Garred *et al.*, 1989). These observations may account for earlier apparent inconsistencies in the literature. Associated IgG_4 is never observed to bind C1q significantly.

Finally, there are now two reports to show that associated rat IgA is capable of binding C1q and of activating C1, but this does not lead to C4 deposition or cell lysis (Bindon *et al.*, 1990; Hiemstra *et al.*, 1990). There are conflicting reports about the ability of human IgA to take part in classical pathway activation (Iida *et al.*, 1976; Burritt *et al.*, 1977; Römer *et al.*, 1980; Jarvis and McLeod Griffiss, 1989).

B. C1 Activation

Attempts to understand how antibody activates C1 are facilitated by increased information on the structure of C1 (reviewed in Cooper, 1985; Weiss *et al.*, 1986; Arlaud *et al.*, 1987; Schumaker *et al.*, 1987). Briefly, there is ample evidence to indicate that the $C1r_2C1s_2$ tetramer is very extended as an isolated molecule but that it compacts considerably when complexed to C1q in C1. Symmetrical models have been proposed in which the C1s–C1r–C1r–C1s tetramer is wound within the stalks of C1q in an S or figure 8 shape (Fig. 12). Such models have the advantage that they bring the catalytic domains of C1r and C1s into contact with one another, thereby making it conceptually easier to understand how activated C1r can activate C1s. An asymmetric model (Fig. 13) that also achieves this feature has the tetramer folded at its midpoint and wrapped around the outside cone of C1q (Cooper, 1985). A further set of asymmetric models has been proposed (Perkins, 1985).

The interesting question for this review is how C1q binding to associated IgG (or antigen-complexed IgM) leads to C1 activation. The evidence seems to favor a distortive model in which binding to an array of Fcs distorts the cone formed by the spreading C1q arms. This leads to autoactivation of C1r, which in turn activates C1s. The best evidence for this viewpoint comes from the description of a mouse monoclonal IgG_1 antibody [or $F(ab')_2$ fragment], which is against an epitope on the collagenous arms of C1q and which is able to activate C1 (Hoekzema *et al.*, 1988).

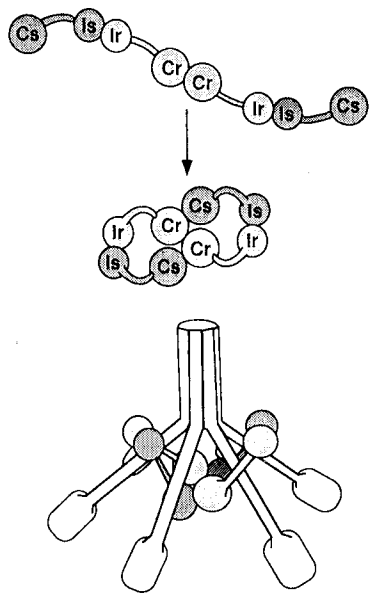


FIG. 12. Model of complement C1 (adapted from Arlaud *et al.*, 1987). The upper diagram shows a model of the extended conformations of $C1r_2C1s_2$ and how a "figure 8-shaped" conformation could be acquired on complexation with C1q. Ir, Is, Interaction domains of C1r and C1s; Cr, Cs, catalytic domains of C1r and C1s. The lower diagram models the C1s–C1r–C1r–C1s tetramer interacting with C1q.

This study shows that (1) bivalency of the antibody is a requirement for C1 activation but not for binding to C1q; (2) increasing the segmental flexibility of the antibody by reduction and alkylation of hinge disulfides destroys the ability to activate C1; (3) an antibody against the C1q heads inhibits C1 activation by associated antibody but not by the anti-C1q arm antibody; (4) isolated C1q stalks (C1q with the heads digested away) are still activated by the monoclonal antibody, indicating the heads are not the origin of the activating signal; and (5) C1 activation is optimal at a monoclonal antibody : C1q ratio of 3:1. The data can be readily interpreted in terms of a symmetrical model wherein the monoclonal antibody distorts a pair of C1q arms to bring C1r and C1s catalytic subunits together in space. In this context it is known that dimers of IgG will activate complement (Wright *et al.*, 1980) and therefore by implication the binding of two heads on the same C1q molecule is sufficient for C1 activation.

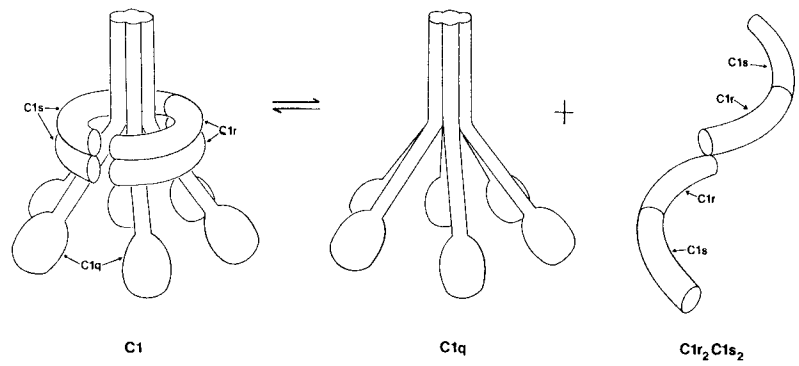


FIG. 13. Alternative model of C1 (adapted from Cooper, 1985). The $C1r_2C1s_2$ tetramer wraps around the arms of C1q rather than being intertwined.

A second suggestion has been that associated antibody may activate C1 by release from the action of C1 inhibitor. According to this theory C1 inhibitor, normally regarded as functioning by actively disassembling activated C1 to give a C1Inh–C1r–C1s–C1Inh complex, binds to unactivated C1 and prevents activation. Antibody displaces C1 inhibitor and therefore activation proceeds. However, the ability of C1 inhibitor to bind to unactivated C1 has been questioned and earlier observations have been interpreted in terms of C1 inhibitor binding solely to activated C1 (Bianchino *et al.*, 1988). Generally, it seems that C1 can autoactivate by both inter- and intramolecular catalysis, but there has been controversy over the years about how much importance to attach to the mechanisms. A recent study (Hosoi *et al.*, 1987) suggests that intramolecular spontaneous activation is very slow but intermolecular activation can be rapid. In this second case, typically, contaminating proteases convert a little C1 to activated C1, which then cleaves further C1 molecules. C1 inhibitor acts solely on the latter process.

Tight C1q binding is not a guarantee of C1 activation. For example, in the case of erythrocytes sensitized with IgG or IgM, both bind C1 equally well but the rate of activation of C1 is far greater in the latter case (Colten *et al.*, 1969). Glutaraldehyde-cross-linked IgG binds C1q as effectively as immune complexes but fails to active C1 (Folkerd *et al.*, 1980). One of the most detailed investigations of the relationship between C1q binding and C1 binding/activation is that of Bindon *et al.* (1988b). These authors compared the ability of rat IgG isotypes binding to the human lymphocyte antigens CAMPATH-1, MHC class I, and LCA (leukocyte common antigen) to bind C1q, bind and activate

C1, and mediate cell lysis. These antigens have comparable surface densities but show marked differences in lytic ability (CAMPATH-1, MHC class I >> LCA). It was found that C1q binding was roughly proportional to antibody binding and dependent on antibody isotype. However, the lytic antibodies were able to bind and activate more C1 than were the poorly lytic ones. C3 activation and whole lysis patterns then propagated these C1 activation differences. The authors suggested two factors that might play a role in this "antigen effect," i.e., preferential C1 binding associated with antigens promoting lysis. One was the possibility of antibody–$C1r_2C1s_2$ interactions (see below). The other was the mode of presentation of antigen-complexed antibody to C1. The authors noted the lower arm flexibility of C1 relative to C1q (Schumaker *et al.*, 1987), which might place more rigorous binding requirements on the former.

Therefore, C1q binding and C1 activation need not correlate when comparing different antigens. For a given antigen, from the limited studies to date, they generally do correlate, e.g., human IgG isotypes binding to hapten-coated red cells (Bindon *et al.*, 1988a), rat IgG isotypes binding to LCA (Bindon *et al.*, 1987), and rat IgG isotypes binding to hapten-coated red cells (Bindon *et al.*, 1990). In particular, human IgG_3 binds C1q and activates C1 more effectively than does IgG_1.

As above, a feature of C1 activation receiving some interest is the possibility of an interaction between antibody and the $C1r_2C1s_2$ tetramer. A review of the literature in 1985 (Burton, 1985) showed no direct evidence for such an interaction involving IgG, and this is still so. Similar conclusions have been reached for IgM (Poon and Schumaker, 1991). However, a number of interesting observations have been made. First, as described initially by Reid *et al.* (1977), the binding affinity of C1q for $C1r_2C1s_2$ is increased by about an order of magnitude on binding to immune complexes (Cooper, 1985). Second, the dissociation rate of C1q in the activated C1 complex from sensitized red cells is about 10-fold slower than that for C1q alone. This applies to a high antibody density, the difference narrowing at lower densities (Okada *et al.*, 1985). Third, the rate of C1 activation on a red cell surface is dependent on antibody density and is independent of antigen or C1 density (Hughes-Jones *et al.*, 1985). Fourth, the lowered dissociation rate for C1q in activated C1 is not found when rabbit Facb (lacking the $C_\gamma3$ domains) is used instead of IgG. The dissociation rate of C1q alone is the same for IgG and Facb (Okada *et al.*, 1985). Fifth, the Facb-bound activated C1 complex is more susceptible to C1 inhibitor inactivation than is the IgG-bound complex. The results have been interpreted to indicate a direct, albeit weak, interaction between $C1r_2C1s_2$ and the $C_\gamma3$ domains of IgG, with these domains protecting the activated tetramer from C1 inhibitor (Okada and Utsumi, 1989).

An alternative is that in a C1-activating situation the conformations of both antibody (IgG) and C1q (in C1) become constrained in the mutual interaction. The antibody becomes arranged in a defined array in which Fc–Fc interactions play a part so that Facb is less effective. Array formation is facilitated at higher antibody densities. The C1q undergoes a distortion that increases its affinity for $C1r_2C1s_2$. The distortion is different depending upon the presence or absence of $C1r_2C1s_2$ bound to C1q, leading to a difference in dissociation rates as described above. These two alternatives are not mutually exclusive, i.e., there could be tetramer binding to antibody and array formation.

Finally, recent sedimentation studies show that activated C1 binds much more tightly to IgM than does C1q alone (Poon and Schumaker, 1991). It is argued that the binding of activated $C1s_2$ to C1q, either alone or together with activated $C1r_2$, induces a conformational change in C1q that results in additional C1q heads binding to complementary sites on IgM. Cryptic sites on IgM, transitorily exposed by random thermal motion, might be "captured" by activated C1, forming a new complex that could mimic activated C1 attached to cell-bound IgM.

C. C4 Activation, C3 Activation, and Cell Lysis

After C1 activation, the next step in the classical complement pathway is the activation of C4 through proteolytic cleavage by activated C1s (schematic structures for these molecules are shown in Fig. 14). The major fragment, C4b, can attach covalently to a suitable surface via an activated acyl group. The Fab of IgG (Campbell *et al.*, 1980; Alcolea *et al.*, 1987) and the antigen (Garred *et al.*, 1990) have been implicated as the surface for C4b deposition in immune complexes. Studies on antibody-coated red cells have found C4 deposition primarily at the cell membrane (Circolo and Borsos, 1982; Bindon *et al.*, 1988a). A number of studies have investigated isotype patterns of C4 activation. Bindon *et al.*, (1988a) looked at the amount of C4b deposited on NIP-coated red cells in complement activation by chimeric human anti-NIP antibodies. They found that IgG_1 deposited far more C4b than did IgG_3 under conditions in which IgG_3 was more efficient at C1q binding and C1 activation, as described earlier. The authors showed that the poor C4b deposition was due to poor C4 activation rather than inability to reach the cell surface, i.e., C1s activated by IgG_3 appeared inefficient at C4 activation. Possible explanations suggested included easier access of C1 inhibitor in the IgG_3 case, favored association of C4

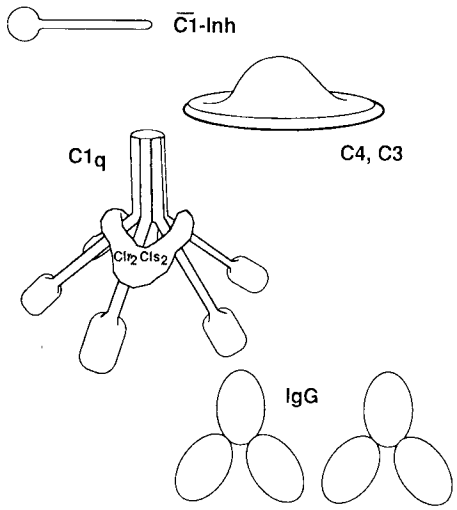


FIG. 14. Schematic representation of the molecules involved in the classical pathway activation of complement. The molecules are drawn roughly to scale. Dimensions and shapes are taken from Reid and Porter (1981), Perkins (1985), Perkins *et al.* (1990a,b), and Odermatt *et al.* (1981).

in the IgG_1 case, and restricted access of C4 to C1s in the IgG_3 case. No C4b binding was detected with either IgG_2 or IgG_4.

Garred *et al.* (1989) looked at C4b deposition on NIP–BSA immune complexes formed with the chimeric human anti-NIP antibodies. In particular, they studied the IgG subclass patterns as a function of epitope density (NIP:BSA) and the concentrations of NIP–BSA, antibody, and complement. They found that IgG_1 and IgG_3 were comparable in C4b deposition at higher epitope density and BSA–NIP concentration but that IgG_1 was far less effective at lower values of these parameters. IgG_2 produced significant C4b deposition at higher epitope density and BSA–NIP concentration but IgG_4 was ineffective under any conditions. In this study, C4b deposition patterns followed those of C1q binding. It is unclear why the two studies produce a different rank order for IgG_1 and IgG_3. It could be that IgG_1 is generally more effective for cell surface activation and IgG_3 for immune complex activation. This interpretation is rendered unlikely by the studies of Michaelsen *et al.* (1991) on isotype patterns in cell lysis (see below). A more probable explanation lies, at least in part, in the manner of presentation of the hapten, and we shall return to this point.

Bindon *et al.* (1990) looked at C4b deposition on NIP-coated red cells using different isotypes of rat anti-NIP antibody. They found that IgM and IgG_{2b} were both very efficient, reflecting their efficacy in C1q binding and C1 activation. However, of IgG_1, IgG_{2a}, IgG_{2c}, and IgA, which bound C1q and activated C1 roughly equivalently, only IgG_{2a} was effective at C4 activation under the conditions employed.

