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The effector functions of immunoglobulins: implications for therapy

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Summary The effector functions of immunoglobulins of the G class (IgGs) are essential for their effective use in therapy. The functions that operate following complex formation with cognate antigen involve binding to C1q (to mediate complement fixation) and the Fc receptors, FcγRI, II and III. Another class of functions that is independent of antigen binding encompasses the transfer of antibodies across the placenta and maintaining the levels in the serum. All effector functions of IgGs are conferred by sequences in the Fc region of antibodies, and this review discusses the localisation of the functions to specific amino acid residues. Such knowledge is of use for the further improvement of IgCs for therapy.

Keywords antibody, catabolism, complement, effector function, Fc receptor, immunoglobulins, maternal transfer

Abbreviations

ADCC	antibody dependent cell mediated cytotoxicity
C1q	first components of complement system
CD4	membrane glycoprotein on T helper cells
CH2/CH3	domains of Fc region
Fc	C-terminal region of IgG heavy chain – encompassing CH2 and CH3 domains
FcR	Fc receptor
HIV	human immunodeficiency virus
IgG	immunoglobulin G
IVIG	intravenous immunoglobulin
MHC	major histocompatibility complex
NK	natural killer cells
SpA	Staphylococcal protein A
SRBC	sheep red blood cells
$t_{\frac{1}{2}}$	half-life.

Introduction

The immunoglobulin molecule has two distinct functions which are structurally separated in distal regions of the molecule: specific binding of antigen through the combining site localized in the Fv region of the molecule, and recruiting other cells or molecules to remove the antigen through the effector sites localized in the Fc region (Fig. 1).

The effector functions of the Fc region of an antibody can be divided in two categories:

1 Effector functions that operate after the binding of antibody to cognate antigen. These involve the participation of the complement cascade or Fc receptor (FcR)-bearing cells.

2 Effector functions that operate independently of antigen binding. These confer persistence in the circulation and the ability to be transferred across cellular barriers by a process known as transcytosis (Table 1).

Together with the effector functions of an antibody, antigen binding is responsible for the protective effect of immunoglobulins in the body's defence against foreign antigens. Therefore, to be efficient, an antibody should be endowed with both high affinity and specificity for the corresponding antigen, and efficient effector functions for recruiting FcR-bearing cells and complement components following antigen binding. These effector functions result in removal and/or destruction of the foreign antigen. Without the cooperation of these effector functions, the antibody alone has only a neutralization effect that may prevent the binding of the exogenous antigen such as viruses to the endogenous target but does not remove the antigen. Furthermore, of relevance for tumour therapy, there is evidence that besides conventional effector functions, an anti-tumour antibody may have agonist activity, resulting in negative signaling in neoplastic cells leading to cellular arrest and/or apoptosis (1).

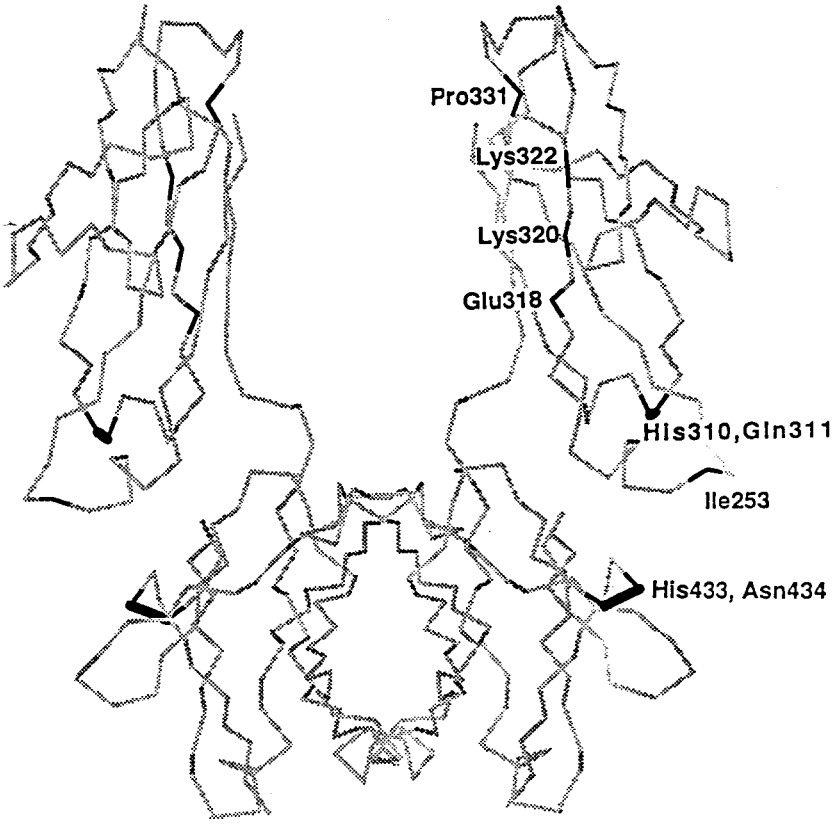

Fig. 1 The structure (α-carbon trace) of the Fc region of IgG (62) with the amino acids involved in effector functions indicated. Residues Ile253, His310, Gln311, Glu318, Lys320, Lys322, Pro331, His433 and Asn434 are shown in black and the remainder of the structure grey. The lower hinge region, involved in binding to FcγRs, is not shown.

Table 1 The effector functions of the immunoglobulin molecule

	Interaction with:	Class of Ig involved	Biological activity
Antigen-dependent			
	C1q component	IgM, IgG	Complement dependent lysis
	FcR* on macrophages, neutrophils, NK cells, lymphocytes, etc.	IgG	Endocytosis of pathogen. Antibody dependent cell mediated cytotoxicity (ADCC)
	FcRn† on epithelial cells	IgG	Transfer across neonatal intestine, placenta, yolk sac
Antigen-independent			
	pIgR‡ on glandular epithelial cells	IgA, IgM	Transport across mucosal epithelia of Ig synthesized locally
	FcRc§ on cells involved in catabolism	IgG	Regulation of serum IgG level

*FcR, receptor that binds to Fc region of IgG molecule (Fc receptor).
†FcRn, Fc receptor identified on neonatal intestine, yolk sac and placenta that is structurally different from FcRs.
‡pIgR, receptor which mediates the transport of polymeric IgA and IgM (poly Ig receptor) across the mucosal epithelium.
§FcRc, protective Fc receptor on putative cells involved in control of IgG catabolism.

Table 2 Intravenous immunoglobulin (IVIG) effects in some human diseases (adapted from (8))

Antibody specificity in IVIG	Type of activity	Effector system engaged	Examples of diseases
Epitopes on bacterial, viral, chlamydial and toxin targets	Anti-infectious	Neutralization, phagocytosis by FcR and complement receptor-bearing cells, complement activation	Primary and secondary immunodeficiencies
Idiotypes	Anti-inflammatory	Pathogenic autoantibody	Immune thrombocytopenic purpura
Nonspecific FcR blockade	Anti-inflammatory	FcR-bearing cells engaged with IVIG not available for destruction of antibody coated platelets	Immune thrombocytopenic purpura

Passively administered immunoglobulins are currently used in clinical settings for three main purposes:
1 To substitute for absent or low concentrations of antibody in the circulation in agammaglobulinaemics and hypogammaglobulinaemics (Table 2).
2 As immunosuppressive agents for the treatment of autoimmune and graft-vs.-host (GVH) diseases, and for the prevention of allograft reactions following transplantation [e.g. OKT3 (2), Xoma-Zyme-CD5 (3)].
3 For the treatment of lymphoid malignancies [e.g. anti-idiotypic (4), CAMPATH- 1 (5)].

The various therapeutic antibodies presently used in the clinic belong to four generations of products that reflect the evolution of techniques for their production:

The first generation is represented by intravenous immunoglobulin (IVIG), comprising primarily gammaglobulins (IgGs) collected from thousands of donors. IVIG contains approximately ten million different antibody specificities per gram. The administration of IVIG for antibody replacement in immunodeficient subjects is a clinical procedure currently used to prevent or treat some infections, chronic inflammations [e.g. LPS-induced immunoinflammatory cascade (6)] and autoimmune disorders [e.g. immune thrombocytopenia purpura (7)] (Table 2).

