Chapter

FIVE

Genetic Manipulation of Antibodies: From Variable Domains to Constant Regions

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INTRODUCTION

Antibodies bind to cognate antigen with high affinity and specificity and are therefore ideal reagents for use in diagnosis and therapy (reviewed in [1]). Recent developments in recombinant DNA technology have resulted in the production of a new generation of "designer" antibodies (reviewed in [2–4]), and it is now possible to rapidly isolate antibodies that recognize an almost unlimited number of different antigens. Two developments in technology have facilitated this: first, the use of the polymerase chain reaction (PCR, [5]) to isolate immunoglobulin variable domain genes and second, the use of prokaryotic (*Escherichia coli*) expression systems [6,7] to assist in the identification of clones producing the desired binding specificity. In addition to methods for the isolation of the optimal binding site, knowledge about the effector functions of each isotype is now available [8,9]. As a result, the variable domains can be linked to constant regions that confer the effector functions of choice. Recent work in our laboratory has identified the region of the murine IgG1 molecule that is involved in controlling the pharmacokinetics of this isotype in mice [10], and extension of this work to human IgGs could result in the production of genetically manipulated IgGs that have predesigned pharmacokinetics.

In this review, developments in the area of the generation of antibody gene libraries and isolation of binding specificities will be described, and this description will encompass the use of *E. coli* as a host and bacteriophage display systems. This will be followed by a discussion of the current understanding of the factors that govern the pharmacokinetics of gammaglobulins (IgGs) and the interrelationship between IgG transcytosis and catabolism. Although the isolation of antibody fragments with the desired binding specificity and IgG catabolism appear to be diverse areas, in our laboratory these studies are interlinked by the use of *E. coli* as a host for the production and analysis of the relevant portions of the IgG molecule.

I. THE STRUCTURE OF THE ANTIBODY MOLECULE

A relatively large number of high resolution structures are now available for antibodies [11–15] and antibody–antigen complexes ([16–21]; reviewed in [22]). From these structures, it is clear that the antibody molecule is made up of strings of domains that are linked to each other by relatively flexible peptides. These domains have β sheet structures and are pinned together by an intramolecular disulfide bridge to form the immunoglobulin (Ig) fold. Within the immunoglobulin superfamily, different members can be subdivided into groups called "sets" [23]. Members of these sets are more similar to each other in structure than those in another set and to date, four sets have been identified [24]. For the variable domains, the ends of six of the β strands are connected to each other by the hypervariable loops (or complementarity determining regions, CDRs), and it is the residues within and flanking these loops that confer antigen binding specificity. Unequivocal experimental proof that antigen binding affinity and specificity is conferred by CDR and flanking residues comes from two types of studies: high resolution structural studies of antibody–antigen complexes involving both X-ray crystallography [16–21] and NMR [25–29], and CDR grafting or humanization of rodent antibodies ([30–35], reviewed in [36]].

The Binding Site: Evolution of the Minimal Unit of Antigen Binding

The minimal unit of antigen binding was originally believed to be the Fv fragment [37,38], which comprises the heavy and light chain variable domains (VH and VL, respectively). However, it has become clear that smaller units of antigen binding exist, such as VH domains (or single domain antibodies; [39–41]), minibodies [42,43] and minimal recognition units (MRUs; [44,45]). VH domains (or single domain antibodies, dAbs) with good affinities for antigen binding were isolated from libraries of these single domains secreted from *E. coli* [39]. More recently, dAbs with other specificities have been described [40,41]. The minibody [42] is a 61 residue β sheet structure and was designed using a VH domain as a template. The β sheet scaffold of this truncated VH domain supports the equivalent of CDR1 and 2. MRUs are CDR-derived peptides that retain antigen binding activity. Thus, the minimal unit of antigen binding has become progressively smaller, and the use of molecular modeling to generate further binding units of this type is an exciting area of protein design.