Fixed C4b interacts with C2 to form the classical pathway C3 convertase, which in turn cleaves C3. The C3b product can bind covalently to an appropriate surface in a manner analogous to C4b. Again this surface can be antibody (Gadd and Reid, 1981b; Brown *et al.*, 1983; Takata *et al.*, 1984), the antigen part of an immune complex (Garred *et al.*, 1990), or a cell membrane (Circolo and Borsos, 1984; Bindon *et al.*, 1988a). The available studies generally indicate a good agreement between efficiency of C4b deposition and efficiency of C3 deposition and cell lysis or terminal complement complex formation (Bindon *et al.*, 1988a, 1990; Garred *et al.*, 1989). However, there is a striking exception. Clark *et al.* (1989a) found that a rat monoclonal anti-CD3 IgG_{2b} antibody with one nonfunctional light chain (and therefore monovalent) generated similar levels of cell-bound C3 but gave more lysis than did the parent divalent antibody. It would seem, therefore, that the triggering antibody molecule can influence the complement cascade at a stage after C3 deposition. Considering the interesting question of the relative abilities of IgG_1 and IgG_3 to mediate cell lysis, the following observations have been made. IgG_1 is considerably more effective than IgG_3 in mediating lysis of hapten (NIP)-coated red cells (Brüggemann *et al.*, 1987; Bindon *et al.*, 1988a). IgG_3 is more effective at mediating lysis of dansyl-coated red cells (Dangl *et al.*, 1988). An IgG_1 directed against a surface antigen is more effective at lysis of lymphocytes than is IgG_3 (Riechmann *et al.*, 1988). An IgG_1 against a tumor cell line mediates lysis (Liu *et al.*, 1987), whereas an IgG_3 against a different cell line does not (Shaw *et al.*, 1987). The picture apparently emerging is therefore of IgG_1 being generally superior for lysis.

However, the studies of Michaelsen *et al.* (1991) on red cells labeled with hapten (NIP)-anti-red blood cell (RBC) F(ab') suggest that the conditions of lysis, i.e., antigen density and antibody and complement concentration, are an important consideration here. Thus, they found anti-NIP IgG_1 to be superior to IgG_3 at high antigen concentration, but this order was reversed at lower antigen concentration. The amount of IgG_3 bound was less than that of the other subclasses so its relative potency is even greater. IgG_2 was only effective at the highest antigen densities. This latter observation is interesting in view of the preponderance of IgG_2 antibodies in anticarbohydrate responses and the

tendency of carbohydrate antigens to be presented in high concentration on the surface of microorganisms. These studies did not determine where in the C1q binding, C1 activation, and C3 and C4 activation steps the relative efficacy of the subclasses is established. The previous studies on immune complexes (Garred *et al.*, 1989) make the C1q-binding step a likely candidate.

Can the various studies on the relative efficacies of IgG_3 and IgG_1 be reconciled simply on the basis of different experimental conditions? We believe probably not and the differences probably arise from differences in presentation of the antigen to the complement system. In particular, Brüggemann *et al.* (1987) and Bindon *et al.* (1988a) used NIP attached to kephalin and therefore, presumably, the hapten was close to the red cell surface, whereas Michaelsen *et al.* (1991) attached NIP to cell surface proteins directly or via Fab′ at greater distances in a different local environment. Therefore, in conclusion, it does not yet seem possible to assert that IgG_1 or IgG_3 is the most effective isotype for complement lysis. This may depend on the antigen involved and the precise experimental conditions employed.

Finally, it should be noted that in the case of lysis mediated by a pair of rat IgGs against the human leukocyte common antigen, efficient lysis required the alternative as well as the classical pathway (Bindon *et al.*, 1987).

D. Antibodies as Activators of the Alternative Pathway

Alternative pathway activation, in its simplest form, involves the generation of the alternative pathway C3 convertase (C3bBb) from C3 and factor B in the presence of factor D. The activation, once initiated, has the potential for positive feedback and amplification. Factors I and H in the fluid phase act to regulate C3 convertase and prevent amplification. Properdin acts as a positive regulator. In the presence of a suitable activating surface, stabilization of the C3 convertase tips the scales in favor of amplification. A typical activating surface is that of a microorganism, although it is suggested that associated antibody may also function in this way (reviewed in Ratnoff *et al.*, 1983).

In particular, it is often assumed that IgA can activate the alternative pathway, although this has been controversial (Kilian *et al.*, 1988). Recent literature maintains the controversy. Hiemstra *et al.* (1987) reported that red cells coated with chemically aggregated human IgA were lysed by the alternative pathway. Rits *et al.* (1988) found that both soluble and insoluble rat IgA immune complexes activated the alternative pathway of homologous rat complement. Hiemstra *et al.* (1988) reported that human IgA_1, IgA_2, secretory IgA, and the $F(ab')_2$ fragment of IgA_1 coated onto microwells were able to activate the alternative pathway. Fab and Fc fragments were not. Schneiderman *et al.* (1990) reported that a series of chimeric rabbit mouse IgA antibodies bound to antigen activated the alternative, but not the classical pathway. Valim and Lachmann (1991) found effective alternative pathway activation by immune complexes formed between BSA–NIP and chimeric human IgA_2 anti-NIP antibodies. The same complexes did not trigger the classical pathway. IgG_2 was also found to activate the alternative pathway less effectively at high epitope density and equivalence or antibody excess.

On the other hand, Imai *et al.* (1988) found that neither human IgA immune complexes nor covalently cross-linked IgA activated the alternative pathway. Russell and Mansa (1989) reported that although human IgA coated onto plastic surfaces activated the alternative pathway in a dose-dependent manner, IgA bound to antigen did not. In contrast, IgG antibodies, either bound to antigen or coated directly onto plastic, activated complement, mainly by the classical pathway. The authors concluded that the complexation of IgA with antigen is insufficient to trigger the alternative pathway and argued rather that denaturation plays a key role in IgA activation. Jarvis and McLeod Griffiss (1989) found that human IgA_1 was unable to mediate alternative pathways lysis of *Neisseria meningitidis* but did mediate classical pathway lysis. The other antibody often associated with alternative pathway activation is rabbit IgG when C3b has been found bound to Fab and the $C_\gamma 3$ domain of IgG (Gadd and Reid, 1981a; Anton *et al.*, 1989).

IV. Human Leukocyte Fc Receptors

Receptors specific for the Fc region immunoglobulins are found on the surface of a variety of human leukocytes. The presence of Fc receptors confers on these immune cells the ability to mediate a number of effector mechanisms important in the humoral response. Recent cloning and sequencing studies have revealed that the large majority of mammalian Fc receptors have evolved as part of the immunoglobulin gene superfamily. Here those Fc receptors specific for IgG (Fc_γ receptors) will be discussed first, followed by those specific for other classes of immunoglobulin.

A. Fc_γ Receptors

Three classes of human Fc_γ receptor have thus far been described: $Fc_\gamma RI$, $Fc_\gamma RII$, and $Fc_\gamma RIII$ (Unkeless *et al.*, 1988). All three ap-

pear capable of mediating a number of protective functions against antibody-coated infectious agents (Pound and Walker, 1990; Van de Winkel and Anderson, 1991). Studies using hybridomas expressing surface anti-$Fc_\gamma R$ monoclonal antibodies as targets have demonstrated that $Fc_\gamma RI$, $Fc_\gamma RII$, and the macrophage/NK cell form of $Fc_\gamma RIII$ can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (Fanger *et al.*, 1989). These same receptors can also mediate phagocytosis (Huizinga *et al.*, 1989c; Anderson *et al.*, 1990a), whereas $Fc_\gamma RI$, $Fc_\gamma RII$, and the neutrophil form of $Fc_\gamma RIII$ have been shown to trigger an oxidative burst (Anderson *et al.*, 1986; Crockett-Torabi and Fantone, 1990; B. A. M. Walker *et al.*, 1991).

1. *$Fc_\gamma RI$*

Human $Fc_\gamma RI$ (CD64) is a 72-kDa glycoprotein expressed constitutively on monocytes and macrophages. It may be induced on neutrophils *in vitro* by treatment with interferon-γ (IFN-γ) (Perussia *et al.*, 1983). IFN-γ treatment also up-regulates $Fc_\gamma RI$ expression on mononuclear phagocytes on the $Fc_\gamma RI$-bearing monocytic cell lines, U937 and HL60 (Guyre *et al.*, 1983).

Three cDNA clones for human $Fc_\gamma RI$ have been isolated using a ligand-affinity cloning technique (Allen and Seed, 1988, 1989). Two clones represent polymorphisms whereas the third has a shorter predicted intracytoplasmic domain (Fig. 15). In each case, the deduced amino acid sequence indicates an integral membrane protein with a single hydrophobic membrane-spanning region. The extracellular portion is composed of three immunoglobulin-like domains, the first two of which exhibit homology to the two extracellular domains of human $Fc_\gamma RII$ and $Fc_\gamma RIII$ (see later). The third domain, nearest to the membrane, is less closely related.

2. *$Fc_\gamma RI$–IgG Interaction at the Molecular Level*

$Fc_\gamma RI$ is sometimes referred to as the high-affinity Fc_γ receptor, because of the three human receptors it is the only one displaying appreciable affinity for monomeric IgG. It binds monomeric human IgG_1 and IgG_3 with a K_a of $\sim 5 \times 10^8\ M^{-1}$ (Fries *et al.*, 1982; Kurlander and Batker, 1982). The affinity for human IgG_4 is approximately 10-fold lower and human IgG_2 does not bind (Woof *et al.*, 1986). Human $Fc_\gamma RI$ appears to bind aggregated IgG, at least in heat or chemically cross-linked forms, with similar affinity to monomeric IgG (Cosio *et al.*, 1981; Carter *et al.*, 1982; Kurlander and Batker, 1982; Woof *et al.*, 1986). This finding, together with the fact that the high serum concentration of monomeric IgG presumably results in constant saturation of

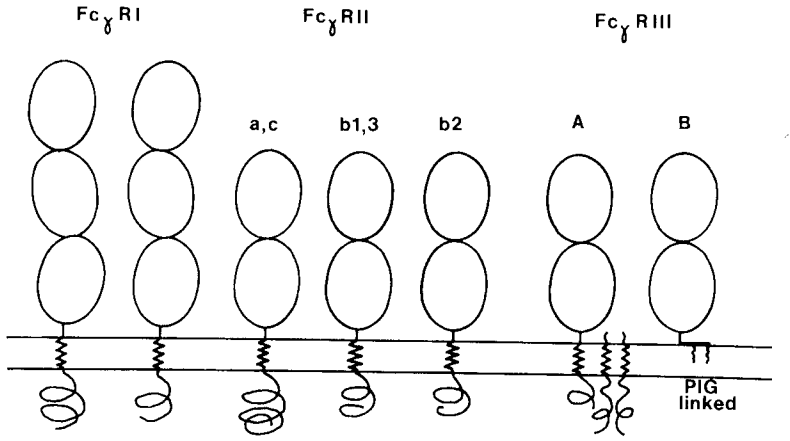


FIG. 15. Schematic representation of the structures of human Fc_γ receptors. The extracellular immunoglobulin-like domains, shown as oval shapes, each have an internal disulfide bond. No structural information is yet available for cytoplasmic domains, but their relative lengths are indicated. PIG, Phosphatidylinositol–glycan.

$Fc_\gamma RI$, raises the question of how $Fc_\gamma RI$ is able to distinguish antibody-coated infectious agents. One possibility is that $Fc_\gamma RI$ may play an important role at tissue sites where monomeric IgG is limiting. Alternatively, up-regulation of $Fc_\gamma RI$ expression by IFN-γ at inflammatory sites may influence the function of the receptor.

The high affinity of $Fc_\gamma RI$ for monomeric IgG has facilitated attempts to localize the $Fc_\gamma RI$ interaction site on IgG. Earlier studies had suggested that the site lay in the $C_\gamma 3$ domain of IgG (Okafor *et al.*, 1974; Ciccimarra *et al.*, 1975). However, the use of highly immunoaffinity-purified IgG fragments and domain-deleted IgG paraproteins indicated that this was not the case (Woof *et al.*, 1984). Further, loss of the N-linked carbohydrate moieties from the $C_\gamma 2$ domain of IgG was shown to result in a marked reduction (>50-fold) in affinity for $Fc_\gamma RI$. This suggested an important role for the $C_\gamma 2$ domain in the interaction, because aglycosylation was deemed more likely to perturb $C_\gamma 2$ structure than that of $C_\gamma 3$ (Leatherbarrow *et al.*, 1985; Walker *et al.*, 1989a). As mentioned earlier, recent NMR studies have verified this assumption (Matsuda *et al.*, 1990).

Direct evidence for the involvement of the $C_\gamma 2$ domain of IgG was provided by experiments in which a panel of antihuman IgG monoclonal antibodies were assessed for ability to inhibit the IgG–$Fc_\gamma RI$ inter-

action and to bind to receptor-bound ligand (Partridge *et al.*, 1988). Only those monoclonal antibodies recognizing epitopes at the N-terminal end of the $C_\gamma2$ domain both blocked IgG binding to $Fc_\gamma RI$ and failed to bind receptor-bound IgG. Recent "domain swap" experiments in which the $C_\gamma2$ and $C_\gamma3$ domains of human IgG_1, either singly or together, substitute for one or both of the homologous $C_\epsilon3$ and $C_\epsilon4$ domains of mouse IgE have generated chimeric IgG/IgE molecules (Shopes *et al.*, 1990). In the series of mutants produced, all those lacking $C_\gamma2$ domains did not inhibit the $Fc_\gamma RI$–IgG interaction, whereas three out of four IgG/IgE constructs possessing $C_\gamma2$ domains did inhibit. These results confirm the importance of the $C_\gamma2$ domain for interaction with $Fc_\gamma RI$. Futher, calculations of the relative energetic contributions of each domain revealed that the $C_\gamma2$ domain contributes about 73% of the overall drop in free energy seen on binding. In contrast, the $C_\gamma3$ domain was shown to contribute maximally about 25% of the free energy drop upon binding. This is consistent with a requirement for the presence of the $C_\gamma3$ domain of IgG for $Fc_\gamma RI$ binding, earlier suggested by the lack of reactivity of a $C_\gamma3$-deleted human IgG_1 paraprotein with the receptor (Woof *et al.*, 1984). The $C_\gamma3$ domains would appear to serve to stabilize the overall structure of the Fc, necessary for optimal $Fc_\gamma RI$ binding.

Another approach assessed the ability of various IgG molecules, from different species and of different subclasses, to inhibit the interaction of radiolabeled human IgG with $Fc_\gamma RI$. Amino acid sequence comparison of the $C_\gamma2$ domains of these IgGs then outlined potential $Fc_\gamma RI$-binding sites that fulfilled the requirements of solvent accessibility and conservation in only the tight binding IgGs. This procedure highlighted a region at the N-terminal end of $C_\gamma2$ comprising residues Leu 234–Ser 239 which appears to be critical for interaction with $Fc_\gamma RI$ (Woof *et al.*, 1986). This region, although encoded by the $C_\gamma2$ exon, structurally forms part of the lower portion of the hinge, lying mostly beyond the extent of the solved crystal structure of Fc, which stops at residue 238 (see earlier).