The second generation of therapeutic immunoglobulins is represented by rodent monoclonal antibodies with a unique specificity that are directed towards epitopes of the pathogen or host cells that are being targeted. To date, several antibody preparations have been used for the therapy of infections [bacterial, viral and chlamydial targets (8)] and as immunosuppressive agents in GVH and allograft rejections (6). However, the use of rodent antibodies in therapy is limited by the immunogenicity of these proteins in xenogeneic hosts (9).

The third generation of therapeutic immunoglobulins consists of chimeric monoclonal antibodies obtained using genetic manipulation to link human constant regions to rodent variable regions (10). These chimeric antibodies are markedly less immunogenic than the rodent counterparts. However, the variable domains of these chimeric antibodies still contain rodent framework sequences which result in the generation of anti-mouse variable domain responses.

The fourth generation of therapeutic antibody comprises humanized or reshaped antibodies which contain rodent hypervariable regions grafted on to human framework sequences (11). These antibodies are less immunogenic than the chimeric antibodies mentioned above, but they may still elicit an anti-idiotypic response due to foreign sequences in mouse hypervariable regions.

By changing some of the amino acid residues in the effector sites of the antibody molecule, modulation of effector functions has been achieved (12), leading either to the increase or decrease/ablation of the targeted effector function. Thus, the survival time of a monoclonal antibody can be altered to increase its residence time in the body or alternatively to accelerate its removal (13). Decreased persistence might be favourable for therapy aimed at rapid removal of the foreign antigen. The FcR-binding function of an antibody might also be improved by modification of the amino acid residues in the effector sites, as suggested by several experiments showing a considerable improvement of antibody dependent cell mediated cytotoxicity of a murine monoclonal antibody with human Fc regions compared with the original murine antibody (14). Furthermore, recent developments in recombinant DNA technology facilitate the affinity improvement of an antibody. For example, antibody variable domain genes can be randomly mutated and higher affinity variants selected using bacteriophage display systems (15). Thus, it is now possible to use recombinant DNA technology to generate the optimal combining site and to link this to constant regions encoding the effector

Table 3 Amino acid sequences in the CH2 domain of IgG proposed to be involved in complement activation (18)

IgG	Position*	318	320	322	331	333	335	337
Human	IgG1	Glu	Lys	Lys	Pro	Glu	Thr	Ser
	IgG2	Glu	Lys	Lys	Pro	Glu	Thr	Ser
	IgG3	Glu	Lys	Lys	Pro	Glu	Thr	Ser
	IgG4	Glu	Lys	Lys	Ser	Glu	Thr	Ser
Mouse	IgG1	Glu	Lys	Arg	Pro	Glu	Thr	Ser
	IgG2a	Glu	Lys	Lys	Pro	Glu	Thr	Ser
	IgG2b	Glu	Lys	Lys	Pro	Glu	Thr	Ser
	IgG3	Glu	Lys	Lys	Pro	Glu	Thr	Ser

*EU nomenclature.

Table 4 Complement activation of mutant mouse IgG2b antibodies (adapted from (19))

Mutants	Haemolysis	C1q binding (nM)
Wild-type	Yes	11
Glu-318 to Ala	No	> 300
Lys-320 to Ala	No	> 300
Lys-322 to Ala	No	> 300
Lys-322 to Arg*	Yes	11
Pro-331 to Gly†	Yes	9–12
Glu-333 to Ala	Yes	9–12
Thr-335 to Ala	Yes	9–12
Ser-337 to Ala	Yes	9–12
Asn-297 to Ala‡	No	31

*In mouse IgG1 position 322 is Arg (see Table 3). Mouse IgG1 does not activate complement.
†Pro-331 was, however, found to be involved in the binding of C1q by other authors (20,21).
‡Point of attachment of N-linked carbohydrate.

functions of choice. These designer antibodies have enormous potential for use in therapy and diagnosis.

In the following pages a complete description of the localization of various effector sites for the subclasses of IgG will be presented. The improvement of antibody affinity using bacteriophage display is not covered in this review, but has been discussed elsewhere (16).

Antigen-dependent effector functions

Complement activation

The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to IgG or IgM antibody complexed with cognate antigen (bacteria, cells, etc.). To activate the complement cascade, it is necessary for C1q to bind to at least two molecules of IgG (but only one molecule of IgM) attached to the antigenic target.

By chemical modification, study of amino acid residue accessibility and sequence comparison, Burton and colleagues (17,18) suggested that the region comprising amino acid residues 318 to 337 might be involved in building the site for the fixation of complement (Table 3). Duncan & Winter (19) used site directed mutagenesis to determine that amino acid residues at positions 318, 320 and 322 are essential for the binding of C1q to murine IgG (Table 4). The experimental model consisted of the measurement of either the binding of ^{125}I-labelled C1q to wild-type and mutant mouse IgG2b anti-hapten (NIP) antibodies coated on NIP-Affigel or the haemolysis of NIP-sheep red blood cells (SRBC) coated with the anti-hapten antibodies. The role of Glu-318, Lys-320 and Lys-322 residues in the binding of C1q was confirmed by the ability of a synthetic peptide containing these residues (Ala-**Glu**-Ala-**Lys**-Ala-**Lys**-Ala) to inhibit complement mediated lysis of NIP-SRBCs by the corresponding anti-NIP antibody (19).

The involvement of Pro-331 in C1q binding has been shown by analysis of the ability of human IgG subclasses to carry out complement mediated lysis. Mutation of Pro-331 to Ser-331 in human IgG3 or IgG1 (Table 3) decreased or abolished complement mediated lysis whilst mutation of Ser-331 to Pro-331 in IgG4 (unable to fix complement) conferred the ability to activate complement on this IgG subclass (20,21). These data clearly indicate that there are at least two different regions involved in the binding of C1q: one on the beta-strand of the CH2 domain bearing the linear determinant Glu-318, Lys-320 and Lys-322 (strand fγ2 in Beale & Feinstein representations) (22) and the other on a turn located in close proximity to the same beta-strand and containing at least one key amino acid residue at position 331 (bend b6 in Beale & Feinstein representations). Residue 330, with Ser in IgG4 and Pro in the other subclasses has been shown not to be involved in binding complement (23). Besides these two spatially close regions involved in C1q binding, some preliminary results were presented indicating that human IgG1 residues Lys-235 and Gly 237 play a critical role in complement fixation and activation (24). These two residues are located in the lower hinge region situated in the neighbourhood of both the beta-strand and the bend containing the key residues 318, 320, 322 and 331 (Fig. 1). Since there are two CH2 domains in IgG, each containing one C1q-binding site, the question arises as to whether both domains or only one are

involved in C1q binding (IgG-C1q-IgG vs. C1q-IgG-C1q). It was shown that only one of the CH2 domains within each individual IgG molecule needs to be active, as hybrid IgGs with Fc regions comprising active IgG2a (or IgG2b) and inactive murine IgG1 were still able to carry out C1q binding and complement mediated lysis (25,26).

Mutation of Asn-297 to Ala also decreased the ability of a murine IgG2b antibody to bind C1q or to mediate complement haemolysis (Table 4). This effect is the consequence of the absence of the carbohydrate moiety (normally anchored to Asn-297) and not of the direct involvement of Asn-297 in complement activation. The N-linked carbohydrate at position 297 separates the two CH2 domains and mediates contact between them. Human aglycosylated IgGs show altered susceptibility to proteolysis and differential reactivity with anti-Fc monoclonal antibodies compared with their glycosylated counterparts. This indicates that IgG lacking the carbohydrate has an altered conformation (27). Aglycosylated human IgG1 and IgG3 are deficient in their ability to activate complement, and at least in part this is a consequence of their decreased capacity to bind C1q (28). The ability of an IgG to activate complement does not only depend upon the presence of carbohydrate, but also its composition. Thus, a chimeric mouse–human IgG1 antibody produced in Chinese hamster ovary cells, containing a truncated carbohydrate (lacking galactose, sialic acid and fucose) was unable to perform complement mediated lysis of antigen coated RBCs (28). In part, this was shown to be due to a reduced ability to bind C1q. This has implications for populations of IgGs, as the carbohydrate attached to the CH2 domain is usually not homogenous and exists in different isoforms (28). Thus, it is possible that even antibody preparations produced by monoclonal hybridomas will exhibit different complement fixation activities. The ability of IgG to bind C1q and activate the complement cascade is therefore determined not only by the amino acid residues of the beta-strand encompassing residues 318, 320 and 322, the turn including residue 331 and possibly the lower hinge region, but also by the presence, absence or modification of the carbohydrate moiety positioned between the two CH2 domains. How the carbohydrate influences the function of the C1q-binding site is not known but it seems that the conformational change in aglycosylated IgG modifies the spatial distribution of the two C1q-binding subsites, impairing the function of the C1q-binding site.