The Constant Region: Effector Functions and Control of in vivo Half-Life

The constant region of the IgG molecule is involved in carrying out the effector functions such as complement fixation and antibody dependent cell mediated cytotoxicity (ADCC). Different isotypes vary in their ability to carry out these functions [8,9], and isotype switching can now be performed in vitro to alter the effector functions that are attached to the variable domains conferring a particular binding specificity (described in more detail in §IV). The constant region is also involved in maintaining serum IgG levels and in transcytosis of IgGs and site-directed mutagenesis has recently been used to identify the region of the murine IgG1 molecule that is responsible for these functions [10,46]. This will be discussed in more detail below.

II. GENERATION OF VH AND VL LIBRARIES AND ISOLATION OF FRAGMENTS WITH ANTIGEN BINDING ACTIVITIES

The Use of the PCR

The PCR can be used to rapidly isolate the genes encoding the VH and VL of an antibody from antibody producing cells [47–52]. In the simplest case, that is, a hybridoma, these antibody producing cells are clonal. If libraries of V genes are to be generated, however, murine splenocytes or human peripheral blood lymphocytes (PBLs) are an ideal source of V genes. There are now primer sequences available to produce diverse libraries of V genes from both mouse and man, and these libraries can be further diversified by either point mutagenesis [53,54] or cassette mutagenesis [55,56]. For cassette mutagenesis, the CDRs and flanking framework residues are usually targeted [55,56], as targeting to other regions could result in the production of a high proportion of misfolded proteins. Mutagenesis of other regions also has no obvious advantage as much structural and biochemical data indicate that residues in the CDRs and flanking framework regions are involved in binding to antigen. It is therefore relatively straightforward to produce diverse repertoires of V genes, but the challenge is now the identification of the clones that produce antibody fragments with the desired binding specificity. This is an area in which the use of *E. coli* has obvious advantages over the use of other expression hosts.

E. coli as an Expression Host

In 1988, two reports [6,7] of expression of Fv and Fab fragments as secreted proteins from *E. coli* opened up new avenues for the isolation of fragments with the desired binding specificity from libraries of Ig fragments. The major advantage of these secretion systems is that the Fv or Fab is produced in either the periplasm or culture supernatant in a functionally active form. This avoids the need for cell lysis followed by refolding, which although possible for individual Fvs or Fabs, is clearly impractical for the screening of large numbers of clones. Using *E. coli* secretion systems, antibody VH domains [39] or Fabs [51] were isolated from expression libraries by screening culture supernatants for the presence of binding activities or probing colony lifts with labeled antigen, respectively. However, for both these approaches the numbers of clones that can be readily screened are significantly less

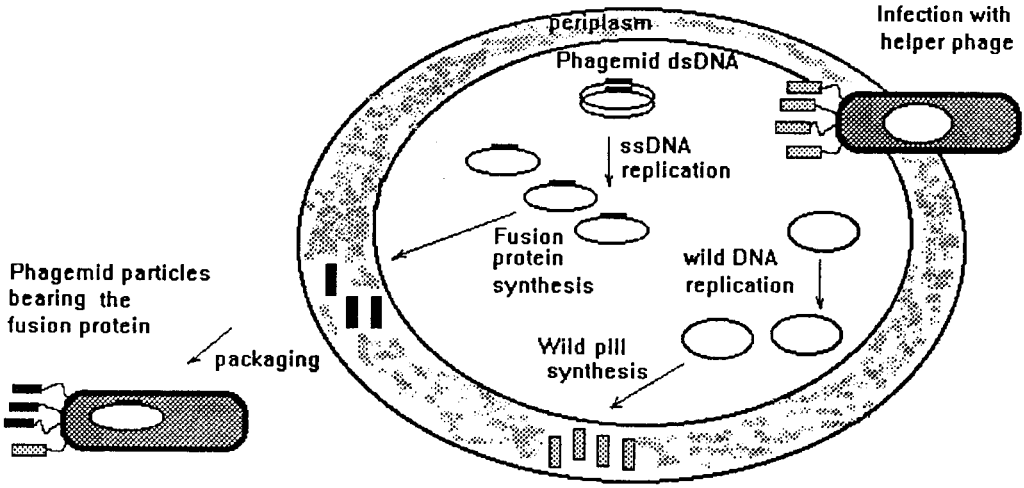

Figure 1. Bacteriophage display. Filled in boxes represent scFvs linked to the coat protein pIII (or pVIII). Helper phage superinfection results in the production of wild type pIII which competes with scFv–pIII fusions for assembly into the phage coat. Thus, phage particles usually bear a mixture of wild type and chimeric pIII-scFv/Fab proteins on their surface.

than the total number of unique V gene combinations that can be generated, and therefore the advantage of producing a diverse library in order to isolate the "best" binder is partially lost. This problem has been overcome by using a selection system based on bacteriophage display in which binders are selected prior to screening.