The proposed site, Leu 234–Leu 235–Gly 236–Gly 237–Pro 238–Ser 239, is present in all IgG isotypes with high affinity for $Fc_\gamma RI$, namely, human IgG_1 and IgG_3, mouse IgG_{2a}, rat IgG_{2b}, and rabbit IgG. In the weaker binding human IgG_4, residue 234 becomes Phe and in mouse IgG_{2b} residue 235 becomes Glu. This latter IgG displays little or no binding to human $Fc_\gamma RI$. However, a mutant mouse IgG_{2b} molecule, generated by site-directed mutagenesis in which Glu 235 is converted to Leu, displayed a greater than 100-fold increase in affinity for the receptor. Indeed, Scatchard analysis of direct binding measurements showed it to have an affinity comparable to that of human IgG_1 (Duncan *et al.*, 1988). More recent mutagenesis experiments have concentrated on the high-affinity human IgG_3 subclass. Substitutions at residues 234–237 inclusive resulted in reductions in ability to inhibit the IgG–$Fc_\gamma RI$ interaction (Lund *et al.*, 1991). The most marked effect is seen with substitution at position 235, with replacement of the Leu with Glu giving a >100-fold decrease in affinity. Substitution of Ala for residues Leu 234, Gly 236, and Gly 237 generated antibodies with affinities reduced ~4-fold, ~4-fold, and ~30-fold, respectively. Rosetting studies with U937 cells confirmed these binding inhibition measurements. Taken together, these findings confirm the importance of the lower hinge region in the interaction of IgG with $Fc_\gamma RI$ (Fig. 2). Evidently, only a single site (i.e., one heavy chain) is required for interaction with a single $Fc_\gamma RI$ molecule (O'Grady *et al.*, 1986; Koolwijk *et al.*, 1989).

Studies using an array of further IgG mutants appear to be in broad agreement with the above-cited work (Canfield and Morrison, 1991). A mutant human IgG_2 molecule, in which the $C_\gamma3$ domains of human IgG_3 were substituted for its own, did not bind to $Fc_\gamma RI$. A human IgG_3 bearing the $C_\gamma3$ domains of human IgG_2, however, did bind $Fc_\gamma RI$, with an affinity comparable to that of wild-type IgG_3. Thus, the critical importance of the $C_\gamma2$ domains in the interaction is reaffirmed. Substitution of Glu for Leu at position 235 in human IgG_3 again resulted in a >100-fold reduction in affinity for $Fc_\gamma RI$. Conversion in human IgG_3 of residue 234 to Phe, as found in human IgG_4, generated a mutant molecule with affinity equivalent to that of wild-type human IgG_4. The reciprocal experiment, in which Phe 234 in human IgG_4 was converted to Leu, generated an IgG_4 molecule with increased affinity for $Fc_\gamma RI$, about threefold lower than that of human IgG_3. This slight shortfall in affinity compared to human IgG_3 prompted the authors to search elsewhere in the $C_\gamma2$ domain for amino acid differences that might serve as an explanation (Canfield and Morrison, 1991). Thus it was proposed that residue 331 (Pro in all tight-binding IgGs but Ser in human IgG_4), located on a loop lying close to the lower hinge site, might make some contribution to the $Fc_\gamma RI$ interaction. The substitution experiments, however, yielded somewhat inconclusive results and further investigation into the possible role of this loop would be interesting.

A second bend, lying close to the lower hinge region in molecular models, may also be of importance. Proton NMR revealed that on aglycosylation of Fc the minor structural changes in the $C_\gamma2$ domain were sensed predominantly by His 268, which lies in this second bend (Matsuda *et al.*, 1990). The marked reduction in affinity for $Fc_\gamma RI$

accompanying aglycosylation may be explained in terms of perturbation of this lower hinge-proximal loop (Lund *et al.*, 1990).

Turning to the interaction site on the receptor, presently rather little information is available. The third domain of $Fc_\gamma RI$ shares less homology with the two extracellular domains of the low-affinity receptors, $Fc_\gamma RII$ and $Fc_\gamma RIII$, than do the first two domains. This perhaps suggests a role of this third domain in conferring high affinity on $Fc_\gamma RI$. Indeed, preliminary studies introducing point mutations into the third domain are reported to confirm this (Allen and Seed, 1989). Further, studies on the mouse homologue, mouse $Fc_\gamma RI$, which also possesses three extracellular domains, suggest that domain 3 confers high affinity and specificity on domains 1 and 2, which form a low-affinity IgG-binding motif (Hulett *et al.*, 1991).

Figure 16 shows a model of the interaction of human $Fc_\gamma RI$ (modeled as three IgG domains) with an IgG_1 molecule simultaneously binding to antigen (hemagglutinin on the surface of a virally infected cell). Although admittedly speculative, such models serve to demonstrate the relative sizes of the molecules involved and highlight conformation constraints imposed by the location of interaction sites on the molecules.

3. *$Fc_\gamma RII$*

Human $Fc_\gamma RII$ (CD32), a 40-kDa glycoprotein, has the most widespread distribution of the three human receptors for IgG, being present on monocytes, macrophages, eosinophils, platelets, neutrophils, basophils, and B cells. Several cDNA clones coding for this receptor predict a transmembrane molecule with two immunoglobulin-like extracellular domains (Stuart *et al.*, 1987, 1989; Hibbs *et al.*, 1988; Stengelin *et al.*, 1988). $Fc_\gamma RII$ is encoded by a minimum of three genes (IIA, IIB, and IIC) that yield six distinct transcripts (Brooks *et al.*, 1989). Gene IIB gives rise to three transcripts, termed $Fc_\gamma RIIb1$, $Fc_\gamma RIIb2$, and $Fc_\gamma RIIb3$, by alternative slicing of cytoplasmic exons or of signal sequence exons. Transcripts of IIB have been demonstrated by a combination of Northern blotting and polymerase chain reaction (PCR) amplification after reverse transcription in macrophages, monocytes, and B lymphocytes, with lower levels found in neutrophils (Brooks *et al.*, 1989). $Fc_\gamma RIIb$ expression has also been demonstrated in placental trophoblasts (Stuart *et al.*, 1989).

The three other $Fc_\gamma RII$ transcripts (two $Fc_\gamma RIIa$ transcripts and one $Fc_\gamma RIIc$) are derived from two further genes referred to as $Fc_\gamma RIIA$ and $Fc_\gamma RIIC$ (Brooks *et al.*, 1989; Stuart *et al.*, 1989). Alternative polyadenylation in $Fc_\gamma RIIA$ gives rise to two transcripts. All three molecules are highly homologous to $Fc_\gamma RIIb$, with differences being restricted to

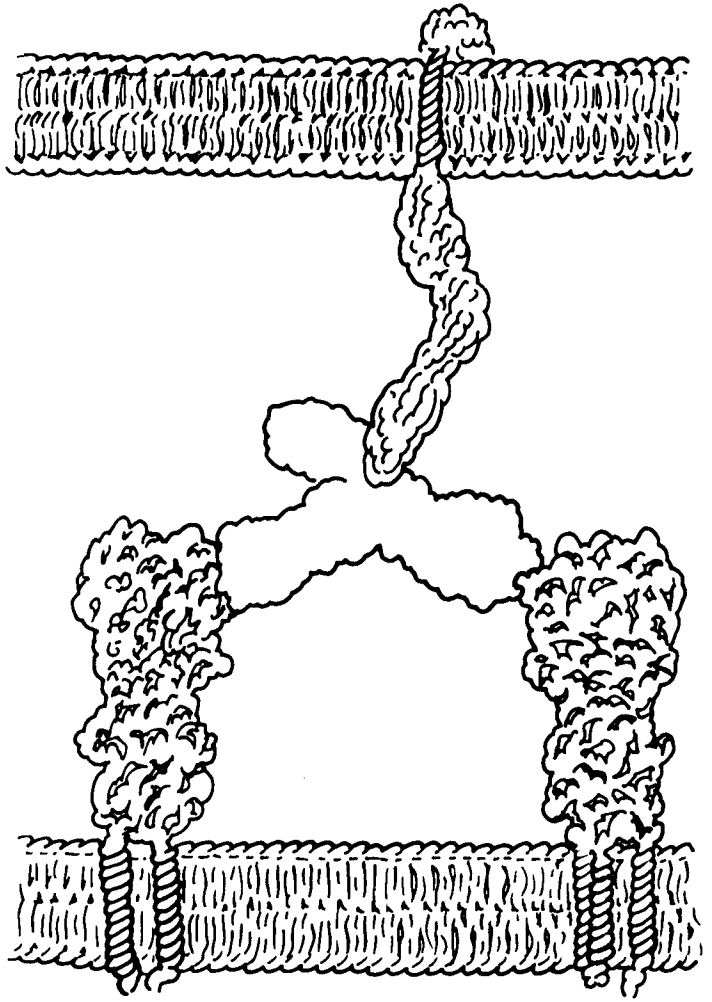


FIG. 16. Antibody as adaptor linking target and effector cells. In the lower part of the picture, the target cell infected by influenza virus is shown expressing two hemagglutinin molecules at its surface. Hemagglutinin (Wilson *et al.*, 1981) is recognized by the Fab arms of the IgG molecule. In turn, the IgG molecule is recognized at the site seen in Fig. 2 by an Fc receptor molecule anchored to the cell membrane of the effector cell. The IgG molecule as shown is dislocated in the sense that the Fc part is in a plane roughly at right angles to that containing the Fab arms. The Fc receptor, FcRI, is known from the work of Allen and Seed (1989) to consist extracellularly of three immunoglobulin-like domains. The structure as shown and antibody binding to the outermost domain are speculative. The effect of opening of the Fab arms (opening the Y) and of dislocation is that the antibody molecule tends to draw the target and effector cells closer together than many other possible arrangements. The use of one of the inner domains of FcRI for antibody binding would enhance this effect still further.

the signal sequence and part of the cytoplasmic domain in $Fc_\gamma RIIa$, and to a portion of the cytoplasmic domain in $Fc_\gamma RIIc$. Ignoring differential polyadenylation, the $Fc_\gamma RIIa$ and $Fc_\gamma RIIc$ transcripts are almost identical apart from their signal sequences. The leader sequence of $Fc_\gamma RIIc$ is homologous to that of $Fc_\gamma RIIb$, whereas that of $Fc_\gamma RIIa$ shows homology to that of human $Fc_\gamma RIII$ (see later). Thus, IIC may have evolved initially by gene duplication and mutation from an ancestral IIB gene, with a later recombination event between IIC and $Fc_\gamma RIII$ genes giving rise to IIA.

Transcripts of IIA have been found readily in monocytes and neutrophils but less prominently in lymphoid cell lines, whereas sensitive PCR amplification techniques have detected $Fc_\gamma RIIc$ transcripts in neutrophils, monocytes, and B cells (Brooks *et al.*, 1989). Thus, there appears to be preferential expression of $Fc_\gamma RIIA$ in neutrophils and $Fc_\gamma RIIB$ in lymphocytes, with both forms being expressed in monocytes (Ravetch and Anderson, 1990).

The mature protein products predicted from the cDNAs of $Fc_\gamma RIIa$, $Fc_\gamma RIIc$, and $Fc_\gamma RIIb$ are closely related. The predicted $Fc_\gamma RIIa$ and $Fc_\gamma RIIc$ proteins are virtually identical (>95%). The extracellular and transmembrane domains of the $Fc_\gamma RIIb$ receptors share a high degree of homology with the predicted $Fc\gamma RIIa$ and $Fc\gamma RIIc$ receptors. However, beyond the first 10–12 residues of the intracellular regions the similarity between $Fc_\gamma RIIb$ and $Fc_\gamma RIIa/IIc$ ends and highly divergent cytoplasmic tails result (Fig. 15). The conserved nature of the extracellular domains together with the diversity of the intracytoplasmic regions suggests that this family of receptors may mediate an array of different functions in response to recognition of the same ligand.

4. *$Fc_\gamma RII$–IgG Interaction at the Molecular Level*

The affinity of human $Fc_\gamma RII$ for monomeric IgG is too low to be easily experimentally determined ($K_a < 1 \times 10^7\ M^{-1}$) (Karas *et al.*, 1982; Kurlander *et al.*, 1984) but binding to complexed IgG is much tighter [for dimers of human IgG_1, $K_a \sim (2–5) \times 10^7\ M^{-1}$] (Karas *et al.*, 1982; Van de Winkel and Anderson, 1991). Recently, the affinity of $Fc_\gamma RII$ for complexed IgG has been shown to increase after treatment with proteolytic enzymes such as pronase and elastase (Van de Winkel *et al.*, 1989b). This process may serve to "activate" $Fc_\gamma RII$ at inflammatory sites (Tax and Van de Winkel, 1990).

Human $Fc_\gamma RII$ is specific for complexes of human IgG_1 and IgG_3 and appears not to bind IgG_4 (Karas *et al.*, 1982; Walker *et al.*, 1989b; Van de Winkel and Anderson, 1991). Reports on the affinity of $Fc_\gamma RII$ for complexed human IgG_2 appear to give conflicting results, with some

workers reporting binding (Van de Winkel and Anderson, 1991) and others not (Walker *et al.*, 1989b). An explanation, now emerging, is that the ability to bind human IgG_2 appears to reside with only one allelic form of $Fc_\gamma RII$, termed low responder (see below) (Warmerdam *et al.*, 1991). $Fc_\gamma RII$ also has affinity for mouse IgG_{2b} but not for mouse IgG_{2a}. A functional polymorphism of $Fc_\gamma RIIa$ has been demonstrated by different assays on monocytes (Tax *et al.*, 1983; Leeuwenberg *et al.*, 1987; Van de Winkel *et al.*, 1987, 1989a; Debets *et al.*, 1990), neutrophils (Leeuwenberg *et al.*, 1990; Gosselin *et al.*, 1990), and alveolar macrophages (Kindt *et al.*, 1991). The two allelic forms of $Fc_\gamma RIIa$ involved differ in their ability to bind complexed mouse IgG_1—the high-responder (HR) form binds whereas the low-responder form (LR) does not (Anderson *et al.*, 1987). A monoclonal antibody, 41H16, has been shown to be specific for the HR form (Gosselin *et al.*, 1990).

Recently, cDNA clones coding for HR and LR forms of $Fc_\gamma RIIa$ have been isolated and sequenced (Clark *et al.*, 1989b; Warmerdam *et al.*, 1990). The alleles appear likely to differ in just two residues; residue 27 in domain 1 is Gln in HR and Trp in LR forms, and residue 131 in domain 2 is Arg in HR and His in LR forms. Domain swap experiments between the two forms indicate that Arg 131 is a critical requirement for the binding of mouse IgG_1 to the HR form (Warmerdam *et al.*, 1991). Arg 131 also appears to form part of the epitope recognized by 41H16. Further, these studies indicate that His at residue 131 in the LR receptor is essential for the binding of this form to human IgG_2.