The role of different hinge sequences in modulating the segmental flexibility and/or spacing between the CH1 and CH2 domains (29), and in turn affecting complement activation, is still an area of investigation. Segmental flexibility was, in an earlier study, considered to be necessary for C1q fixation (30). However, more recent studies have shown that the flexibility and spacing provided by the hinge region are not required for complement activation (23,29). Consistent with the latter study, a series of hinge shuffling experiments indicated that an IgG4 molecule with the flexible hinge of IgG3 was still unable to activate complement (31). Taken together, the data indicate that for complement activation, the presence of a hinge region is not a requirement; the IgG molecule should only have an interheavy chain disulphide bond to constrain the two CH2 domains in the correct conformation for C1q binding and activation (23,29).

When using therapeutic immunoglobulins in replacement therapy or in blocking and/or removing specific targets, it would be of therapeutic value to decrease or increase, respectively, the C1q-binding capacity of the antibody. Thus, an increase in C1q binding activity might be desired when the participation of the complement system is critical for effective treatment, as it is in some bacterial infections or in the removal of immune complexes from the circulation (8). On the other hand, dimeric IgGs present in IVIG are responsible for the beneficial effect of IVIG treatment in immune thrombocytopenic purpura, by blockade of the FcR on mononuclear phagocytes that are responsible for the destruction of autoantibody coated platelets (Table 2) (7). However, dimeric IgG may bind to C1q and initiate the complement cascade, resulting in the unfavourable side-effects observed following IVIG treatment (7,8). Therefore, dimeric IgG with an intact FcR-binding site, but with C1q-binding site inactivated by site directed mutagenesis, would be a preferable immunoglobulin for use in this situation.

Fc receptor interaction

Multiple biological functions can be mediated via the interaction of the Fc region of an IgG antibody with receptors (FcγRs) that are expressed on a wide variety of effector cells. The FcγR receptors, designated FcγRI, II and III, belong to the Ig supergene family (Table 5). All classes of FcγR have been shown to mediate both phagocytosis of immune complexes (removing antibody coated pathogens) and antibody dependent cell mediated cytotoxicity, lysing erythrocytes and various other cellular targets (e.g. tumour cells) coated with the corresponding antibody (32).

Based on studies with myeloma proteins with deleted hinge regions (33) and sequence comparisons of several IgG antibodies with various affinities for FcγR-bearing

Table 5 Characteristics of human FcγR (adapted from (32))

Charactertstics	Receptor class		
	FcγRI (CD64)	FcγRII (CD32)	FcγRIII (CD16)
Molecular mass	72	40	80
Number of Ig-like domains	3	2	2
Number of isoforms	1	3	2
Affinity for monomeric human IgG*	High	Low	Low
Affinity for human IgG isotypes	3 > 1 > 4 ≫ 2	3 > 1 = 2 ≫ 4	1 = 3 ≫ 2 = 4
Affinity for mouse IgG isotypes	2a = 3 ≫ 2a,1	1 > 2b ≫ 2a,3	3 > 2a > 2b ≫ 1
Expression on cells†	MØ, Mo, N ↑	MØ, Mo, PMN, P, B, NK	MØ, Mo, N, E, T
Functions:‡			
Phagocytosis	+	+	+
ADCC	+	+	+
TNFα secretion	+	+	+
IL-6 secretion	+	?	?
Lysozomal enzyme release	?	+	+
Superoxide generation	+	+	+
Regulation of Ig production	–	+	–

*FcγRI binds monomeric IgG with high affinity whilst FcγRII and FcγRIII require multimeric IgG as either soluble immune complexes or coated on particles/cells.
†Abbreviations: B, B-lymphocytes; E, Eosinophils; MØ, monocytes; M0, macrophages; N, neutrophils, N ↑, neutrophils after stimulation with interferon α; NK, natural killer cells; P, platelets; PMN, polymorphonuclear leukocytes; T, a subpopulation of T-lymphocytes.
‡Abbreviations: ADCC, antibody dependent cell-mediated cytotoxicity; IL-6, interleukin 6; TNFα, tumour necrosis factor α.

cells (34), the binding site for these FcγRs on IgG was found to reside mainly in the lower hinge region (Leu-234 to Pro-238) (35). Thus, in human IgG1 or murine IgG2a which both have high affinity for FcγRI (Table 5), the sequence in this region is Leu-234–Leu-Gly-Gly Pro-238. In contrast, for murine IgG2b, which does not bind to FcγRI, the sequence is Leu-234–Glu-Gly-Gly Pro-238. The variation in position 235 of mouse IgG2b vs. IgG2a was shown to be critical for interaction with FcγRI-bearing cells (35). Thus, the binding of ^{125}I-labelled human IgG1 to human U937 cells (bearing FcγRI) was inhibited by a mutated murine IgG2b in which Glu-235 was changed to Leu (as in human IgG1 and murine IgG2a). In contrast, wild-type IgG2b did not interfere with the binding of human IgG1 to U937 cells (35). The participation of Leu-235 in building the FcγRI binding site was confirmed and extended using another system based on the binding of various chimeric mouse–human anti-DNS antibodies to human U937 cells (36). As shown in Table 5, monomeric IgG1 and IgG3 bind to FcγR1 with high affinity (10^9 M) whilst IgG4 binds with a 10-fold lower affinity (10^8 M) and IgG2 does not bind. Comparison of the sequences of the region encompassing residues 233 to 238 indicates significant differences between these isotypes (Table 6).

Table 6 Amino acid sequences in lower hinge region of human IgG proposed to be involved in FcγR binding (36,37)

Isotype position*	233	234	235	236	237	238
IgG1 or 3	Glu	Leu	Leu	Gly	Gly	Pro
IgG2	Pro	Val	Ala	–	Gly	Pro
IgG4	Glu	Phe	Leu	Gly	Gly	Pro

*EU nomenclature.

Mutation of Leu-234 to Phe and Leu-235 to Glu decreased the affinity of IgG3 for FcγRI from 1.8×10^9 M to 1.9×10^8 and 10^7 M, respectively, clearly indicating that in addition to residue 235, residue 234 is also involved in binding to FcγRI (36). The role of other amino acid residues in the 233–238 sequence in building the FcγRI binding site was also investigated by analysing the ability of mutant human–mouse chimeric IgG1 molecules with mutations at position 233 (Glu to Pro), 235 (Leu to Ala) and deletion of Gly 236 to inhibit the binding of ^{125}I-labelled human IgG1 to U937 cells (37).

Both mutations and the deletion completely abolished the cytophilic activity of the IgG1 molecule, indicating that the complete sequence from 233 to 237 is necessary for full FcγRI binding activity. In fact, replacing the entire 233–237 sequence of IgG2 with that of IgG1 yielded a molecule with a binding activity four times higher than that of IgG1 (37).