Bacteriophage Display

The display of peptide libraries on the surface of bacteriophage [57,58] has been extended to the display of single chain Fv (scFv, [59,60]) or Fab fragments in functional form ([61–63]; Figure 1). The genes encoding single chain Fvs (scFvs) or Fabs are linked in translational frame to the coat protein genes (coat protein pIII or pVIII) of filamentous phage. This has led to the generation of large, diverse libraries of antibody fragments which can be displayed on the surface of bacteriophage [64–73]. In a recent report in which *Cre/lox*P mediated recombination was used to recombine heavy and light chains, libraries as large as 10^{10} members were made [74]. This library therefore approaches the size of the immune repertoire of humans (approximately 10^{12}).

The phage that bear recombinant scFvs/Fabs can be "panned" on antigen coated surfaces to isolate phage with binding specificity that can be used to reinfect *E. coli*. The obvious advantage of this system is that the binding specificity in the form of a scFv or Fab is physically connected to the genetic material that encodes it. To an extent, this therefore resembles a B cell with surface antibody. Following several rounds of selection to further enrich for specific binding, the phage "binders" can be used to reinfect *E. coli* and the DNA encoding the scFv or Fab isolated in clonal form. Furthermore, by switching the bacterial host strain [65] or by a straightfor-

ward restriction and ligation step [62], the once surface bound scFv/Fab can be expressed as a soluble secreted fragment. The production of soluble fragments clearly facilitates purification and further characterization.

Using bacteriophage display systems, scFvs and Fabs have now been isolated that recognize a wide array of both protein and hapten antigens [64–74]. Many of these antibody fragments have therapeutic potential, for example, neutralizing activity against viruses [67,68,73]. Bacteriophage display systems were initially used to isolate antibodies from libraries derived from immunized mice or humans. More recently, antibodies of good affinities have been isolated from enormous libraries of human origin [65,69,72,74]. In several cases [72,74], the CDR3s of the heavy chains were generated in vitro. The potential of these systems to isolate antibodies of almost any specificity is now almost unbounded, and possibly the current limitation is the availability of the antigen of interest for use in panning. However, it is clear that the antigen used need not be purified to homogeneity and this is a significant advantage.

Bacteriophage display can also be used as a route to the rapid isolation of binding specificities from a new, powerful way of generating high affinity human antibodies that has recently been described by two independent groups [75,76]. This approach involves the generation of mice that are transgenic for human V and C region loci. Following immunization, such mice produce isotype-switched, high affinity antibodies, and to date these antibodies have been isolated using hybridoma technology. The phage display systems could readily be used in concert with these transgenic mice as an extremely rapid way of isolating antibodies. These methodologies have enormous scope for the production of antibodies for use in therapy.

Isolation of Anti-TCR Antibodies

A major interest of our laboratory is to generate reagents for the diagnosis and therapy of T cell mediated autoimmunity. The oligoclonality of T cell responses in two murine models of T cell mediated autoimmunity, namely experimental allergic encephalomyelitis [77] and collagen induced arthritis [78,79] suggests that anti-TCR V region antibodies have potential both as diagnostic reagents and in treatment. Furthermore, a significant amount of evidence indicates that the T cell responses in multiple sclerosis [80–83] and rheumatoid arthritis [84,85], for which EAE and CIA are instructive models, are oligoclonal. For Vα regions of TCRs, in particular, there are very few family specific antibodies available and this has hindered the analysis of TCR (Vα) expression at disease sites. The reasons for the difficulty in generating anti-TCR V region antibodies are not clear, but prior to the development of expression systems [86–96] for the efficient production of soluble TCRs, the lack of availability of immunogens in pure form was one of the limitations. One way of circumventing the use of soluble TCR protein to raise antibodies is to use murine transfectomas expressing heterologous (human) TCRs as immunogens [97–99]. Despite the current availability of production systems using both mammalian [86–91] and prokaryotic hosts [92–96], there have been relatively few reports of the successful use of soluble TCRs to generate anti-Vβ antibodies (for example, [100–102]). This paucity of antibodies is even more marked for anti-α chain antibodies (for example, [103,104]).