Loss of the N-linked carbohydrate moieties from the $C_\gamma 2$ domain of human IgG_1 and IgG_3 molecules results in a marked reduction in their ability to interact with $Fc_\gamma RII$ (Walker *et al.*, 1989b). This effect is reminiscent of that seen with $Fc_\gamma RI$ and therefore suggests the importance of the $C_\gamma 2$ domain in the $Fc_\gamma RII$–IgG interaction. The localized perturbation on aglycosylation particularly in the vicinity of His 268, as assessed by the proton NMR methods mentioned earlier (Matsuda *et al.*, 1990), perhaps further points toward a similarity between $Fc_\gamma RI$ and $Fc_\gamma RII$ interaction sites. In order to assess this possibility, the earlier described panel of mutant IgG_3 molecules with point substitutions in residues 234 to 237 was utilized. The ability of these antibodies to form rosettes with the Daudi cell line, which expresses $Fc_\gamma RII$ only, was determined and compared to that of wild-type human IgG_3 (Lund *et al.*, 1991). Substitutions of Leu → Ala 234, Leu → Ala 235, Leu → Glu 235, Gly → Ala 236, and Gly → Ala 237 in each case reduced the number of rosettes formed with Daudi cells. The most marked effect was seen with Leu → Ala substitution at residue 234, where rosette formation was reduced to <10% of that of the wild-type

IgG_3. The fact that point substitutions in this lower hinge region obviate, at least to some degree, both $Fc_\gamma RI$ and $Fc_\gamma RII$ binding suggests that the receptors recognize overlapping interaction sites. However, the subtle differences seen in the rosetting profiles of the two receptors with the mutant IgG_3 panel indicate that the interaction sites do not appear to be strictly identical. Hence, the presence of Leu 234 would seem to be most critical for $Fc_\gamma RII$ recognition whereas that of Leu 235 appears to be more important for $Fc_\gamma RI$ binding.

Recently, as mentioned earlier, chimeric IgG/IgE molecules have been generated in which the $C_\gamma 2$ and $C_\gamma 3$ domains are interchanged either singly or together, with the $C_\epsilon 3$ and $C_\epsilon 4$ domains (Shopes *et al.*, 1990). The results obtained when these chimeras were assessed for ability to mediate rosette formation with the $Fc_\gamma RI^-$, $Fc_\gamma RII^+$ K562 cell line may at first appear to be in conflict with the above. Only molecules bearing both $C_\gamma 2$ and $C_\gamma 3$ domains were capable of mediating rosette formation, suggesting a requirement for both domains in the interaction. The $C_\gamma 3$ domain would thus appear to be contributing more than merely a stabilizing effect on the structure of the $C_\gamma 2$ domain because substitution with the homologous $C_\epsilon 4$ domains in the $\gamma/C_\epsilon 4$ chimera failed to produce a molecule capable of rosetting. However, the contribution of $C_\epsilon 4$ to the overall stability of the chimeric Fc may be less than that from the native $C_\gamma 3$. The reduced affinity of $\gamma/C_\epsilon 4$ for $Fc_\gamma RI$ would indeed be consistent with this idea. Hence, if the $\gamma/C_\epsilon 4$ chimera had even a slightly reduced affinity for $Fc_\gamma RII$ this may take it below the threshold necessary to mediate rosette formation in this purely qualitative assay. Therefore, contrary to first appearances, the results with these chimeric molecules may still be consistent with a critical role for the lower hinge region of the $C_\gamma 2$ domain in the $Fc_\gamma RII$–IgG interaction.

5. *$Fc_\gamma RIII$*

The third class of human Fc receptor for IgG, $Fc_\gamma RIII$ (CD16), is a glycoprotein present on neutrophils, macrophages, cultured monocytes, and NK cells. Recently, reactivity with anti-$Fc_\gamma RIII$ monoclonal antibodies (3G8, CLB Gran 1, and B73.1) has indicated the presence of this receptor on kidney mesangial cells and on placental trophoblasts (Sedmak *et al.*, 1990, 1991). $Fc_\gamma RIII$ has also been demonstrated on a small subpopulation of freshly isolated monocytes (Passlick *et al.*, 1989; Anderson *et al.*, 1990b). On sodium dodecyl sulfate (SDS) gels it appears as a broad band of 50–80 kDa.

In humans, $Fc_\gamma RIII$ is encoded by two genes, termed $Fc_\gamma RIII$-A and $Fc_\gamma RIII$-B, each giving rise to a single transcript (Ravetch and Perussia, 1989). Alternatively, the two genes have been termed $Fc_\gamma RIII$-1 and $Fc_\gamma RIII$-2, where III-1 is equivalent to III-B and III-2 is equivalent to III-A. III-B is expressed exclusively in neutrophils, wherein the receptor is anchored to the plasma membrane via a phosphatidylinositol–glycan linkage. III-A is expressed in NK cells, macrophages, and cultured monocytes and encodes a transmembrane molecule with a 25-amino acid cytoplasmic region (Huizinga *et al.*, 1988; Selvaraj *et al.*, 1988, 1989; Scallon *et al.*, 1989). Both forms of receptor possess two extracellular immunoglobulin-like domains (Fig. 15). The two genes are, in fact, nearly identical, with only nine nucleotide substitutions. These give rise to six amino acid differences in the two proteins, as well as the loss of 21 residues from the C-terminus of the III-B receptor. A single amino acid substitution lying at the site of phosphatidylinositol–glycan (PIG) linkage accounts for the alternative topological forms of $Fc_\gamma RIII$. Thus, Ser at position 203 in III-B results in a PIG-linked receptor whereas Phe 203 in III-A does not permit this mode of anchoring to occur. A transmembrane form of $Fc_\gamma RIII$ therefore results (Hibbs *et al.*, 1989; Kurosaki and Ravetch, 1989; Lanier *et al.*, 1989a).

The structural differences between $Fc_\gamma RIII$-A and III-B manifest important functional distinctions. For example, $Fc_\gamma RIII$-A receptors on NK cells and macrophages can mediate phagocytosis and ADCC, but reports suggest that $Fc_\gamma RIII$-B cannot (Fanger *et al.*, 1989; Selvaraj *et al.*, 1989; Anderson *et al.*, 1990a). $Fc_\gamma RIII$-B on neutrophils may therefore serve as a highly mobile "trap" for multivalent ligand, allowing efficient presentation to $Fc_\gamma RII$ with subsequent mediation of effector functions. However, recent reports suggest that $Fc_\gamma RIII$-B may, by as yet undefined means, independently initiate certain functions (Kimberly *et al.*, 1990). Multivalent ligation of $Fc_\gamma RIII$-B, for example, results in a $[Ca^{2+}]_i$-dependent increase in actin filament assembly, known to be a prerequisite of phagocytosis and secretion (Salmon *et al.*, 1991).

Two allelic forms of $Fc_\gamma RIII$, termed NA1 and NA2, exist on neutrophils. $Fc_\gamma RIII$ on NK cells and macrophages, however, is restricted to the NA2 allele (Tetteroo *et al.*, 1988; Huizinga *et al.*, 1989b; Salmon *et al.*, 1990; Kindt *et al.*, 1991). Comparison of nucleotide sequences coding for the allelic forms revealed minor differences, which result in the loss of glycosylation sites in NA1 (Ory *et al.*, 1989; Ravetch and Perussia, 1989).

$Fc_\gamma RIII$-A has been demonstrated to associate with a dimer of another small polypeptide, either the γ subunit of the high-affinity Fc receptor for IgE ($Fc_\epsilon RI$; see later) or the CD3ζ chain of the T

cell receptor (Lanier *et al.*, 1989b; Ra *et al.*,1989a). These smaller molecules form part of a family of related proteins that associate as disulfide-linked dimers (Orloff *et al.*, 1990). The interaction of Fc_γRIII-A with homo- or heterodimers of γ and ζ appears to be critical for optimal expression, as demonstrated by experiments in COS cells (Hibbs *et al.*, 1989; Kurosaki and Ravetch, 1989). The sites of interaction between Fc_γRIII-A and γ or ζ lie in their transmembrane regions (Farber and Sears, 1991; Lanier *et al.*, 1991). Recently, "activation" of Fc_γRIII in NK cells by interaction with complexed IgG has been shown to result in phosphorylation of the associated ζ chain. Hence, the ζ chain acts as a transducing element in mediation of responses of Fc_γRIII-A occupation by complexed IgG (O'Shea *et al.*, 1991; Vivier *et al.*, 1991).

6. *IgG–Fc_γRIII Interaction at the Molecular Level*

The affinity of Fc_γRIII-B on neutrophils for monomeric IgG is too low to be readily experimentally determined. Studies using IgG dimers of different human subclasses have indicated that Fc_γRIII-B binds IgG_1 and IgG_3 but not IgG_2 or IgG_4 (Huizinga *et al.*, 1989a). Estimates of affinity constants for human IgG_1 dimers range from $3.6 \times 10^6 M^{-1}$ (Kurlander and Batker, 1982) to $11 \times 10^7 M^{-1}$ (Huizinga *et al.*, 1989a). The former value may be lower due to the glutaraldehyde treatment used to cross-link IgG molecules in the experiment. The authors of the latter investigation highlight the possibility that their measurements may be a composite value, with contributions from both Fc_γRIII and Fc_γRII.

Recently, studies of Fc_γRIII-A on NK cells and macrophages suggest that this form of the receptor may have a higher affinity for human IgG than does Fc_γRIII-B (Van de Winkel and Anderson, 1991). The anti-Fc_γRIII monoclonal antibody, 3G8, can be displaced from Fc_γRIII-A by monomeric IgG, but not from Fc_γRIII-B (Anderson *et al.*, 1990b). Further, different glycoforms of Fc_γRIII-A may vary in their affinity for ligand. The high-mannose-containing Fc_γRIII-A on NK cells appears to have a higher affinity for IgG than does Fc_γRIII-A on monocytes because the latter receptor, which lacks high-mannose oligosaccharides, requires higher concentrations of IgG to displace 3G8 (Kimberly *et al.*, 1991). Studies on NK cells show a specificity profile for mouse IgG subclasses of $IgG_3 > IgG_{2a} > IgG_{2b} >> IgG_1$ (Kipps *et al.*, 1985; Anasetti *et al.*, 1987).

Fc_γRIII is able to interact with certain lectin molecules, probably via its high-mannose-type oligosaccharides. Thus, the phagocytosis of Con A-treated erythrocytes by neutrophils is inhibitable by aggregated IgG, 3G8, and monosaccharides such as D-mannose (Salmon *et al.*, 1987; Kimberly *et al.*, 1989). Thus, the oligosaccharide moieties recognized by Con A have been suggested to contribute to the integrity of the IgG-binding site on Fc_γRIII.

Turning to the interaction site on IgG, early experiments indicated that the integrity of the Fc region was required for binding to neutrophil Fc_γRIII. Hence, neither a pFc' fragment nor a tryptic $C_\gamma2$ domain fragment could inhibit the interaction (Barnett-Foster *et al.*, 1978). The importance of the $C_\gamma2/C_\gamma3$ interface was suggested by the reported ability of protein A, which binds in this region, to block the binding of IgG to the receptor (Barnett-Foster *et al.*, 1982). However, other workers found an anti-IgG monoclonal antibody, which recognizes an epitope in the $C_\gamma2/C_\gamma3$ interface region, unable to inhibit the binding of IgG to Fc_γRIII on monocyte-depleted peripheral blood mononuclear cells (Sarmay *et al.*, 1985). This same study, using a panel of anti-IgG monoclonal antibodies and the same effector cells, proposed that the Fc_γ receptor involved, assumed to be Fc_γRIII, binds to a region in the $C_\gamma3$ domain of IgG and that a second region in the $C_\gamma2$ domain is critical for triggering of ADCC via this receptor. More recently, aglycosylation of Fc of human IgG_3 has been reported to render it incapable of mediating ADCC via Fc_γRIII on K cells (T plus Null cells) (Lund *et al.*, 1990). Currently, however, our understanding of how IgG and Fc_γRIIII interact at the molecular level seems to require further clarification.

B. Fc_ϵ Receptors

Two classes of receptor specific for the Fc region of IgE, termed Fc_ϵRI and Fc_ϵRII, have been described and will be discussed here in turn.

1. *Fc_ϵRI*

Human Fc_ϵRI is found exclusively on the surface of mast cells and basophils, where it binds IgE with high affinity. Upon aggregation of Fc_ϵRI–IgE complexes by interaction with multivalent antigen the cell degranulates, releasing mediators of the allergic response (Metzger *et al.*, 1986; Metzger, 1988). Fc_ϵRI is a multichain glycoprotein consisting of one α subunit, one β subunit, and two γ subunits (Metzger *et al.*, 1983; Alcarez *et al.*, 1987) (Fig. 17). The α subunit, which contains the binding site for IgE, is an integral membrane protein with a single membrane-spanning region and two immunoglobulin-like extracellular domains. cDNA clones for the human α subunit have been isolated (Kochan *et al.*, 1988; Shimizu *et al.*, 1988). The predicted product

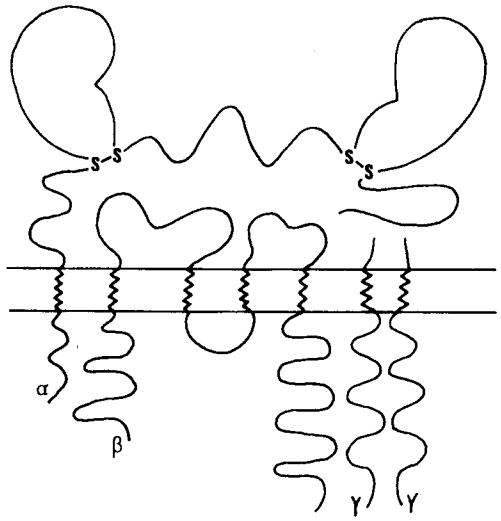


FIG. 17. Schematic representation of $Fc_\epsilon RI$. The receptor is a tetramer of one α subunit, one β subunit, and two γ subunits.

shares a considerable degree of homology to human $Fc_\gamma RIII$-A. Between species, the most highly conserved region is the transmembrane portion, with the cytoplasmic tail showing most divergence (Kinet, 1989). These observations may suggest that the α subunit intracellular region is not involved in a key function, whereas the membrane-spanning portion serves some specific role, perhaps involving interaction with the β or γ subunits (Kinet and Metzger, 1990). Indeed, recent results from experiments expressing wild-type and mutant forms of the rat $Fc_\epsilon RI$ in COS cells support these suggestions and will be discussed a little later.