Residues 233–237 of IgG are also necessary for interaction with FcγRII (38,39) and FcγRIII (40,41). By using cells that express either FcγRII (Daudi, K562) or FcγRIII (NK) and antibody coated target cells, it was shown that mutations in positions 234, 235 and 237, but not 236, resulted in the greatest reduction of binding to FcγRII and FcγRIII (38,40). Taken together, the results suggest that all three FcγRs recognize a binding site on IgG in the lower hinge region (233–237). However, each receptor sees this common site in a different way (38,41). IgG4 has a pair of serine residues replacing the alanine and proline at positions 330 and 331 of IgG1, 2 and 3 (Table 3) and as these residues are predicted to be in close proximity to the lower hinge region (36), Pro-331 in IgG3 was changed to Ser and the binding of this mutant to target cells analysed. The affinity of this mutant was sixfold lower than that of the unmutated IgG3, clearly indicating that the bend b6 is also involved in FcγRI binding (28,36). The other polymorphic position (330) of this region is, however, not involved in FcγRI binding since the change of Ala-330 to Ser in IgG3 had no effect (20,23).

In addition to these two FcγR-binding sites, a third was located by studying the interaction of some mutants of a murine anti-NIP IgG2b antibody using NIP-SRBCs and the FcγRII-bearing mouse P388D1 cell line (39). It was shown in this assay that, in contrast to the wild-type, two mutants (Gly 237 to Ala and Glu-318 to Ala) were not able to form rosettes with P388D1 cells. This not only confirmed that Gly 237 is essential for the binding to FcγRII, but also indicated that another region located on the fγ2 beta-strand might be involved in building the FcγRII-binding site, at least for murine IgG2b (39). The data concerning the localization of the FcγR binding site on the IgG molecule indicate that in addition to the region of IgG (234–238) that is involved in binding all three FcγR classes, there are two spatially close regions that are involved in binding FcγRI (Pro-331) and FcγRII (Glu-318) (Fig. 1). It is obvious that there are not enough data to claim that the FcγR-binding region of IgG is located in three distinct regions irrespective of the class of FcγR recognized. However, if this were the case, it is interesting to note that both antigen-dependent effector functions of IgG, namely the binding of C1q and FcγR-bearing cells, involves the same regions of the molecule, i.e. fγ2 beta-strand, b6 bend and the lower hinge region (Fig. 1). Moreover, these regions are adjacent to each other in three dimensional space. The overlap of the C1q and FcγR-binding sites does not mean that there is not a distinct 'footprint' for these structurally different ligands, but suggests that the binding of C1q and FcγR to IgG might compete with each other under physiological circumstances. If this is so, it follows that once an immune complex binds C1q, it is unable to interact with FcγR bearing cells and vice versa. In fact, there is a close correlation between the ability of human or mouse IgG subclasses to bind either C1q or FcγR (Table 7). The data presented in the Table suggests that both functions may involve the same region of IgG and that their function might be mutually exclusive.

Table 7 Interaction of human and murine IgG isobpes with human C1q and homologous FcγR

	Interaction with:*			
Isotope	C1q	FcγRI	FcγRII	FcγRIII
Human				
IgG1	+	+	+	+
IgG2	±	–	–	–
IgG3	+	+	+	+
IgG4	–	–	–	–
Mouse				
IgG1	–	–	+	+
IgG2a	+	+	+	+
IgG2b	+	–	+	+
IgG3	–	–	–	–

*The interactions were scored as: +, strong positive; ±, weak positive and –, negative.
The same type of interaction (+ or –) was observed for human IgG1 and IgG3 and murine IgG2a and IgG3 with C1q and all three classes of FcγR. For the other human and murine isotypes the same type of interaction was seen only for some classes of FcγR and C1q.

Another common feature of the interaction of IgG with C1q and FcγR is the dependency of these two effector functions on the presence of the carbohydrate moiety in the CH2 domain. Aglycosylation of mouse IgG2b, by mutating Asn-297 to Ala, considerably reduces the ability of the antibody to form rosettes with P388D1 cells (39). This is in agreement with previous studies with aglycosylated chimeric mouse–human IgG1, in which the aglycosylated molecule (by mutating Asn-297 to Gln) does not bind to human FcγRI and does not activate complement (27,28). Also, the number of sites per IgG molecule are the same for C1q and

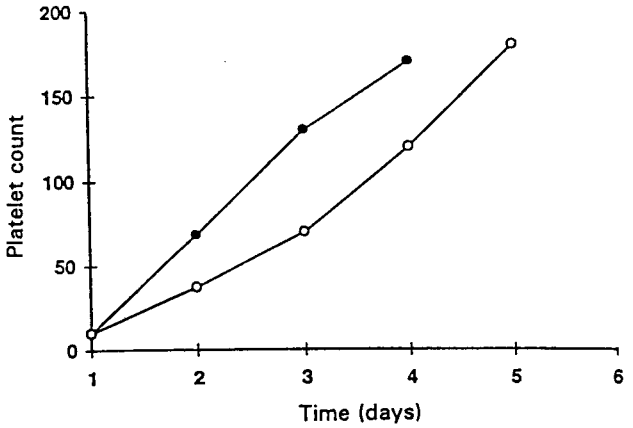

Fig. 2 Comparative platelet increase after treatment with IVIG or anti-FcγRIII mouse monoclonal antibody (7). (○) IVIG; (●) anti-FcγRIII.

FcγR binding. As discussed above, one C1q-binding site in the two CH2 domains is sufficient for C1q fixation and similarly only one heavy chain is necessary for the IgG–FcγR interaction (42,43). This was clearly demonstrated by showing that a monomeric hybrid mouse IgG1/IgG2a and IgG2b/IgG2a, with only one functional FcγRI binding site per IgG, bound to human FcγRI as efficiently as the parental IgG2a/IgG2a homodimer (43).

The clinical effect of IVIG treatment in patients with immune thrombocytopenic purpura occurs via FcR blockade (7,8) by IgG molecules present in dimeric or oligomeric form in IVIG preparations (44). As a result of FcγR blockade the platelet count of the patient rises, since autoantibody coated platelets are no longer destroyed by FcγR-bearing cells of the mononuclear phagocytic system. Consistent with the effect of IVIG being due to FcγR blockade, the use of an anti-FcγRIII monoclonal antibody is an effective treatment for refractory thrombocytopenia (45) (Fig. 2). Clinical studies with murine anti-FcγRIII antibody infusions have shown that this treatment can induce not only a transient increase in platelet counts (Fig. 2), but also long-term improvement without the need for further treatment (7). The long term effect might be due to the release of some bioactive mediators (see Table 5) as a result of the *in vivo* stimulation of FcγR-bearing cells, with some beneficial effects on the immune status of the patients by inducing a diminished level of anti-platelet antibody.

There is also evidence that antibody mediated complement lysis and cellular cytotoxicity can induce anti-tumour effects, with certain IgG isotypes being more effective than others (46–49). Thus, the murine IgG2a isotype appears to be the most effective antibody for inhibition of human tumour growth in nude mice through interaction with FcγR-bearing effector cells. This was clearly shown by complement depletion of these nude mice, which did not affect the anti-tumour function of this antibody (49). Two rat antibodies directed towards a glycoprotein on lymphocytes belonging to the IgG2a and IgG2b isotype (CAMPATH-1) were used for the treatment of B-cell chronic lymphocytic leukaemia patients. The IgG2b isotype, in contrast to IgG2a, produced a long-lasting depletion of leukaemic lymphocytes from the blood and bone marrow without any changes in complement levels (48). This effect probably reflects the ability of rat IgG2b to interact with human FcγR-bearing effector cells rather than complement fixation, since both IgG2a and IgG2b are lytic with human complement, but only IgG2b binds to FcγR and mediates ADCC (48). Furthermore, the humanized version of some murine monoclonal antibodies containing human Fc regions were able to induce ADCC with human effector cells, a function absent from the original mouse antibody (46). These results convincingly show that by genetic engineering of the FcγR-binding site of IgG it is possible to produce antibody molecules with a higher therapeutic efficacy due, for example, to increased affinity for FcγR. The fact that an IgG molecule which does not bind in monomeric form to FcγRI (mouse IgG2b) can be rendered cytophilic by changing a single amino acid (35) suggests that engineered antibodies with higher affinity for FcγR-bearing effector cells may soon be applied in therapy.