We therefore decided to apply two relatively new technologies to the production of anti-Vα antibodies: i) the use of an *E. coli* expression system [92,94] for the production of milligram quantities of single chain TCRs (scTCRs, comprising the Vα domain linked to the Vβ domain by a synthetic peptide linker) and Vα domains (designated Vαs) and ii) a bacteriophage display system to isolate scFvs that recognize soluble TCRs, in particular Vαs. Mice have been immunized with soluble TCRs (Vα or scTCRs) derived from two different TCRs that are representative of the TCRs borne by oligoclonal pathogenic TCRs in EAE (designated 1934.4, [105]) and CIA (designated qcII85.33, [106]). The antibody VH and Vκ genes have been isolated from the splenocytes of these mice and used in two different approaches to generate anti-Vα antibodies, as described below:

VH shuffling to convert an scFv of anti-hen egg lysozyme specificity to one that recognizes a TCR Vα

In many antibody: antigen complexes that have been structurally solved (for example, [16–21]), the VH residues contribute more to antigen binding than those of the VL. The major role of the VH in binding to antigen is also substantiated by the observation that VH domains (or dAbs) with good affinities for binding to antigen could be isolated from VH libraries made from splenocytes of immunized mice [39]. Furthermore, camels have antibodies that are made up solely of heavy chains [107], indicating that the added diversification of the immune repertoire by light chains is not necessary for this species. The expression of VH libraries as phage displayed domains is technically difficult, however, presumably due to the relative hydrophobicity of VHs in the absence of Vκ partners (G. Winter, pers. commun.). This prompted us to generate a VH gene library from a mouse that had been immunized with a TCR Vα (1934.4 Vα). This VH library was used to replace the VHD1.3 (anti-lysozyme) gene in a phagemid containing the VH and Vκ genes of the D1.3 antibody (Figure 2; [108]). This VHx-VκD.13 library was used as a source of scFv genes for expression in a bacteriophage display system. The recombinant bacteriophage were panned against soluble purified 1934.4 Vα and one scFv, designated VH14:VκD1.3, was isolated that bound to the Vα with reasonable affinity (Table 1). The amino acid sequence of VH14 is shown in Figure 3a. To improve the affinity further, a light chain shuffling experiment using a Vκ library from the same immunized mouse was carried out [108]. The affinity of the antibody was improved 30-fold (Table 1). Sequencing of the Vκ (Vκ9) gene that in combination with VH14 led to this improved affinity gave a slightly unexpected result. The Vκ gene had the same sequence as the D1.3 Vκ with the exception of two point mutations which resulted in amino acid changes in two CDRs (Figure 3b). Thus, the origin of Vκ9 is not clear, but it is most likely derived from PCR contamination with the D1.3 Vκ (albeit at very low levels as controls gave no evidence of such a possibility) followed by mutation due to misincorporation by Taq polymerase. This indicates that the same result could have been reached by PCR mutagenesis followed by selection using bacteriophage display [53,54].

Several questions emerge from this study. First, why was only one VH isolated, which in combination with VκD1.3 produced an antibody that recognized the Vα? Possible reasons are that in this type of experiment, there is a requirement for the