No sequence data is currently available for the human β subunit. However, cDNA clones of both the rat and mouse β chains have been isolated (Kinet *et al.*, 1988; Ra *et al.*, 1989b). The predicted protein products share an extremely high degree of identity (83%), suggesting that the human counterpart may also do so. The predicted topology of the β subunit comprises four membrane-spanning regions with both N- and C-termini in the cytoplasm.

Both cDNA and genomic clones of the human γ subunit have been isolated (Küster *et al.*, 1990). The γ subunit appears to be well conserved, with 86% identity between the predicted polypeptides of human, mouse, and rat γ chains. This small third $Fc_\epsilon RI$ subunit spans the membrane once to give a short N-terminal extracellular region of just 5 amino acids and a longer cytoplasmic tail (42 amino acids) (Küster *et al.*, 1990). As mentioned earlier, the γ subunit displays homology to the ζ chain of CD3 and both are members of a family of proteins that associate as disulfide-linked dimers (Orloff *et al.*, 1990). Recently it has been shown that human CD3ζ can substitute for the γ subunit in assembly and functional expression of rat Fc_ϵ RI in a *Xenopus* oocyte expression system (Howard *et al.*, 1990). Similarly, both γ and ζ dimers may associate with human $Fc_\gamma RIII$-A (see earlier).

A series of experiments in which component subunits of the receptor were cotransfected into COS cells to reconstitute rodent, human, or chimeric $Fc_\epsilon RI$ molecules have yielded interesting information on requirements for efficient cell surface expression. In both the rat and mouse systems, cotransfection of the α, β, and γ subunits are necessary for expression of $Fc_\epsilon RI$ at the cell surface (Blank *et al.*, 1989; Ra *et al.*, 1989b). However, cotransfection of human α and γ subunits is sufficient to give efficient expression of a molecule capable of binding IgE (Küster *et al.*, 1990). Addition of rodent β chain did not increase expression efficiency. Chimeric receptors of human α subunit plus rat β and γ of either rat or mouse have also been expressed and shown to bind IgE with affinity comparable to that of $Fc_\epsilon RI$ on normal cells (Miller *et al.*, 1989; Ra *et al.*, 1989b).

Site-specific mutagenesis of the α, β, and γ subunits of a rat $Fc_\epsilon RI$ expressed in COS cells revealed that removal of cytoplasmic tails from any or all of the subunits had little effect on surface expression. However, even minor changes within the transmembrane regions led to reduced expression (Varin-Blank and Metzger, 1990). In the rat receptor, therefore, the membrane-spanning regions of the subunits appear critical for optimal expression. By contrast, cotransfection of human α subunit and a truncated rat γ subunit lacking a cytoplasmic domain resulted in no expression of human α, suggesting that the human and rodent receptors may assemble differently (Varin-Blank and Metzger, 1990). Models to describe the molecular interaction between transmembrane segments of the subunits are now emerging (Varin-Blank and Metzger, 1990; Farber and Sears, 1991).

2. *$Fc_\epsilon RI$–IgE Interaction at the Molecular Level*

Human $Fc_\epsilon RI$ binds human IgE with high affinity ($K_a \sim 1 \times 10^{10}$ M^{-1}) and interacts somewhat more weakly with rat and mouse IgE (Conrad *et al.*, 1983). A chimeric α subunit consisting of the extracellular portion of the human α subunit fused to the transmembrane and

cytoplasmic domains of the p55 IL-2 receptor has recently been shown to bind IgE with high affinity. Thus, the interaction site on the receptor appears to lie in the extracellular portion of the α subunit, with no apparent contribution from the other subunits (Hakimi *et al.*, 1990). Using the same chimeric receptor, it has been further demonstrated that monoclonal antibodies recognizing epitopes in the second domain of the α subunit (adjacent to the cell membrane) can inhibit the interaction with IgE (Riske *et al.*, 1991). This may suggest that IgE also binds directly to the second α-subunit domain. Alternatively, the inhibiting antibodies may exert their effect by steric hindrance of a site distal to their point of recognition or by induction of conformational changes in the IgE-binding region. The carbohydrate moieties of the α subunit appear not to be involved in the IgE interaction because rat $Fc_\epsilon RI$ on basophilic leukemia cells cultured in the presence of tunicamycin, an inhibitor of N-linked glycosylation, are still able to bind IgE (Hempstead *et al.*, 1981).

Turning to the site of interaction on IgE, an excellent review of the various studies attempting to localize the binding site appeared some 3 years ago (Metzger, 1988). Here, therefore, we will concentrate on advances made since then. The binding site for $Fc_\epsilon RI$ is known to lie in the Fc portion of IgE, which is composed of the paired $C_\epsilon 2$, $C_\epsilon 3$, and the $C_\epsilon 4$ domains (Fig. 8) (Ishizaka and Ishizaka, 1975). The importance of the $C_\epsilon 2/C_\epsilon 3$ interface was earlier suggested by the finding that interaction of rat IgE with rat $Fc_\epsilon RI$ protected that region of the antibody, in particular from tryptic proteolysis (Perez-Montfort and Metzger, 1982). Further mapping of the human $Fc_\epsilon RI$ site has made use of recombinant peptides of human IgE expressed in *Escherichia coli*. The lack of glycosylation of Fc_ϵ fragments generated in this manner does not impede their ability to interact with $Fc_\epsilon RI$ (Ishizaka *et al.*, 1986). This is consistent with an earlier report that nonglycosylated intact rat IgE still bound to rat $Fc_\epsilon RI$ (Kulczycki and Vallina, 1981). A monomeric recombinant peptide of 76 amino acids, comprising residues 301–376 spanning the $C_\epsilon 2/C_\epsilon 3$ interface, was reported to interact with human $Fc_\epsilon RI$ with an affinity similar to that of intact IgE (Helm *et al.*, 1988). Use of a series of peptides subsequently demonstrated that residues 363–376 in the above peptide are not essential for $Fc_\epsilon RI$ binding (Helm *et al.*, 1989). Further, an epitope lying within this peptide recognized by an anti-IgE monoclonal antibody demonstrates a sensitivity to heat and alkylation similar to that displayed by IgE when interacting with $Fc_\epsilon RI$ (Del Prado *et al.*, 1991).

A recent report suggests that an octapeptide lying at the $C_\epsilon 3/C_\epsilon 4$ interface within the peptide above (residues 345–352) is capable of

inhibiting histamine release by human basophils (Nio *et al.*, 1990). However, this result should perhaps be questioned, as the octapeptide only appears to inhibit at molar concentrations several orders of magnitude greater than those required with intact human myeloma IgE. A series of resonance energy transfer studies provide further evidence for the possible involvement of the $C_\epsilon 2/C_\epsilon 3$ interface region in $Fc_\epsilon RI$ binding (Holowka and Baird, 1983; Holowka *et al.*, 1985; Zheng *et al.*, 1991). Distances between fluorescent donor probes, placed at specific sites on IgE and anti-IgE antibodies, and acceptor probes at the cell membrane surface were determined. These measurements indicate IgE bound to $Fc_\epsilon RI$ has a bent conformation, with the $C_\epsilon 2$ and $C_\epsilon 3$ domains lying closest to the cell membrane and therefore presumably to the receptor (see later).

A number of recent studies have utilized a panel of chimeric IgE molecules to assess the relative contribution of each domain. One group found that mutant mouse IgE molecules lacking either 45 amino acids from the carboxy end of $C_\epsilon 3$ or almost the entire $C_\epsilon 4$ domain no longer bound to rat $Fc_\epsilon RI$ (Schwarzbaum *et al.*, 1989). Further, the sites of recognition of two anti-IgE antibodies earlier shown to inhibit the IgE–$Fc_\epsilon RI$ interaction (Baniyash and Eshhar, 1984; Baniyash *et al.*, 1988) were localized to the $C_\epsilon 3$ domain. A third anti-IgE antibody, known not to inhibit receptor binding, was shown to bind the $C_\epsilon 4$ domain. These observations were interpreted as indicating that the $C_\epsilon 3$ domain plays a key role in $Fc_\epsilon RI$ recognition, whereas the $C_\epsilon 4$ domain, although not directly involved in the interaction, serves to stabilize the conformation of Fc_ϵ necessary for $Fc_\epsilon RI$ binding.

A further study made use of the ability of human IgE to bind to human $Fc_\epsilon RI$ but not to rat $Fc_\epsilon RI$, in contrast to the reactivity of mouse IgE with both receptors (Nissim *et al.*, 1991). Chimeric human/mouse IgE molecules, in which single or multiple mouse domains substituted for human ones, were assessed for binding to either rat $Fc_\epsilon RI$ or a reconstituted human $Fc_\epsilon RI$ expressed in COS cells. When the $C_\epsilon 2$ domains of human IgE were replaced by those of mouse IgE, the resultant molecule bound human but not rat $Fc_\epsilon RI$. In contrast, a chimeric human IgE containing mouse $C_\epsilon 3$ domains (CHM3) bound both receptors. Furthermore, deletion of $C_\epsilon 2$ from CHM3 produced no impairment of binding to rat $Fc_\epsilon RI$. Again, these results suggest that the $C_\epsilon 3$ domain is the principal region involved in interaction with $Fc_\epsilon RI$ (Nissim *et al.*, 1991).

A second group of investigators has generated chimeric mouse IgE in which one or more IgE domains are substituted by homologous regions from human IgG_1 (Weetall *et al.*, 1990). An IgE molecule in

which the $C_\epsilon4$ domains were replaced by IgG $C_\gamma3$ domains bound rat $Fc_\epsilon RI$ with affinity comparable to that of wild-type mouse IgE. All other chimeric molecules tested did not contain both $C_\epsilon2$ and $C_\epsilon3$ and were unable to bind rat $Fc_\epsilon RI$. The favored interpretation was that both $C_\epsilon2$ and $C_\epsilon3$ domains are necessary for the binding interaction (Weetall *et al.*, 1990). However, the observations mentioned earlier (Nissim *et al.*, 1991) suggest that the role of $C_\epsilon2$ here may be in stabilizing the conformation of $C_\epsilon3$ necessary for optimal $Fc_\epsilon RI$ interaction. A substituted human IgG_1 hinge may not be able to perform this role adequately. This possibility may explain the inability of a chimeric human IgG molecule, in which mouse $C_\epsilon3$ domains substitute for $C_\gamma2$ domains, to interact with rat $Fc_\epsilon RI$ (Weetall *et al.*, 1990). The current weight of evidence, therefore, would seem to support the idea that the recognition site for $Fc_\epsilon RI$ lies (1) within the region encoded by the $C_\epsilon3$ exon and (2) within the peptide Gln 301–Leu 363. The region of Fc_ϵ fulfilling both these criteria lies between Asp 330 and Leu 363. Of particular pertinence is the emergence of a new molecular model for the Fc region of IgE (Helm *et al.*, 1991). This revised model incorporates the finding that the inter-$C_\epsilon2$ disulfide bonds involving Cys 238 and Cys 241 are parallel, rather than crossed as in the earlier models (Padlan and Davies, 1986; Pumphrey, 1986). The most pronounced consequence of modeling parallel disulfide bridges is the appearance of an exposed segment of approximately 2.4 nm in length, comprising residues 329–335 lying between $C_\epsilon2$ and $C_\epsilon3$ (Fig. 3). This region may constitute the structural equivalent of the lower hinge region in IgG and hence it is tempting to speculate that the $Fc_\epsilon RI$ interaction site may lie here. Should further mutagenesis experiments verify this possibility, a common theme of Ig-like Fc receptor domains interacting with a flexible lower hinge region (or its equivalent) in immunoglobulins may emerge.

3. *$Fc_\epsilon RII$*

The second class of receptor for the Fc region of IgE ($Fc_\epsilon RII$ or CD23) has lower affinity for its ligand than does $Fc_\epsilon RI$ and hence is sometimes referred to as the low-affinity receptor. $Fc_\epsilon RII$ is present on inflammatory cells, including monocytes, eosinophils, and platelets, and on B lymphocytes. cDNA clones coding for human $Fc_\epsilon RII$ have been isolated and shown to bind IgE when expressed in mammalian cell systems (Kikutani *et al.*, 1986; Ikuta *et al.*, 1987; Lüdin *et al.*, 1987). Unlike all other leukocyte Fc receptors described here, $Fc_\epsilon RII$ is not related to the immunoglobulin gene family of proteins. Rather it displays homology to a family of animal lectins, which includes the human and rat asialoglycoprotein receptors.

On SDS gels human $Fc_\epsilon RII$ has a molecular weight of about 43,000 and is composed of a 321-amino acid polypeptide core of ~36,000 molecular weight and both O- and N-linked oligosaccharides (Delespesse *et al.*, 1989). The receptor spans the membrane once in a rather unusual orientation, because the short 23-amino acid N-terminal domain lies inside the cell and the much longer C-terminal region is found to the exterior. The domain exhibiting homology to animal lectins spans about 120 residues of the extracellular portion and includes three cysteine pairs. Close to the C-terminus lies an Arg-Gly-Asp (RGD) sequence in reverse, i.e., DGR (Kikutani *et al.*, 1986). A number of molecules that bind to the integrin family of receptors contain this RGD motif, suggesting that $Fc\epsilon RII$ may be able to interact with adhesion molecules (Gordon *et al.*, 1989).

More recently, a second species of human $Fc_\epsilon RII$, termed $Fc_\epsilon RIIb$, has been identified and differs from the earlier described receptor ($Fc_\epsilon RIIa$) only in the first few amino acids at the N-terminus (Yokota *et al.*, 1988). mRNA for $Fc_\epsilon RIIa$ is constitutively expressed in B cells alone. In contrast, $Fc_\epsilon RIIb$ is expressed in monocytes, eosinophils, and B cells only after stimulation with IL-4 (Yokota *et al.*, 1988). Functionally, $Fc_\epsilon RIIa$ appears to be involved in the regulation of B cell development (Gordon *et al.*, 1989), whereas $Fc_\epsilon RIIb$ plays a role in IgE-dependent cytotoxity against parasites such as schistosomes (Capron and Dessaint, 1985).

4. *$Fc_\epsilon RII$–IgE Interaction at the Molecular Level*

Monomeric human IgE binds to $Fc_\epsilon RIIb$ with a K_a of about $3 \times 10^7\ M^{-1}$ (Anderson and Spiegelberg, 1981; Joseph *et al.*, 1986). Dimeric IgE may have a slightly higher affinity for the receptor than monomers, at least in the rat system (Finbloom and Metzger, 1982). The natural occurrence of soluble proteolytic cleavage products of $Fc_\epsilon RII$, termed $sFc_\epsilon RII$, soluble CD23, or IgE-BF, which are capable of binding IgE, indicates that the site of interaction lies in the C-terminal part of the extracellular portion (Letellier *et al.*, 1989). Indeed, expression of recombinant soluble $Fc_\epsilon RII$ has localized the binding site within a 172-amino acid stretch at the C-terminus (Uchibayashi *et al.*, 1989). This C-terminal portion also includes the region of homology with animal lectins. Despite the implication that $Fc_\epsilon RII$ may therefore interact with the oligosaccharide chains of IgE, this is not the case. Certain recombinant human ϵ-chain fragments synthesized in *E. coli* and therefore devoid of carbohydrate are still able to bind to human $Fc_\epsilon RII$ (Vercelli *et al.*, 1989). Further, enzymatically deglycosylated myeloma IgE interacts with the receptor. In fact, it appears to bind slightly more tightly to the receptor than does the

parent IgE. Moreover, high concentrations of mono- and disaccharides do not inhibit the interaction between mouse IgE and $Fc_\epsilon RII$. The interaction does, however, share a dependence on calcium and pH with other lectin proteins (Richards and Katz, 1990).