Antigen-independent effector functions

Catabolism

Plasma IgG levels are tightly regulated by a process that is responsive to the concentration of IgG in the blood. The concentration of IgG in the blood or other body fluids is constant, since under normal conditions the rate of catabolism is equal to the rate of synthesis. The catabolic rate of plasma proteins (e.g. IgG) is related to the half-life ($t_{\frac{1}{2}}$) that directly correlates with the persistence of that protein in the circulation (catabolic rate = $0.69/t_{\frac{1}{2}}$). The usual measurement of IgG half-life is performed by injecting the subject with a known amount of radiolabelled IgG and following the decrease of plasma radioactivity as a function of time. Graphical representation of the logarithm of remaining radioactivity in plasma (in %) vs. time results in a biphasic curve from which the biological half-life can be calculated (Fig. 3). Determination of the biological half-life of IgGs in humans has shown that IgG catabolism is

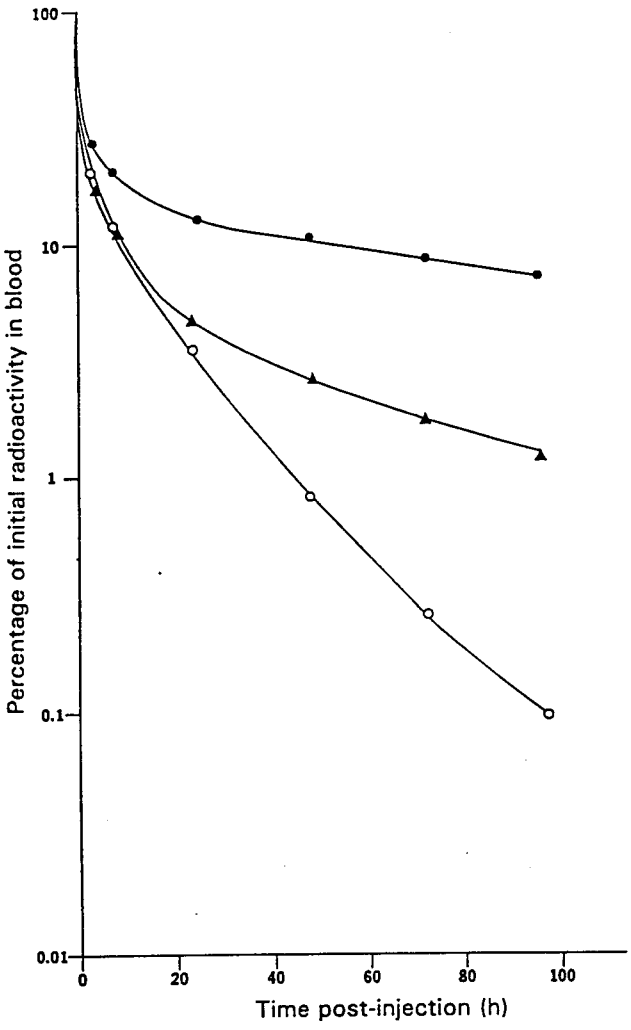

Fig. 3 Clearance curves of some Fc derivatives from the circulation of mice (59). (●) Fc WT; (○) Fc H310A-Q31 1N/H433A-N434Q mutant; (▲) Hybrid Fc consisting of one polypeptide chain from wild-type Fc and the other from H310A-Q311N/H433A-N434Q mutant.

accelerated in patients with increased IgG levels (short half-life) (e.g. in myelomas) and conversely decreased in patients with low serum concentration (long half-life) (e.g. in a- and hypogammaglobulinaemics) (50,51). This concentration–catabolism effect for IgG was also observed in germ-free and hyperimmunized mice (50,51). To explain the dependency between the rate of IgG catabolism and its plasma concentration, Brambell and colleagues (52,53) hypothesized that IgG is internalized by fluid phase endocytosis into the cells involved in catabolism, and a proportion of ingested IgG is bound to intracellular receptors that recognize the Fc region. Receptor-bound IgG molecules are 'protected' from lysosomal degradation and recycled into the circulation. In contrast, unbound molecules are degraded. Since the number of receptors is limited the increase in IgG concentration in serum will result in a shorter half-life as, due to saturation of the receptors, the proportion of internalized unbound IgG molecules is increased. To date, our knowledge of the cellular site where this process takes place, as well as the physico-chemical nature of the putative Fc receptors, are rudimentary (51). The participation of the gastrointestinal tract, liver, kidney and/or other cellular systems (e.g. reticuloendothelial system) is still an area of investigation and dispute (13,51), whilst little is known about the putative Fc receptors beyond the observation that the 'classical' FcγRs (I, II and III) are not involved in the regulation of the catabolism of IgG (54).

The participation of the Fc region of IgG in the control of its catabolism ('the catabolic site') was demonstrated by using various proteolytically derived fragments of the IgG molecule and measuring their persistence in the circulation (13,55). Further investigation concerning the localization of the catabolic site gave contradictory results, as some authors claimed only involvement of CH2 domain residues (56), whereas other data indicated possible involvement of residues in both the CH2 and CH3 domains (57). More recent data have demonstrated that the catabolic site encompasses residues at the CH2 and CH3 domain interface, overlapping with the protein A(SpA)-binding site (58–61). The amino acid residues of human IgG involved in binding to a monovalent fragment of SpA are clustered in three hydrophobic patches located at the CH2 and CH3 domain interface (Table 8) (Fig. 1) (62,63). Injection of murine IgG complexed with SpA or its fragment into mice resulted in rapid clearance, suggesting that residues located at or near the SpA-binding site are involved in regulating the catabolic rate of IgG (64–66).

To better define the site(s) of IgG involved in the control of catabolism, site-directed mutagenesis was used to generate mutant Fc fragments with the amino acids involved in SpA-binding (Table 7) altered as indicated in Table 9. These Fc fragments were derived from murine IgG1 and expressed in aglycosylated form in *Escherichia coli* (58). The pharmacokinetics of these mutants were investigated in mice. The data (Table 9) clearly demonstrate that the mutation of residues located at the CH2 and CH3 domain interface have significant effects on the half-life of the recombinant Fc fragments (58). Mutations in the CH2 domain (Ile-253 to Ala, His-310 to Ala and Gln-311 to Asn) have a more marked effect than those in the CH3 domain (His-433 to Ala, Asn-434 to Gln) and the mutant with both CH2 and CH3 domain mutations is cleared even more

Table 8 Staphylococcal protein A (SpA) -binding sites on murine and human Fc regions

Isotope		Amino acid residues in Fc involved in SpA binding*		
		252–253–254	308–309–310–311–312	433–434–435–436
Murine	IgG1	Thr-Ile-Thr	Ile-Met-His-Gln-Asp	His-Asn-His-His
	IgG2a	Met-Ile-Ser	Ile-Gln-His-Gln-Asp	His-Asn-His-His
	IgG2b	Met-Ile-Ser	Ile-Gln-His-Gln-Asp	Lys-Asn-Tyr-Tyr
	IgG3	Met-Ile-Ser	Ile-Gln-His-Gln-Asp	His-Asn-His-His
Human	IgG1	Met-Ile-Ser	Val-Leu-His-Gln-Asp	His-Asn-His-Tyr
	IgG2	Met-Ile-Ser	Val-Val-His-Gln-Asp	His-Asn-His-Tyr
	IgG3	Met-Ile-Ser	Val-Leu-His-Gln-Asp	His-Asn-Arg-Tyr
	IgG4	Met-Ile-Ser	Val-Leu-His-Gln-Asp	His-Asn-His-Tyr

*EU nomenclature.

Table 9 Half-life and SpA-binding activities of recombinant Fc derivatives (58,59)

Fc derivative*	Half-life (h)	SpA-binding activity†
IgG1	85.3 ± 7.3	100
Wild-type Fc	82.9 ± 10.0	100
H285A	76.0 ± 14.6	ND
I253A	20.0 ± 0.6	22.6
H310A-Q311N	17.5 ± 1.6	12.0
H433A-N434Q	50.3 ± 2.9	24.9
H310A-Q311N/ H433A-N434Q	15.6 ± 0.8	9.7

*All recombinant Fcs contain the hinge region. Designation of mutants: H285A = His-285 is substituted by Ala, H310A-Q311N = His-310 and Glu-311 are substituted by Ala and Asn, respectively, etc.
†Percentage of IgG1 binding activity.

rapidly than any of the other mutants. The half-life of an Fc fragment with a mutated histidine residue distal to the CH2 and CH3 interface (His-285) does not have a significantly lower value than the wild-type (Table 9), indicating that the mutation of histidine *per se* does not result in more rapid clearance of the Fc fragment.