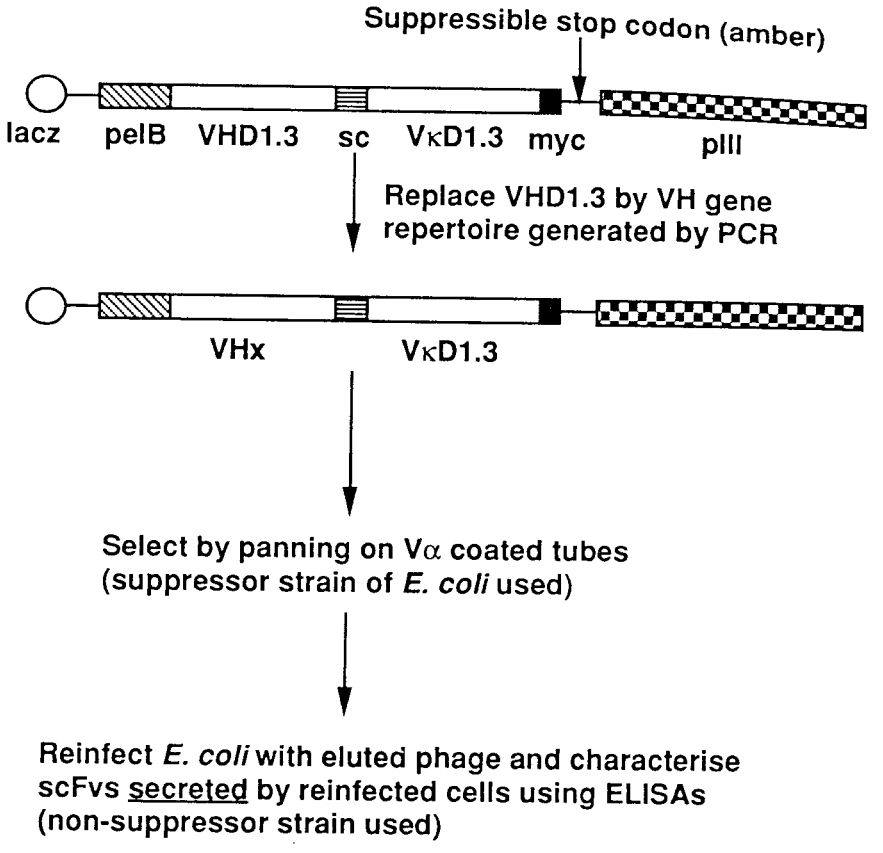

Figure 2. Strategy for VH shuffling experiment to convert a scFv of anti-hen lysozyme specificity to one that recognizes a TCR Vα. Open circle = lacz promoter, pelB = pelB leader sequence for export into periplasm, open boxes = VH and VL genes, horizontal lines = single chain (sc) linker sequence and chequered box = gene encoding pIII. Suppression of the amber codon results in the production of scFv-pIII chimeras.

VH to not only bind to the antigen, but also to pair well with a single Vκ (VκD.13 in this case). Although promiscuity of VH: Vκ pairing has been observed [64,71,109, 110], it can vary from one VH/Vκ to another [64,71]. In addition, the library was relatively small, and if larger libraries are constructed it may be possible to isolate more VHs that in association with the VκD1.3 change the binding specificity. Second, why was only one Vκ (a close homolog of VκD1.3.) found that could combine with the VH to produce an antibody with improved affinity? This suggests that the limitation is the lack of promiscuity of VH14. In other words, in the VH shuffling experiment, a VH (VH14) has been selected for good pairing with VκD1.3 or a close homolog, such as Vκ9.

Taken together, the data from this series of experiments indicate that VH shuffling can be used to change the specificity of an antibody, again emphasizing the role of VH in antigen recognition and specificity. A more specific feature of this experiment, which clearly may not be general to other Fv interactions with antigen,

Table 1. Kinetic Parameters for Fvs That Recognize 1934.4 Vα

	VH14:VκD1.3	VH14:Vκ9 (a)	VH14:Vκ9 (b)
k_{on} (M^{-1} s^{-1})	3.8×10^4	1.8×10^6	1.5×10^6
k_{off} (s^{-1})	3.1×10^3	4.4×10^{-3}	4.9×10^{-3}
K_a (M^{-1})	1.2×10^7	4.1×10^8	3.1×10^8

(a) and (b) are datasets for two independently prepared batches.

a)

CDR1
*QVKLQQSG*AELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEW

CDR2
IGEINPSNGRTNYNEKFKSKATLTVDKSSSTAYMRLSSLTSEDSAVYYC

CDR3
ARGSWFAYW*GQGTTVTVSS*

b)