Use of recombinant Fc_ϵ fragments mentioned above has allowed further localization of the $Fc_\epsilon RII$ interaction site on IgE (Vercelli *et al.*, 1989). A fragment (rE2–4) corresponding to the whole of the Fc_ϵ (paired $C_\epsilon 2$, $C_\epsilon 3$, and $C_\epsilon 4$ domains) bound to $Fc_\epsilon RII$ on a human B cell line with comparable affinity to intact IgE. A shorter fragment, comprising paired $C_\epsilon 3$ and $C_\epsilon 4$ domains, also bound but with much lower affinity. An intermediate peptide (rE2′–4) with the 30 C-terminal residues of $C_\epsilon 2$ plus $C_\epsilon 3$ and $C_\epsilon 4$ was almost as active as the whole Fc_ϵ. In contrast, removal of $C_\epsilon 4$ (leaving $C_\epsilon 2$ plus $C_\epsilon 3$) generated an inactive peptide. Thus, receptor recognition appears to require the presence of all three domains. The $C_\epsilon 4$ domain, however, may be substituted for by the $C_\gamma 3$ domain of mouse IgG_{2b} without considerable loss of reactivity with $Fc_\epsilon RII$. The indirect role of the $C_\epsilon 4$ domain would thus appear to be to promote the dimerization of the two ϵ chains, necessary for receptor binding. This is borne out by the demonstration that replacement of Phe 506, lying at the interface between the paired $C_\epsilon 4$ domains, with Arg generates a monomeric form of the Fc_ϵ chain that is unable to bind to $Fc_\epsilon RII$ (Vercelli *et al.*, 1989). Unlike $Fc_\epsilon RI$, binding of $Fc_\epsilon RII$ therefore has a clear requirement for both heavy chains in Fc_ϵ. One possibility is that two $Fc_\epsilon RII$ molecules may interact simultaneously with one IgE molecule. The observation that dimers of mouse $Fc_\epsilon RII$ may preexist adds weight to this idea (Peterson and Conrad, 1985).

Anti-IgE monoclonal antibodies against epitopes lying between residues 307–315 in $C_\epsilon 2$ and residues 367–370 in $C_\epsilon 3$ exhibit marked abilities to inhibit the IgE–$Fc_\epsilon RII$ interaction (Chrétien *et al.*, 1988). These two regions may lie close to one another in three-dimensional space (Fig. 3). An attractive hypothesis, incorporating the findings with recombinant peptides and monoclonal antibodies alike, is that the $Fc_\epsilon RII$ site may lie close to residues 367–370 in the $C_\epsilon 3$ domain (Vercelli *et al.*, 1989). The anti-$C_\epsilon 2$ antibodies might then exert their effect by steric hindrance.

C. Fc_α Receptors

The presence of receptors for IgA has been reported on human monocytes, macrophages, and neutrophils (Fanger *et al.*, 1980; Maliszewski *et al.*, 1985; Chevailler *et al.*, 1989), T cells (Briere *et al.*, 1988; Millet *et al.*, 1988), B cells (Gupta *et al.*, 1979; Millet *et al.*, 1989),

eosinophils (Abu-Ghazaleh *et al.*, 1989), and NK cells (Kimata and Saxon, 1988). Purification of the receptor from neutrophils and monocytes has revealed a heavily glycosylated protein of about 60 kDa (Albrechtsen *et al.*, 1988; Monteiro *et al.*, 1990).

Fc_α receptors on monocytes and neutrophils are capable of mediating phagocytosis of IgA-coated target cells (Fanger *et al.*, 1983; Gorter *et al.*, 1987; Yeaman and Kerr, 1987). They may also serve to promote ADCC by synergism with Fc_γ receptors (Shen and Fanger, 1981). Interaction of IgA, aggregated either artificially or at a cell surface, with monocyte and polymorphonuclear (PMN) Fc_α receptors can trigger both the release of inflammatory mediators such as leukotrienes and prostaglandins and the generation of superoxide (Ferreri *et al.*, 1986; Gorter *et al.*, 1987; Stewart and Kerr, 1990; Padeh *et al.*, 1991). Cross-linking of Fc_α receptors in this way may also result in neutrophil degranulation (Albrechtsen *et al.*, 1988).

Recently, a cDNA clone coding for a human Fc_α receptor has been isolated from a monocyte-like cell line cDNA library (Maliszewski *et al.*, 1990). COS cells transfected with the cDNA clone readily bind IgA-coated erythrocytes. The deduced amino acid sequence indicates an integral membrane protein with a peptide core of about 30 kDa. The remainder of the mass is contributed by carbohydrate moieties attached at up to six potential extracellular N-glycosylation sites and perhaps further O-glycosylation sites. The N-terminal 206 amino acids, lying outside the cell, comprise two immunoglobulin-like domains that display homology to the extracellular regions of $Fc_\gamma RI$, $Fc_\gamma RII$, $Fc_\gamma RIII$, and the α-subunit of $Fc_\epsilon RI$. A single transmembrane segment of 19 hydrophobic amino acids is then followed by a C-terminal cytoplasmic domain of 41 amino acids. Northern blot analysis revealed that mRNA coding for the Fc_α receptor was present in peripheral blood monocytes and neutrophils. No message was detected in tonsillar B or T cells, suggesting either that the IgA receptor reported in these cell types is structurally distinct, or that receptor expression was not induced under the particular conditions used.

$Fc_\alpha R$–IgA Interaction at the Molecular Level

The human monocyte/PMN Fc_α receptor appears to bind human serum IgA_1 and IgA_2 with similar affinity (Chevailler *et al.*, 1989; Stewart and Kerr, 1990). Estimates using solubilized receptor indicate that both subclasses give half-maximal inhibition of the receptor–IgA interaction at concentrations of 4.8×10^{-7} M, suggesting that the affinity constant lies around 5×10^7 M^{-1} (Mazengera and Kerr, 1990). Human secretory IgA of both subclasses also exhibits very similar

inhibitory abilities. Thus, the receptor interacts equally well with monomeric serum IgA and the dimeric IgA, in complex with J chain and secretory component, of secretory IgA. The site of interaction lies entirely in the Fc region of IgA, because Fc_α can inhibit the binding of polymeric IgA to the receptor, as shown in an indirect immunofluorescence assay (Monteiro *et al.*, 1990). Further, the rebinding of purified receptor to IgA–Sepharose beads is inhibited by Fc_α (Mazengera and Kerr, 1990). The above results with secretory IgA therefore suggest that the presence of either J chain or secretory component does not impede interaction of $Fc_\alpha R$ with the Fc region of secretory IgA.

A pepsin digestion product of IgA, lacking the $C_\alpha 3$ domains, retains a somewhat reduced ability to interact with the neutrophil Fc_α receptor (Mazengera and Kerr, 1990). This may suggest that the presence of both the $C_\alpha 2$ and $C_\alpha 3$ domains is necessary for full reactivity with the receptor. It is possible, however, that the $C_\alpha 3$ domain serves the indirect role of maintaining the conformation of $C_\alpha 2$ as we have seen with homologous Ig domains in the $Fc_\gamma RI$–IgG and $Fc_\epsilon RI$–IgE interactions earlier. Site-directed mutagenesis experiments on human IgA should, in the future, help to elucidate the precise molecular requirements for binding to Fc_α receptors (Woof *et al.*, 1992).

D. Fc_μ Receptors

Subpopulations of human and mice B and T lymphocytes are reported to express functional Fc_μ receptors (Moretta *et al.*, 1975; Ferranini *et al.*, 1977; Mathur *et al.*, 1988a,b). Biochemical analysis has revealed an IgM-binding protein of about 60 kDa on activated human B cells (Sanders *et al.*, 1987). This molecule was not, however, detected on T cells, monocytes, or granulocytes. More recently, binding inhibition experiments have confirmed that this protein binds to the Fc portion of IgM and hence is a true Fc_μ receptor (Ohno *et al.*, 1990). It appears to be expressed throughout the various stages of B cell differentiation and, in this respect, differs from the Fc_μ receptor on murine B cells (Mathur *et al.*, 1988b). The human receptor is anchored to the membrane via a phosphatidylinositol–glycan linkage and possesses O-linked but not N-linked oligosaccharides.

Human B cell Fc_μ receptor interacts with human and mouse IgM and, as mentioned previously, $Fc_{5\mu}$ fragments generated by hot trypsin digestion (Ohno *et al.*, 1990). As the major trypsin cleavage site lies in the $C_\mu 2$ domain, the $Fc_{5\mu}$ fragments consist primarily of paired $C_\mu 3$ and $C_\mu 4$ domains. The same report details experiments using mouse IgM domain deletion mutants, which help to further localize the $Fc_\mu R$ binding site on IgM. Loss of the $C_\mu 1$ domain did not impair binding to the receptor. Deletion of both domains $C_\mu 1$ and $C_\mu 2$ resulted in reduced but still significant binding. However, a mutant lacking domains $C_\mu 1$, $C_\mu 2$, and $C_\mu 3$ and another lacking $C_\mu 4$ no longer bound $Fc_\mu R$. Hence, $Fc_\mu R$ seems to require the presence of both $C_\mu 3$ and $C_\mu 4$ domains for recognition. Further study will be necessary to determine whether each domain contributes directly or indirectly to binding. It is perhaps of interest to note that the $C_\mu 3$ domain of mouse IgM appears to play the major role in binding to $Fc_\mu R$ on murine T and B cells (Mathur *et al.*, 1988a,b).

E. Fc_δ Receptors

Receptors for human IgD have been detected on human B and T cells (Sjöberg, 1980; Rudders and Andersen, 1982; Tamma and Coico, 1991). The receptors on B cells, at least, interact with the Fc portion of human IgD. No significant degree of information on the molecular basis of the interaction, in the human system, is currently available.

F. Effector Cell-Target Cell Interaction Mediated by Leukocyte Fc Receptors

We will now attempt to consider the interaction of antibodies and Fc receptors in a more physiological situation. First, we will discuss how antibody array formation at cell surfaces may facilitate interaction with Fc receptors. Second, we will consider the multiple factors that influence the "linkage" of effector cell and target cell by antibody molecules.

The possibility of antibody array formation on target cell surfaces would appear an attractive hypothesis. Earlier, we mentioned the potential interaction of arrays of antibody molecules, possibly stabilized by Fc–Fc interactions, with C1q. Arrays of dislocated IgG molecules, for example, might also be expected to interact advantageously with Fc receptors for two main reasons. First, multiple Fc receptors could bind simultaneously to an array of antibody molecules. The receptors would thus be effectively cross-linked, constituting a trigger for subsequent effector function. Second, dislocation of IgG molecules would serve to maximize access to the $Fc_\gamma R$ interaction sites(s) lying in the lower hinge region by rotation of Fc regions perpendicular to Fab arms (Burton, 1986). Similar arguments may also apply to IgE. Indeed, experimental evidence suggests that IgE bound to $Fc_\epsilon RI$ has a bent conformation in which the $C_\epsilon 2/C_\epsilon 3$ interface, interacting with the receptor, lies about 45 Å away from the cell surface. The remainder of the Fc lies about 55 Å away from the membrane whereas the tips of the Fab arms extend some 100 Å away from the cell

surface (Holowka and Baird, 1983; Holowka *et al.*, 1985; Zheng *et al.*, 1991). Further experimental evidence for antibody arrays was discussed earlier.

We will now turn to the factors influencing recognition of antibody-coated target cells by FcR-bearing effector cells. Because many FcR^+ cells express more than one class of receptor, and an opsonized target cell may be coated with multiple antibody isotypes, the *in vivo* situation is presumably rather complex. It seems likely, for example, that different types of Fc receptor may act synergistically to trigger effector mechanisms (Crockett-Torabi and Fantone, 1990; Kimberly *et al.*, 1990; Koolwijk *et al.*, 1991). In order to simplify matters here we will discuss a model system involving the interaction of erythrocyte targets, coated with a single antibody isotype, with Fc_γ receptor-bearing effector cells. This simplification allows assessment of the cell–cell interaction by rosette formation, the microscopically visible binding of several erythrocytes to an effector cell.