The effect of the mutations on the binding of the recombinant Fc derivatives to SpA-Sepharose (Table 9) correlates well with the effect on biological half-lives (Spearman correlation coefficient 0.85), demonstrating that both the catabolic site and the SpA-binding site are overlapping. Furthermore, the recombinant wild-type Fc fragment has the same half-life as intact IgG1, indicating that at least for this isotype the presence/absence of the carbohydrate moiety does not affect catabolism, in agreement with earlier results (27). The amino acid residues that are involved in the catabolic site are highly conserved in IgGs of mouse and man (67). Polymorphism of some of these amino acid residues in mouse IgG2b (Lys-433) and IgG2a (His-433) and human IgG3 (Arg-435) and IgG1 (His-435) (Table 8) might explain the differences in the half-lives of these murine and human isotypes. Thus, IgG2a has a half-life of 5 days whilst the half-life of IgG2b is much shorter (3.5 days) (13,68). Similarly, human IgG3 has a much shorter half-life (7 days) than IgG1 (21 days) (13,50).

The question as to whether one or two of the CH2 and CH3 domain interfaces are necessary for maintaining the IgG1 molecule in the circulation was addressed by constructing a hybrid Fc fragment comprising one wild-type heavy chain (with long $t_{\frac{1}{2}}$) and one mutant heavy chain (H310A-Q311N/H433A-N434Q) (with short $t_{\frac{1}{2}}$). The half-life of the hybrid molecule (37.9 h) was significantly less than that of the wild-type Fc (82.9 h) but longer than that of the mutant (16 h) (Fig. 3) (59). This indicates that both catabolic sites located at the CH2 and CH3 interface are probably necessary for a Fc/IgG molecule to be maintained in the circulation with the half-life of a wild-type Fc/IgG. The data are consistent with a study in which it was shown that an SpA fragment–mouse IgG complex conjugated at a molar ratio of one SpA fragment molecule to one IgG molecule was cleared rapidly from the circulation (65).

The segmental flexibility and spacing between the CH1 and CH2 domains conferred by the hinge region appears to be unnecessary for the functioning of the catabolic site (60). As described above for complement fixation (see section on complement fixation), by substituting the hinge region attached to the Fc fragment (Val-Pro-Arg-Asp-Cys-Gly Cys-Lys-Pro-Cys-Ile) with a 'synthetic' hinge, introducing a single disulphide bond between the two heavy chains (Gly-Cys-Gly Ala-Ser), the engineered Fc has a half-life (72.0 ± 7.0 h) which is not statistically different from that of wild-type Fc-hinge (79.8 ± 9.5 h) (60). On the other hand, another recombinant Fc fragment (48.7 ± 7.8 h) and Fc

obtained by enzymatic digestion (56.0 ± 5.8 h), both lacking the hinge region and therefore devoid of disulphide bridging between the two heavy chains, have half-lives that are significantly shorter than those of recombinant Fc fragments with 'natural' and synthetic hinge regions (60). Limited proteolysis was used to demonstrate that the Fc fragments without the disulphide bridges between the heavy chains have different conformations in the region of the CH2 and CH3 domain interface. This is most likely due to increased mobility of the CH2 domain which in turn results in structural perturbations in the region of the catabolic site (69).

The half-life of a therapeutic antibody is a critical clinical parameter as it determines the amount of IgG to be infused and how often the infusion should be repeated. The repeated administration of IVIG at intervals more frequent than its half-life (usually around 21 days) will result in the increase of its concentration until it reaches the steady state level determined by the concentration–catabolism relation (70). When murine therapeutic antibodies are administered to humans, two experimental obstacles have to be overcome: the short half-life and the immunogenicity (71,72). The half-life of a murine IgG antibody in humans is very short (15–30 h) (13) and therefore requires frequent administration. In turn, repeated administration results in a human anti-mouse antibody response with consequent elimination of the antibody. In view of the conservation of the residues of the catabolic site in both man and mouse (Table 8), the short half-life of murine antibodies in humans is surprising. Furthermore, human IgGs have the same half-lives as murine IgGs in mice (73). The short half-life of murine IgG is related to the presence of some natural antibodies in the human plasma that recognize an epitope located on the carbohydrate moiety of murine IgG (74). Therefore, the rapid elimination of therapeutic murine antibodies is due to immune clearance. As a consequence, elimination of the carbohydrate from murine IgG by either enzymatic deglycosylation or by DNA technology (e.g. conversion of Asn297 to Gln) might increase the half-life of these antibodies in humans, at least of those belonging to the IgG1 isotype. Another experimental way to increase the half-life and to reduce the immunogenicity is to use chimeric mouse/human antibodies containing the Fc region derived from human IgG. These antibodies appear to have a half-life between that of murine IgG and human IgG (i.e. six times as long as murine IgG and one-fifth as long as human IgG) and, as expected, are less immunogenic (71). The fact that chimeric mouse/human IgG antibodies with human Fc regions have a half-life that is still shorter than that of human IgG (4 days vs. 21 days) does not mean that regions outside the Fc control the catabolism of the chimeric IgG molecule, but rather suggest that polyreactive autoantibodies recognizing epitopes on the variable region of the mouse IgG might cause the more rapid elimination of the chimeric IgG as compared with human IgG.

The data concerning the localization of the catabolic site of IgG—in addition to the demonstration that two sites per Fc and an interheavy chain disulphide bond are needed for optimal serum persistence—has implications for the engineering of therapeutic proteins, by tagging them with recombinant IgG fragments. Clearly, serum persistence analogous to that of IgGs can currently only be achieved by linking a complete, dimeric Fc fragment to the protein of interest.

Maternal transmission

In mammals, maternal IgGs are transmitted to the foetus via the placenta (humans, primates) or yolk sac (rabbits) and to the neonate from the colostrum via the small intestine (ruminants). Rodents use both methods of transfer, with maternal–foetal transfer across the yolk sac playing a minor role, and transfer from mother's milk via the small intestine being the major route (75). Immunization of the mother can be used to protect the offspring against infectious disease, as protective IgGs induced in the mother will be transferred (75). This is important, as the immune system of the new-born is not fully developed (75). This procedure has been applied in the clinic to protect new-born infants (especially pre-terms) against a variety of infectious diseases (76,77).

The transfer of IgG antibody across the cellular barrier of the placenta, yolk sac or intestinal brush border involves the process of transcytosis. This process is mediated by Fc receptors (in mice and rats called FcRn) that—particularly from rodent intestinal brush borders—have been well characterized. Rodent FcRn comprises a heterodimer of two polypeptide chains with different molecular masses (50 kDa and 14 kDa) (78–80). The heavy chain shares sequence homology with the heavy chain (alpha) of MHC class I molecules, whilst the light chain is, as in an MHC class I heterodimer, beta-2-microglobulin (78–80). The X-ray crystallographic structure of rat FcRn revealed that the alpha-1 and alpha-2 domains have a very similar backbone structure to MHC class I counterparts (81,82). However, a significant difference is that the alpha helices are not separated and therefore the groove that binds peptides in the MHC class I molecule is absent in FcRn. These structural differences reflect the distinct

functions of the two classes of protein. Rat FcRn binds to the rat Fc fragment at the edge of the alpha-1/alpha-2 domain region, which is distinct from the sites of interaction of Class I with either peptides, CD8 or T-cell receptors (82). Beta-2-microglobulin of FcRn may also make additional contacts with the Fc (81,82).