The interaction of the opsonized target with the effector cell in the rosette, in energetic terms, can be seen as the result of two opposing contributions, described by the following equation (Walker *et al.*, 1989b):

$$\Delta G(\text{rosette formation}) = \Delta G(\text{Ab–FcR})\text{interaction} + \Delta G(\text{nonspecific cell–cell interaction})$$

The first term [ΔG(Ab–FcR)interaction], promoting rosette formation, is associated with the free energy of occupation of Fc receptors by antibody molecules. The second opposing term [ΔG(nonspecific cell–cell interaction)], militating against rosette formation, is associated with the repulsive forces of bringing together two cell surfaces close enough to allow bridging by antibody molecules. Hence, a sufficient input of free energy from antibody–receptor interactions to overcome cell–cell repulsion is necessary to allow rosette formation (see experimental examples in Fig. 18). These two terms appear to depend on a number of factors, each of which may influence rosette formation. Thus, the rosette-promoting term is governed by both the antibody isotype and the Fc receptor involved and by the number of antibody–receptor interactions. The opposing term depends on the net surface charge of the two cells and the geometries of the antigen epitope and the Fc receptor. Both antigen and receptor must be "accessible" and suitably orientated to give optimal interaction with the antibody molecule. Finally, the relative structure of the antibody is important. For example, human IgG_3 molecules, with their extended hinge regions,

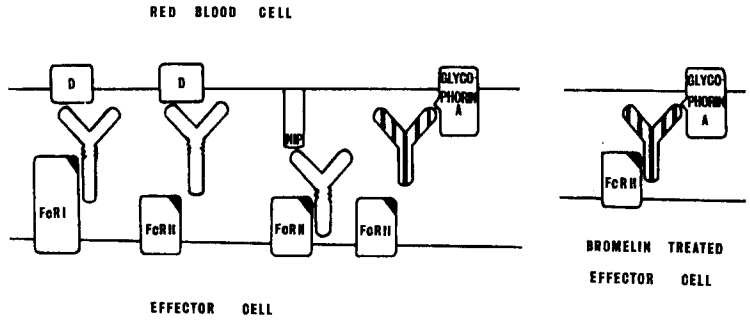


FIG. 18. Schematic representation of effector cell–target cell interactions. Human IgG_3 antirhesus D or anti-NIP antibodies (open Y shapes) and mouse IgG_1 antiglycophorin A antibodies (striped Y shapes) interact with their respective antigens on the surface of a human rhesus D^+ erythrocyte. Under favorable conditions, these antibodies may also interact simultaneously with the IgG-binding sites (dark areas) on Fc_γ receptors on a neighboring effector cell. The antibodies then serve to "bridge" between the two cell types, a situation that, under the microscope, would appear as a rosette, with several erythrocytes bound per effector cell. Five different combinations of antigen, antibody, and Fc_γ receptor are shown here to illustrate the theoretical considerations discussed in the text. In the first case, on the left, a human IgG_3 anti-D antibody bridges between $Fc_\gamma RI$ and the somewhat buried D antigen in the erythrocyte membrane. Thus, this illustrates the fact that an effector cell bearing $Fc_\gamma RI$ (e.g., U937) can form rosettes with IgG_3 anti-D-sensitized erythrocytes. In the second case, however, human IgG_3 is unable to bridge between the relatively inaccessible D antigen and $Fc_\gamma RII$. This represents the finding that an effector cell bearing $Fc_\gamma RII$ alone (e.g., Daudi) cannot form rosettes with IgG_3 anti-D-sensitized erythrocytes. In the third combination, the antigen, NIP, is at a more accessible location on the red blood cell surface. This allows the IgG_3 anti-NIP antibody to bind both to NIP and to $Fc_\gamma RII$. Hence, Daudi cells are able to form rosettes with NIP-derivatized erythrocytes coated with IgG_3 anti-NIP antibodies. In the fourth example, as in the second, the antibody, in this case a mouse IgG_1 molecule, is unable to bridge between the antigenic epitope on the red cell and $Fc_\gamma RII$ on the effector cell. Hence, no rosettes are seen with this combination. However, when the $Fc_\gamma RII^+$ effector cell is pretreated with the enzyme bromelin, as in the fifth example, this same combination of antigen, antibody, and Fc receptor may simultaneously interact. Bromelin treatment results in cleavage of surface glycoproteins, thereby reducing the net surface charge of the cell. The two cell surfaces may therefore approach each other more closely as mutual repulsion is reduced. Thus, Daudi cells, after bromelin treatment, become able to form rosettes with erythrocytes coated with the mouse IgG_1 antiglycophorin A antibody.

appear to mediate rosette formation more readily than IgG_1 molecules do, when all other parameters are the same (Walker *et al.*, 1988, 1989b). However, this interaction process is merely the first stage of target cell destruction and some evidence suggests that optimal interaction may not always lead to optimal destruction. Hence, although human IgG_3

molecules more readily promote target cell–effector cell interaction than do human IgG_1 molecules, it is the latter antibody that mediates most effective ADCC against target cells once interaction has occurred (Brüggemann *et al.*, 1987).

In terms of the "design" of antibodies for therapy, a consideration of the multiple parameters influencing interaction of target cell and effector cell would seem pertinent. Thus, in order to generate a monoclonal antibody effective at Fc receptor-mediated target–cell killing, we must take into account not only the more obvious factors of antibody isotype, class of Fc receptor, and type of effector cell(s) involved, but also antigen density, epitope accessibility, and surface charge on the target cell. We must ensure that we have an antibody capable of both specifically recognizing a target cell, and of "bridging" efficiently between that cell and an effector cell, prior to triggering target cell destruction.

V. Catabolism of Antibodies

Although not an effector function, the survival of antibodies in blood has important consequences in understanding the biochemistry of these molecules and for their potential therapeutic and diagnostic uses. The subject has recently been reviewed by Zuckier *et al.* (1989) and we shall concentrate on points relating to molecular aspects.

In humans, antibodies of the IgG class have the longest half-lives of any of the serum proteins (average $t_{1/2} = 21$ days), with the other classes having shorter half-lives: IgA, 6 days; IgM, 5 days; IgD and IgE, 3 days. IgG_3 demonstrates a significantly shorter $t_{1/2}$ (7days) than the other IgG subclasses whereas the two IgA subclasses have similar $t_{1/2}$. The unique feature of IgG catabolism in humans and in other species studied is that the catabolic rate is proportional to the serum concentration. Thus serum IgG is degraded much more rapidly in hypergammaglobulinemic individuals and much more slowly in hypogammaglobulinemics. This has led Brambell *et al.* (1964) to propose that there are a limited number of receptors that complex IgG and protect it from degradation in blood. When levels of IgG are high, the receptors will be saturated, making more IgG available for degradation. Conversely, when levels are low, most of the IgG will be protected, prolonging serum survival. There is no direct evidence for this mechanism but it has been considered the best available (Waldmann and Strober, 1969).

An alternative occurring to us is one based on an equilibrium between monomeric and associated IgG species. Higher serum concentrations of IgG would favor aggregate formation that might be catabolized more rapidly than monomer. Such an aggregated population might form only a small proportion of the total IgG, and therefore be difficult to observe, but be catabolized very rapidly. The site(s) regulating the catabolism of IgG (whatever the molecular mechanism involved) is believed to be in the Fc part of the molecule because Fc fragments are catabolized at the slow rates characteristic of IgG, in contrast to Fab fragments, which are rapidly cleared (Spiegelberg and Weigle, 1965a,b; Wochner *et al.*, 1967; Zuckier *et al.*, 1989). Furthermore, infusion of Fc fragments can mimic IgG in accelerating the catabolism of circulating IgG in mice (Fahey and Robinson, 1963). Interestingly, the Fc fragments of all four human IgG subclasses appear to have identical fractional catabolic rates, implying that structures outside the Fc region are responsible for the accelerated catabolism of IgG_3 (Spiegelberg and Fishkin, 1972).

A number of studies have sought to localize the site more precisely. Yasmeen *et al.* (1976) reported that a $C_\gamma2$ fragment from a human IgG_1 protein was cleared from rabbit circulation with a $t_{1/2}$ similar to intact IgG and Fc and much longer than that for Fab, pFc′, or $C_\gamma3$ fragments. Arend and Webster (1977) reported that rat pFc′ was rapidly catabolized in rats compared to Fc. These studies imply a crucial role for the $C_\gamma2$ domain. In contrast, Pollock *et al.* (1990), using mutant mouse IgG molecules, have obtained evidence that deletion of any of the constant domains has an effect on clearance in the mouse. Further, using $IgG_{2b/2a}$ hybrid molecules, they suggest that sequences at the C-terminal end of $C_\gamma2$ or within the $C_\gamma3$ domain, or conformations controlled by these sequences, are important in catabolism.

Recently Wawrzyncak *et al.* (1992a) looked at the rates of clearance, from the circulation of mice, of mutant mouse IgG_{2b}s used in the C1q-binding and Fc receptor studies described earlier. They found no significant differences between $t_{1/2}$ values for mutant and wild-type IgGs, implying that clearance is independent of the ability to bind C1q or mouse FcRI (mutation of Glu to Leu at position 235 was shown to generate IgG_{2b} binding to mouse as well as human FcRI).

Though there has been debate about the role of carbohydrate, the present consensus seems to imply that the carbohydrate moieties have only a limited effect on serum half-life (Waldmann and Strober, 1969; Tao and Morrison, 1989; Zuckier *et al.* 1989; Wawrzynak *et al.* 1992b). However, the terminal galactose residues on IgA molecules, and in some cases on other Ig classes, do target them to the hepatic galactose receptor and thus can play a decisive role in their catabolism (Zuckier *et al.*, 1989).

VI. Bacterial Fc Receptors

Certain bacteria express on their surface proteins capable of binding specifically to the Fc region of mammalian immunoglobulins. These so-called bacterial Fc receptors have been demonstrated on many staphylococcal and streptococcal strains (Boyle and Reis, 1987). Those receptors specific for the Fc of IgG have currently been classified, according to their functional reactivity with different species and subclasses of IgG, into six groups or types. The nature of the interaction of each of type with human IgG will be dealt with in turn. Relatively little is currently understood, in precise molecular terms, about the interaction of bacterial Fc receptors with other classes of immunoglobulin. These types of receptors will not, therefore, be discussed here.

A. Type I Fc Receptor

Type I Fc receptor, frequently termed protein A, is found on the surface of the majority of strains of *Staphylococcus aureus* (Forsgren and Sjöquist, 1966). This very extended protein has been cloned and found to consist of five homology units, each capable of binding the Fc of IgG and a sixth region that does not bind Fc but binds to cell walls (Moks *et al.*, 1986). Active 7-kDa fragments, each corresponding to a homology domain, can be generated by trypsin digestion. It is possible for two such fragments to bind simultaneously to one Fc molecule. A typical binding affinity ($K_a \sim 3 \times 10^6\ M^{-1}$) is demonstrated by fragment B binding to rabbit Fc (Lancet *et al.*, 1978).

Earlier crystallographic data (Deisenhofer *et al.*, 1976, 1978) have been refined to produce a model of the complex of human Fc and fragment B at 2.8 Å resolution (Deisenhofer, 1981). Fragment B forms two contacts with Fc molecules in the crystals but one of these is argued to be merely a crystal contact. In the other, thought to exist in solution, fragment B binds at the interface between the $C_\gamma2$ and $C_\gamma3$ domains of IgG (Fig. 19). The residues involved comprise parts of two hydrophobic patches on Fc, one on the $C_\gamma2$ domain (Met 252, Ile 253, Ser 254, Leu 309, His 310, and Glu 311) and the other on the $C_\gamma3$ domain (His 433, His 435, Tyr 436, and Asn 434).

Protein A binds the human subclasses IgG_1, IgG_2, and IgG_4, and also IgG_3 molecules of the allotype $IgG_3m(15, 16)$, characteristic of mongoloid populations (Recht *et al.*, 1981). Each of these proteins has a histidine residue at position 435 involved in the protein A interaction. By contrast, in IgG_3 molecules of the Caucasian allotypes $IgG_3m(5)$ and $IgG_3m(21)$, this histidine is replaced by arginine. Model building (Deisenhofer, 1981) reveals that the inability of such proteins

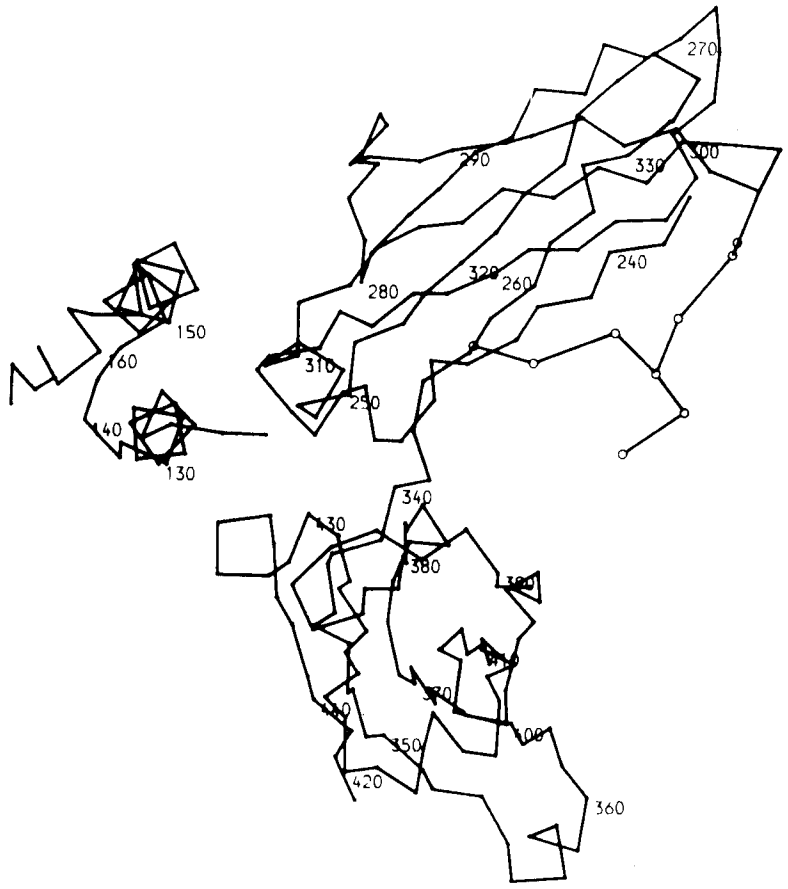


FIG. 19. Structure of the complex of fragment B of protein A with human Fc. Approximate centers of carbohydrate hexose units are shown (○). The α-carbon trace of only one Fc heavy chain is shown on the right. The interaction involves the contact of two α-helices of fragment B (left) with a hydrophobic patch in the $C_\gamma2/C_\gamma3$ interface (after Deisenhofer, 1981).

to bind protein A probably results from the prevention of favorable IgG–protein A contact formation by the lengthy side chain of arginine.

Loss of the N-linked carbohydrate moieties from the $C_\gamma2$ domains of IgG appears not to affect the interaction, as aglycosylated forms of both mouse IgG_{2a} and IgG_{2b} still bind to protein A (Leatherbarrow and

Dwek, 1983; Nose and Wigzell, 1983). It has been demonstrated recently, by use of ^1H NMR, that aglycosylation results in only a small and localized structural change in the vicinity of the His 268 reporter group at the N-terminal end of the $C_\gamma2$ domain (Lund *et al.*, 1990). Hence it is hardly surprising that loss of the carbohydrate residues from Fc does not affect the interaction with protein A. Similarly, neither reduction and alkylation nor hinge deletion of human IgG_1 perturbs the $C_\gamma2$–$C_\gamma3$ interface region, as demonstrated by the continued reactivity of these modified IgGs with protein A.

B. Type II Fc Receptor

This second class of receptor is associated with certain strains of group A streptococci. There is, however, a considerable degree of heterogeneity in the IgG subclass binding profiles among different group A isolates. Further, the binding profile of a particular isolate may vary with passage and even individual colonies within that isolate may express a variety of IgG-binding capacities (Yarnall *et al.*, 1984). Thus binding of IgG to these bacteria is complex and appears to be a function of several different type II Fc receptors. A recent study has defined five subtypes of the receptor based on their IgG species and subclass specificities (Raeder *et al.*, 1991a).

Type IIa receptor binds human IgG_1, IgG_2, and IgG_4, but not IgG_3. It also binds rabbit, pig, and horse IgGs. Immunoblotting techniques with the strain 64/14 have demonstrated a molecule of ~50 kDa with these binding properties (Yarnall and Boyle, 1986a). More recently, an IgG-binding protein, cloned from the group A strain CS110 (Heath and Cleary, 1987, 1989), displayed the IgG-binding profile of type IIa receptor (Cleary and Heath, 1990). A study assessing the ability of various dipeptides to inhibit the binding of radiolabeled IgG to strain 64/14 provided information on the localization of the type IIa receptor site on IgG (Yarnall and Boyle, 1986b). The dipeptides glycyltyrosine and glycylhistidine the binding of human IgG_1, IgG_2, and IgG_4 and rabbit and pig IgG to 64/14. This result, taken with the human IgG subclass specificity profile of this receptor, suggests that the IgG-binding site for the type IIa receptor may be very similar to that of the type I receptor. Histidine and tyrosine residues would appear to be important in the interaction. Interestingly, an IgG_3 molecule capable of binding protein A, and therefore presumably of an allotype with histidine at position 435, was found to bind the type IIa receptor on 64/14 (Yarnall and Boyle, 1986a). Reactivity of only certain IgG_3 allotypes with the cloned receptor was also noted (Cleary and Heath, 1990).

The type IIb Fc receptor has been demonstrated on the group A strains 64/14, A992S, and 11434 and substrain A928 A2 (Raeder *et al.*, 1991a) with a molecular weight of ~35,000 in the two former strains and ~47,000 in the two latter. It binds solely to human IgG_3 (Raeder *et al.*, 1991a; Yarnall and Boyle, 1986a). The largest differences between IgG_3 and the other human subclasses reside in its very extended hinge region, suggesting that a potential interaction site for type IIb receptor may lie in this part of the Fc. Attempts to inhibit the interaction with monoclonal antibodies specific for the hinge of human IgG_3 (Lowe *et al.*, 1982) might prove informative.

Type IIc receptor, a 116-kDa protein demonstrated on the strain A992S, has been defined as displaying specificity for human IgG_1 and rabbit, pig, and horse IgGs, and weak reactivity with human IgG_4. Sequence comparison of the $C_\gamma2$ and $C_\gamma3$ domains between the human IgG subclasses reveals that all residues conserved in both human IgG_1 and IgG_4 are also conserved in IgG_2 and IgG_3. Thus the specificity of type IIc receptor is difficult to explain by sequence differences in the ligands. Rather, the idea that distinct IgG-binding reactivities may be the sum of a number of independent binding units coming together in different combinations (Raeder *et al.*, 1991a) may be more appropriate.

Type IIo receptor, present on the substrain A928 A1, binds all human IgG subclasses as well as rabbit, pig, and horse IgGs (Raeder *et al.*, 1991a). This specificity is associated with a 47-kDa protein. The fifth receptor subgroup, type II'o, is a variant of the above and binds only human IgG (all four subclasses) and rabbit IgG. A protein displaying this specificity has been cloned from strain AP1 and given the alternative name of protein H (Åkesson *et al.*, 1990; Gomi *et al.*, 1990). It has a molecular weight of ~40,000 and shows homology to protein Arp, an IgA-binding streptococcal protein, but not to protein A, protein G (see later), or type IIa Fc receptor. Protein H is reported to block the binding of both protein A and protein G to IgG, suggesting it may also interact with the $C_\gamma2/C_\gamma3$ interface (Åkesson *et al.*, 1990).

C. Type III Fc Receptor

Type III Fc receptor is expressed on the surface of most human group C and group G streptococci. This receptor, more frequently termed protein G, exhibits a remarkably wide reactivity, interacting with all human IgG subclasses and IgG from rabbit, goat, cow, sheep, rat, mouse, guinea pig, horse, and pig. It displays a considerably higher affinity ($K_a \sim 1 \times 10^9 M^{-1}$ for human IgG subclasses) than does protein A (Reis *et al.*, 1984; Åkerström and Björck, 1986). Type III receptor is reported to have some affinity for Fab fragments of IgG mediated at a

site distinct from that binding to Fc (Erntell *et al.*, 1988). In addition, the receptor has affinity for human serum albumin.

The type III receptor has been successfully cloned from two different group G sources (Fahnestock *et al.*, 1986; Guss *et al.*, 1986; Olsson *et al.*, 1987). The deduced amino acid sequence shares features in common with that of protein A. Starting at the C-terminus, there is a region responsible for cell wall anchorage, followed by six repeated elements predicted to adopt a conformation of linearly arranged domains. The capacity to bind serum albumin is mediated by the N-terminal half of the domain structure (Guss *et al.*, 1986), whereas the site for IgG lies in the C-terminal half of the molecule (Åkerström *et al.*, 1987; Sjöbring *et al.*, 1988). Despite the similarity in overall organization of type III receptor and protein A, there is no homology between their IgG-binding regions, suggesting that they arose by convergent evolution.

In order to localize the type III receptor site on Fc, two different approaches have been used. First, the ability of enzymatically derived fragments of IgG to interact with the receptor was assessed in direct binding and inhibition studies (Schröder *et al.*, 1986; Stone *et al.*, 1989). The pFc′ fragment ($C_\gamma3$ dimer) of both human and rabbit IgGs did not bind to the receptor. Both human and rabbit F(ab′)$_2$ fragments failed to inhibit binding of radiolabeled Fc to group C and G streptococcal strains. Further, the $C_\gamma3$-lacking rabbit Facb fragment showed little inhibitory ability. In the second approach, chemical modification of IgG implicated the involvement of IgG tyrosines in the interaction (Stone *et al.*, 1989). These results suggest that type III receptor binds to an interaction site very similar to that of protein A, especially since the monovalent fragment D of protein A inhibits the binding of type III protein to IgG. Indeed, type III receptor was also shown to be capable of inhibiting the binding of IgM rheumatoid factors, which generally bind to the $C_\gamma2$–$C_\gamma3$ domain interface on IgG. However, a marked difference between protein A and type III receptor is that the former is sensitive to the His 435 → Arg interchange in the IgG_3 molecules of most Caucasians, but the latter is not. This suggests that this residue, clearly central in protein A binding, is on the periphery of the type III receptor site. Subtle differences in the recognition processes of the two receptors may also explain the differences in pH optimum for IgG binding to protein A (~pH 8) and type III receptor (pH 4–5) (Åkerström and Björck, 1986). X-Ray crystallographic analysis of the complex of Fc and type III receptor (or a fragment of it) would perhaps now allow definite identification of the interaction on both ligand and receptor.

D. Type IV Fc Receptor

Type IV Fc receptors are associated with some β-hemolytic bovine group G streptococci. They are capable of binding human IgG_1, IgG_3, and IgG_4 and IgG from several other species, particularly rabbit (Reis *et al.*, 1990; Raeder *et al.*, 1991b). Sequence analysis of the $C_\gamma2$ and $C_\gamma3$ domains of rabbit IgG and the human IgG isotypes reveals only three residues, which are conserved in rabbit IgG, human IgG_1 and IgG_4, but not in human IgG_2. These are residues Leu 235, Gly 236, and Ala 339, all in the $C_\gamma2$ domain, the latter residue being located on the interior surface of the domain. Hence, one might speculate that a potential interaction site may encompass residues 235 and 236, which, as mentioned earlier, form part of the lower hinge site for the high-affinity human $Fc_\gamma RI$. Attempts to inhibit the binding to one receptor with soluble fragments of the other might be an illuminating future experiment.

E. Type V Fc Receptor

This fifth bacterial receptor for the Fc of IgG is found on certain strains of *Streptococcus zooepidemicus*. It has a binding specificity very similar to that of protein A, displaying affinity for human IgG_1, IgG_2, and IgG_4 and pig, guinea pig, and rabbit IgGs. Only weak reactivity is reported with cow, sheep, goat, horse, rat, dog, and cat IgGs (Myhre and Kronvall, 1980). More recently, a subtype of this receptor has been described (Yarnall and Widders, 1990). The subtype protein has a molecular weight of ~45,000 and similar specificity to the original type V receptor except that it has strong reactivity with cat and horse IgGs. This subtype V receptor may recognize a site on Fc distinct from the protein A site because protein A apparently still bind to IgG after binding of subtype V receptor in blotting experiments. This is somewhat surprising considering the similar binding profiles of the two receptors, especially with the human IgG subclasses. Further binding inhibition studies with native receptors might aid clarification of the situation.

F. Type VI Receptor

An IgG-binding protein of ~46 kDa, present on the surface of *S. zooepidemicus* strain S212, has been designated type VI Fc receptor (Reis *et al.*, 1988). It has specificity for rabbit, pig, sheep, goat, and cow IgGs and weaker reactivity with human, mouse, and rat IgGs. This affinity for rat IgG is, however, the greatest displayed by any bacterial Fc receptor thus far described. An explanation of this binding profile in terms of IgG site localization is, with present knowledge, difficult.

VII. Conclusions

Considerable advances have been made in the past few years in delineating the sites on antibodies interacting with effector molecules. Although far from complete, such information allows much to be done in terms of rational design of effector function. For instance, single point mutations can be used to eliminate C1q and Fc receptor (FcRI and FcRII) binding from an IgG molecule. More subtly, point mutations can be used to eliminate complement lysis while leaving C1q binding unaffected or to eliminate FcRI binding with small effects on FcRII. In the opposite experiment, a single point mutation can be used to convert an IgG displaying no measurable affinity for FcRI into one with fully functional affinity. Generally, the introduction of function to an inactive antibody is likely to be more demanding of the level of our understanding, and here we have further to go.

A recurring theme in this review is that binding of the appropriate antibody isotype to antigen is a necessary but not sufficient criterion for effector triggering. Examples abound of antibodies, for instance, that bind C1q but do not sustain a later step in the complement cascade. Similarly, there are antibodies that link effector and target cells effectively but do not lead to target damage. There would appear to be extra requirements associated with the antigen. Further, this does not appear to be simply a question of antigen density. One explanation might be that certain antigens are able to trigger allosteric changes whereas others are not. We have discussed why we think this unlikely. The explanation we favor is that antibodies linking arrays of antigen and effector molecules have preferred arrangements for optimal effector function. Some antigens, or their local environment, would preclude the formation of such arrangements and so they would bind antibody but be unable to activate the effector function. This hypothesis could accommodate the independent sensitivities of many of the steps of the complement cascade. For instance, a particular antibody arrangement could be sufficient for C1q binding and C1 activation but inappropriate for C4 activation.

Accepting for a moment the notion of preferred arrangements, how might they look? We have described some circumstantial reasons for favoring the formation of IgG hexamers involving dislocated IgG molecules with Fc–Fc interactions, but have no hard data to support this as yet. Certainly IgM appears to function best with respect to complement activation in a "preferred arrangement" (hexamer) with Fc–Fc interactions. Dislocation of antibody molecules is a feature that is difficult not to embrace given the localization of Fc receptor binding

regions to the middle part of the antibody IgG and IgE molecules. In simple terms it is difficult to envisage the topology of the bridging of two cells by antibody without invoking movement of Fc out of the plane of the two Fab arms. The available physical data support the notion that such movements should be allowed. Indeed, flexibility of the antibody molecule could be useful in a number of ways when linking antigen and effector. For instance, dislocation and Fab arm opening will both serve to bring effector molecules closer to the antigenic surface. Thus, for example, the surface of an effector cell will be brought closer to that of the target cell (Fig. 16), facilitating target cell destruction, and the generation of activated C4b and C3b will occur closer to the target cell membrane, facilitating complement lysis. Again, flexibility is expected to be advantageous given the diversity of antigens that must be linked to the common effector systems.

Clearly, much of this discussion is conjecture. One would like to have experimental measurements on the interacting triumvirate of antigen, antibody, and effector molecule. As discussed earlier this is a tall order, but it is to be hoped that appropriate methodologies will be developed.

An interesting feature of antibody effector function is the occurrence of the human IgG subclasses. Typically one wonders about the role of the IgG_2 and IgG_4 subclasses given their very poor reputations in this area. We would suggest the following propositions: IgG_1 and IgG_3 both appear to mediate effector functions, although the relative efficacy of the two may vary according to the conditions of effector triggering. IgG_3, with its long hinge, would seem to have an advantage in bringing antigen and effector closer together in that repulsive forces, e.g., between cell surfaces, would be minimized. IgG_1, on the other hand, by bringing antigen and effector closer together, may facilitate target damage. It could be that, *in vivo*, the two function cooperatively. IgG_2 does activate complement under conditions of high epitope density and this provides a "rationale" for the preponderance of IgG_2 anticarbohydrate antibodies (Michaelsen *et al.*, 1991) because carbohydrates are often presented at high density on microbial surfaces and at lower densities more ubiquitously. IgG_2 does not generally interact with Fc receptors except for one form of FcRII as discussed. IgG_4 does not appear to activate complement under any circumstances. It does interact with FcRI, albeit more weakly than IgG_1 and IgG_3, but not with FcRII or FcRIII. It may be that there are situations, e.g., blocking of the function of certain viruses, wherein it is desirable to have antibody binding without, e.g., cellular uptake.

Many effector studies are now carried out with monoclonal antibod-

ies, although *in vivo*, of course, the response is polyclonal. Because the effector systems tend to recognize arrays of antibodies that could differ depending on the composition of the antibodies involved, the distinction may be important. An example is provided by the synergistic effect of two monoclonal antibodies on complement activation described previously. As more human monoclonal antibodies become available (Burton, 1991; Burton *et al.*, 1991; Persson *et al.*, 1991) this is an area which should be explored. It is our guess that we shall find that antibodies work best as mixtures, both in terms of epitope specificity and subclass.

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The Development of Functionally Responsive T Cells

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I. Properties of Mature T Cells: The Destination of the Process

T (thymus-derived) lymphocytes play central roles in immune responses and in the regulation of cell growth for several hematopoietic lineages. The importance of these cells is derived from their unique integration of specific antigen recognition with an impressively versatile, highly regulated array of possible effector functions. The developmental processes that endow T cells with their functional responsiveness and couple this to an appropriate repertoire of recognition specificities have been of great interest for many years to immunologists and to cell and molecular biologists alike. In the past decade, these cells have become one of the best understood cell types in vertebrates.

The enormous progress toward understanding immunological recognition on both the cell and the population level has dominated the field of T cell development in recent years. Antigen-specific recognition and antigen-specific tolerance are, after all, the classic features of immunocytes that both T and B cells share. There is an important distinction between the roles of B and T cells in the immune system, however, that goes beyond their different target-structure specificities. B cell responses consist of secreting immunoglobulin (Ig) molecules that are simply variants of the recognition structures through which the cells are triggered. Thus, both specificity and effector function can be understood through the regulation of Ig gene expression. By contrast, T cell effector function is based on the expression of genes that encode growth factors, differentiation factors, or directly cytolytic structures, a different set of genes from those that encode the molecular components of the T cell receptor (TCR) for antigen. Thus, a well-rounded view of T cell development encompasses the regulation of T cell signaling and response genes, as well as the assembly and selection of the TCR.

At one level, throughout this review, we will use a checklist of mature T cell characteristics as a way to stage the progress of pre-T cells toward maturity. For example, the transition from a TCR^- to a TCR^+ state may be seen as the *outcome* of a developmental process. At