FcRn is expressed on the surface of jejunal epithelial cells (brush borders) on their apical face. The binding takes place at the pH of the intestinal lumen, ranging from 6 to 6.5, and the FcRn–IgG complex is endocytosed and transcytosed to the basolateral face of the cells where IgG dissociates due to the higher pH of the blood (83,84). The pH dependence of IgG binding has been clearly demonstrated by studying the interaction of recombinant soluble FcRn with IgG (85). The receptor involved in IgG transcytosis across the placenta or yolk sac is not as well characterized as FcRn, but the available data indicate that the placental FcR is similar if not identical to FcRn (86,87). However, a major difference between the intracellular localization of FcRn on enterocytes or yolk sac has been reported (86). In contrast to FcRn, which is localized on the apical surface, the yolk sac FcR is localized only on the endocytic vesicles in the immediate vicinity of the apical membrane of yolk sac endodermal cells (86). This finding is in agreement with the fact that no pH gradient was observed between the maternal and foetal faces of placenta or yolk sac, suggesting that if the FcR is similar to FcRn, pH dependent binding should occur in a slightly acidic intracellular compartment. Consistent with this, the yolk sac FcR is located in an intracellular compartment, suggesting that binding of IgG takes place after this protein is internalized by nonspecific fluid phase endocytosis (86). It should be noted that this possibility was suggested earlier by Brambell (53,75) in his unifying hypothesis on the mechanism of IgG transmission and catabolism.

Localization of the FcRn binding sites in the IgG molecule using proteolytic fragments yielded contradictory results, indicating involvement of either solely the CH2 domain (88) or both CH2 and CH3 domains (89,90). The Brambell hypothesis postulates that both catabolism and transcytosis take place through the same site of IgG, being recognized by FcRn or putative Fc receptors involved in catabolism control (75). If this assumption is correct, the catabolic site should overlap with the FcRn binding site. As a consequence, IgGs with a high catabolic rate should have low transmission and vice versa. This was verified experimentally by studying the intestinal transmission in neonatal mice of recombinant mouse Fc fragments (wild-type and mutants) that have half-lives ranging from short (16 h) to long (83 h) (Tables 9 and 10). Intestinal transfer of

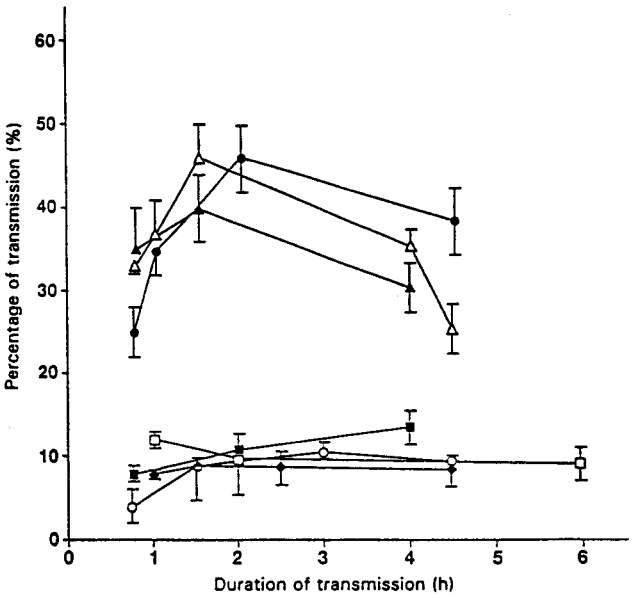

Fig. 4 Intestinal transfer in neonatal mice of radiolabelled murine IgG1, Fab and various Fc derivatives (91). Neonatal mice were force fed with radiolabelled proteins and bled at different times following feeding. The percentage of radioactivity transferred from gut to circulation was calculated using a blood volume equal to 7.2% of body weight. (○) Fab; (●) mIgG1; (△) Fc-papain; (▲) WT Fc-hinge; (□) H310A-Q311N/H433A-N434Q; (■) WT Fc-hinge in adult mice; (◆) Hybrid Fc.

radiolabelled recombinant Fc fragments from the proximal intestine to the circulation was analysed by force feeding neonates with the radiolabelled proteins (91). Approximately 45% of an Fc fragment obtained by papain digestion was transferred into the circulation (Fig. 4). Similar levels of transfer were seen for a recombinant wild-type Fc fragment. In contrast, only 10% of the H310A-Q311N/H433A-N434Q mutant Fc and the hybrid Fc (with one heavy chain from the wild-type Fc and the other from the mutant Fc) is transferred. This value is close to that of both an Fab fragment and wild-type Fc fragment in adult mice used as negative controls in these experiments (Fig. 4) (91).

Since the H310A-Q311N/H433A-N434Q mutant Fc is cleared from the circulation at a much higher rate than the wild-type Fc (Table 9) it is conceivable that the lower level of radioactivity in plasma might be due to more rapid clearance rather than lower transmission. To eliminate this possibility, experiments measuring the ability of various nonradioactive Fc derivatives to inhibit the transfer of radioactive IgG were carried out, with the aim of obtaining data independent of the catabolic rate. Consistent with the direct transfer results, the inhibition data clearly show that the wild-type

Table 10 Inhibition of intestinal transmission of radiolabelled murine IgG1 by recombinant Fc derivatives (91)

Conipetitor (unlabelled)	Transmission (%)	Inhibition of transmission*
None	54.1 ± 9.7	–
IgG1	26.3 ± 5.7	51.4
Fc-papain	20.3 ± 7.1	62.5
Wild-type Fc	24.2 ± 4.3	55.3
H310A-Q311N	49.3 ± 7.3	8.9
I253A	43.2 ± 5.3	20.2
H433A-N434Q	30.1 ± 4.4	44.4
H310A-Q311N/ H433A-N434Q	51.2 ± 8.3	5.4
Hybrid Fc†	42.5 ± 5.9	21.5
BSA	49.3 ± 5.1	8.9

*Relative to transmission of radiolabelled IgG without any competitor.
†Consisting of one polypeptide chain from wild-type Fc and the other from H310A-Q311N/H433A-N434Q mutant.

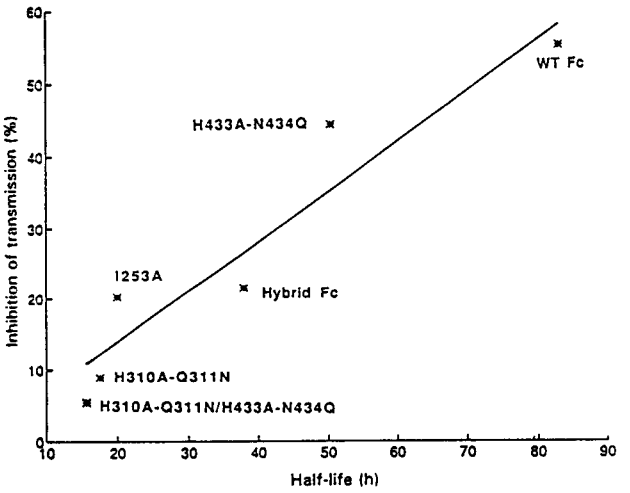

Fig. 5 Correlation between the inhibition of transmission of radiolabelled mouse IgG1 by Fc fragments and their beta-phase half-life (58,91). The data were taken from Tables 9 and 10.

Fc and Fc-papain have similar inhibitory capacities as unlabelled IgG1, whereas the mutant Fc fragments and the hybrid Fc inhibit transfer at much lower levels (Table 10) (91). These results were confirmed by an *in vitro* experiment investigating the binding of the wild-type Fc and the mutant Fc to brush borders isolated from neonatal mice (91). The data strongly support the concept that both catabolic and transcytosis sites encompass the same amino acid residues at the CH2 and CH3 domain interface. This finding is supported by the excellent correlation (Spearman correlation coefficient 0.95) between the inhibition of transmission of radiolabelled IgG1 by a particular Fc fragment and its half-life (Fig. 5). The involvement of amino acid residues at the CH2 and CH3 interface in maternal transmission of IgG (91) was confirmed by showing that an SpA fragment can inhibit the binding of FcRn to Fc (92) and by solving the three-dimensional structure of the FcRn–Fc fragment complex at 6.5 Å resolution (81,82). This structure shows that histidine residues at positions 310, 433 and 435 potentially contact regions in the alpha-1 and alpha-2 domains of FcRn that contain no histidines, thus confirming the earlier finding that only histidine from the IgG CH2 and CH3 interface, and not from FcRn, are responsible for the pH-dependency of IgG transepithelial transfer. Furthermore, the pH dependency of the FcRn–Fc interaction in a range covering the pK_a values of histidine is consistent with the involvement of these residues in the interaction (91)

The data for the hybrid Fc fragment indicate that for an IgG to be effectively transmitted across the intestinal barrier, two functional CH2–CH3 domain interface sites per molecule are necessary (91) (Table 10). Bivalency at the level of the Fc region appears to be necessary for binding to FcRn and/or transfer, and this is consistent with *in vitro* studies showing that two recombinant FcRn molecules are bound to one rat Fc fragment (81,82,85). The inhibition data (Table 10) show that the Fc hybrid *does* retain higher affinity for interaction with FcRn than the CH2 domain mutants, however, and this suggests that the primary deficiency of this molecule is in transcytosis. By analogy, dimeric IgA is transcytosed with higher efficiency than monomeric IgA (93), and this may be a manifestation of the need for the transcytotic receptors to form dimers to traffic across cells.

The wild-type Fc fragment was transcytosed in a similar manner to an Fc fragment derived by proteolysis from the glycosylated IgG1 molecule. This suggests that the conclusions concerning the location of the FcRn binding site drawn from experiments using aglycosylated Fc fragments can be extended to glycosylated IgG (91). This is in agreement with earlier results showing that removal of the carbohydrate from a mouse IgG2b antibody by site-directed mutagenesis (Asn297 to Ala) had no effect on its binding to FcRn *in vitro* and *in vivo* (94).

Immunoglobulin G is the only plasma protein that can cross the placental (yolk sac) barrier and be transferred from mother to foetus (75). For transfer from mother to foetus, this unique property might be used for transporting polypeptides of therapeutic value that

normally cannot cross the placental barrier. To achieve this goal, IgG (or its Fc fragment) should be used as a 'carrier' for the therapeutic polypeptide. An excellent example of this possibility is the synthesis of 'immunoadhesins' containing an adhesion molecule (e.g. CD4) linked to the immunoglobulin Fc fragment (95–97). The immunoadhesin consists of a portion of the CD4 molecule (amino acid residues 1–180) fused to human IgG1 heavy chain sequences commencing at the first residue in the IgG1 hinge region, Asp-216 (95,96). Due to the dimeric nature of the Fc region, two CD4 fragments are present in this chimeric molecule. The half-life of the CD4–Fc dimer in the circulation of rabbits was 48 h vs. 0.25 h for CD4 and 113 h for human IgG1 (95). The active transport of the immunoadhesin across the placenta of pregnant Rhesus monkeys occurred at a rate comparable to that of human IgG1, achieving a concentration that was 3% of that in the circulation of the mother (96). These data suggest that the Fc portion of the immunoadhesin retains its affinity for FcRn on the apical surface of the syncytiotrophoblast and can be transferred efficiently across the placenta (96). In addition, the immunoadhesin can concentrate in the foetal tissue and, thereby, protect the foetus from HIV infection *in utero* (96). Finally, the immunoadhesin mediates the *in vitro* killing of HIV infected cells by ADCC, thus indicating that the Fc portion of the CD4–Fc dimer has retained its ability to bind FcγRI (96).

These studies have clearly indicated the feasibility of using the Fc portion of IgG as a carrier for various bioactive agents. The construction of chimeric proteins containing the Fc region not only results in passage across the placenta to the foetus, but also a much longer residence time in the foetus compared to the persistence of the nonconjugated bioactive agent. In addition, these experiments have convincingly shown that for effective transmission and a long half-life, the Fc portion should contain the complete Fc region tethered in the N-terminal hinge region by one or more disulphide bonds, in agreement with the data concerning the common localization of the catabolic and transcytosis sites of murine IgG1 (61,91).

Conclusions

The data presented in this review indicate that the two classes of effector functions of the IgG molecule, the antigen-dependent and antigen-independent, are localized in well defined and separate regions of the IgG molecule (Fig. 1). The antigen-dependent functions, manifested by the activation of the humoral (C1q binding) and cellular (FcR binding) systems are localized solely in the CH2 domain, in proximity to the lower hinge region. The extent of overlap between the C1q binding site and the FcR binding site are not yet clearly defined, but there is evidence that three sequentially distant, but spatially close peptide stretches are involved in building both C1q and FcR interaction sites. Thus, the lower hinge region containing the amino acid residues in positions 233–237 is involved in both FcR and C1q binding. The bend b6 encompassing residue 331 which stretches between beta-strands fy2 and fy3, adjacent in space to the lower hinge region, is also involved in interacting with both C1q and FcRs. Finally, the fy2 beta-strand, in proximity to the b6 bend and lower hinge region contains amino acid residues that are involved in C1q (318, 320 and 322) and FcR binding (318). Both the humoral and cellular functions of IgG are dependent on the presence of the carbohydrate bridging of the two CH2 domains of Fc. The removal of the carbohydrate may induce conformational changes in the lower hinge region, b6 bend or fy2 beta-strand which alters the conformation of the binding sites. A logical conclusion of these findings seems to be that these two functions are mutually exclusive, in so far as an antibody molecule bound to the antigenic target can bind either C1q or FcR-bearing cells, but not both simultaneously. Suggestive evidence for this possibility resides in the ability/inability of an antibody belonging to certain IgG isotypes to activate complement or to induce ADCC (Table 7). Experiments showing that immune complexes cannot bind to FcR-bearing cells once they are attached to C1q or vice versa are necessary to demonstrate that these two functions are mutually exclusive. This suggests that it will be difficult to genetically engineer an IgG antibody to abolish complement activation whilst retaining FcγR binding.

The antigen-independent functions of the IgG molecule (control of catabolism and transcytosis) are localized in a distinct site of the molecule, namely the CH2 and CH3 domain interface (Fig. 1). In this hydrophobic interface there are three regions which are sequentially distant (residues at positions 253, 310–311 and 433–434) belonging to both CH2 and CH3 domains, but spatially close, building a conformational site that interacts with the transcytotic receptor FcRn. The site is distinct from that involved in interacting with the 'classical' FcγRs (FcγRI, II and III). FcRn, or a homologue, has been isolated from the cells involved in maternal transmission of antibody to offspring in rodents, and more recently, in humans (87). The Fc receptors involved in regulating the clearance rates of antibodies have yet to be identified, but based on the close overlap between the transcytosis and catabolic sites of the antibody they may

be very similar to FcRn. However, the cellular location of the FcRs involved in the two processes may differ so that their pH-dependent binding might be exerted in the endosomal system of the catabolic cells (as in placental transfer) and not on the cell surface (as in intestinal transfer). The tissue distribution of heavy chain mRNA encoding an FcRn homologue has indicated expression in human heart, liver, lung, muscle, kidney and pancreas in addition to placenta, suggesting that some of these organs might be involved in IgG catabolism (87).

In contrast to antigen-dependent functions, catabolism and transcytosis do not depend on the presence of the CH2-linked carbohydrate in the IgG molecule. This again supports the idea that the antigen dependent and independent effector functions are carried out by distinct regions of the IgG molecule. The CH2 and CH3 interface is a region with multiple functional abilities besides controlling catabolism and transcytosis. Thus, amino acid residues that are involved in catabolism and transcytosis also play a role in the binding of SpA (67), Streptococcal protein G (98) and some rheumatoid factors (99;100) suggesting that in certain pathological conditions (e.g. Staphylococcal and Streptococcal infections, Rheumatoid arthritis) the half-life, in addition to the transmission of endogeneous IgGs, might be altered.

Finally, the application of current techniques of genetic manipulation suggest that the manufacture of antibodies 'to order' will be possible in the near future. For example, using bacteriophage display (16), high affinity binding sites for an almost unlimited number of antigens can be generated. The variable domains can then be linked to constant regions that have been genetically modified to endow the optimal effector functions for a particular therapeutic situation. The enormous potential of such antibodies should provide us with a powerful and highly specific armoury for use in immunotherapy.

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