CDR1
*DIELTQSP*ASLSASVGETVTITCRASGNIRNYLAWYQQKQGKSPQLLVY

CDR2 CDR3
YTTTLADGVPSRFSGSGSGTQYSLKINSLQPEDFGSYYCQHLWSTPRTFG

G*GTKLEIKR*

Figure 3. a) Amino acid sequence of VH14. b) Amino acid sequence of Vκ9. Differences between this sequence and that of VκD1.3 (190) are indicated by arrows. For both sequences, CDRs are indicated by underlining. Primer encoded sequences are shown in italics.

is that the D1.3 Vκ and in particular, its Vκ9 homolog, appear to play a role in contacting the Vα. This demonstrates that a Vκ in combination with two different VHs can recognize two very different antigens, hen egg lysozyme and a TCR Vα, with appropriate specificity and suggests some plasticity of antibody V regions in immune recognition.

However, neither VH14:VκD1.3, nor the higher affinity derivative, recognize Vαs on the surface of T cells and therefore do not have obvious uses in T cell repertoire analysis nor in therapy. Our more recent experiments have been directed towards making scFvs that recognize native TCRs. For this, the bacteriophage display system has been employed in a more conventional way.

Isolation of scFvs that recognize native Vαs

Mice were immunized with purified scTCR derived from the qcII85.33 hybridoma (designated 85.33), that is in turn derived from an arthritogenic T cell clone [106]. The splenocytes were used as a source of VH and Vκ genes which were assembled as scFv gene libraries using the PCR [111]. A bacteriophage display system with panning on 85.33 Vα coated surfaces was used to isolate scFvs that recognized the recombinant 85.33 Vα. Four scFvs were characterized in detail using ELISAs and all bound the 85.33 Vα, but varied in their ability to bind to the 85.33 scTCR [111]. Several of these scFvs also showed cross-reactivity with the 1934.4 Vα [77,105], which shares 30% amino acid homology with the 85.33 Vα ([106], M. Ciubotaru and E.S.W., submitted for publication). Flow cytometric analyses using the 85.33, 1934.4 and as a negative control, the 4G4 (TCR^-) hybridomas showed that three of these scFvs stained the 85.33 cells (Figure 4). No staining of the 4G4 cell line was observed, and scFvs 34 and 142 also showed some weak staining of 1934.4. cells, consistent with these scFvs showing the highest levels of crossreactive recognition between the 85.33 and 1934.4 Vαs in ELISAs. Although the shifts are relatively small (compared with staining by the F23.1 antibody, for example, which recognizes the Vβ of the 85.33 hybridoma), they indicate specific recognition of surface Vαs by three of these scFvs. Smaller shifts for anti-Vα versus anti-Vβ antibodies have been observed by other authors [103]. There are several possible reasons for the relatively small shifts: first, that the affinity of the scFvs are low, and this is currently being investigated. If so, the affinities can be improved using the methods described below. Second, the Vα epitope may be partially masked on the T cell surface, by either CD3 polypeptides or glycosylation and this may account for the difficulty in making anti-Vα antibodies. Nevertheless, the data show that soluble recombinant TCR V domains can be used as immunogens, and scFvs that recognize native TCR V regions isolated using bacteriophage display. Clearly, this approach could be extended to the isolation of scFvs that recognize other TCR V region families and in particular, could be useful for generating reagents that bind to human T cells. Such antibodies could be of value in diagnosis, and in the longer term, in therapy of T cell mediated autoimmunity.

III. IMPROVING THE AFFINITY OF RECOMBINANT ANTIBODIES

For use in therapy and diagnosis it is desirable to have high affinity antibodies at hand, and the bacteriophage display system can be combined with several approaches to improve affinities of existing antibodies. Prior to the development of bacteriophage display systems, the screening of large numbers of mutants limited attempts to improve affinities by a random mutagenesis approach, that is, mimicking in vivo affinity maturation [112,113]. An alternative way to increase the avidity, and hence affinity of an Fv or Fab fragment is to generate multivalent Fvs/Fabs. Clearly, for a particular antibody the two approaches can be combined to produce high affinity, high avidity reagents.

Mutagenesis or Chain Shuffling Followed by Selection

VH and Vκ gene can be subjected to rounds of the PCR under conditions designed to insert random point mutations (Figure 5), and the resulting mutated genes

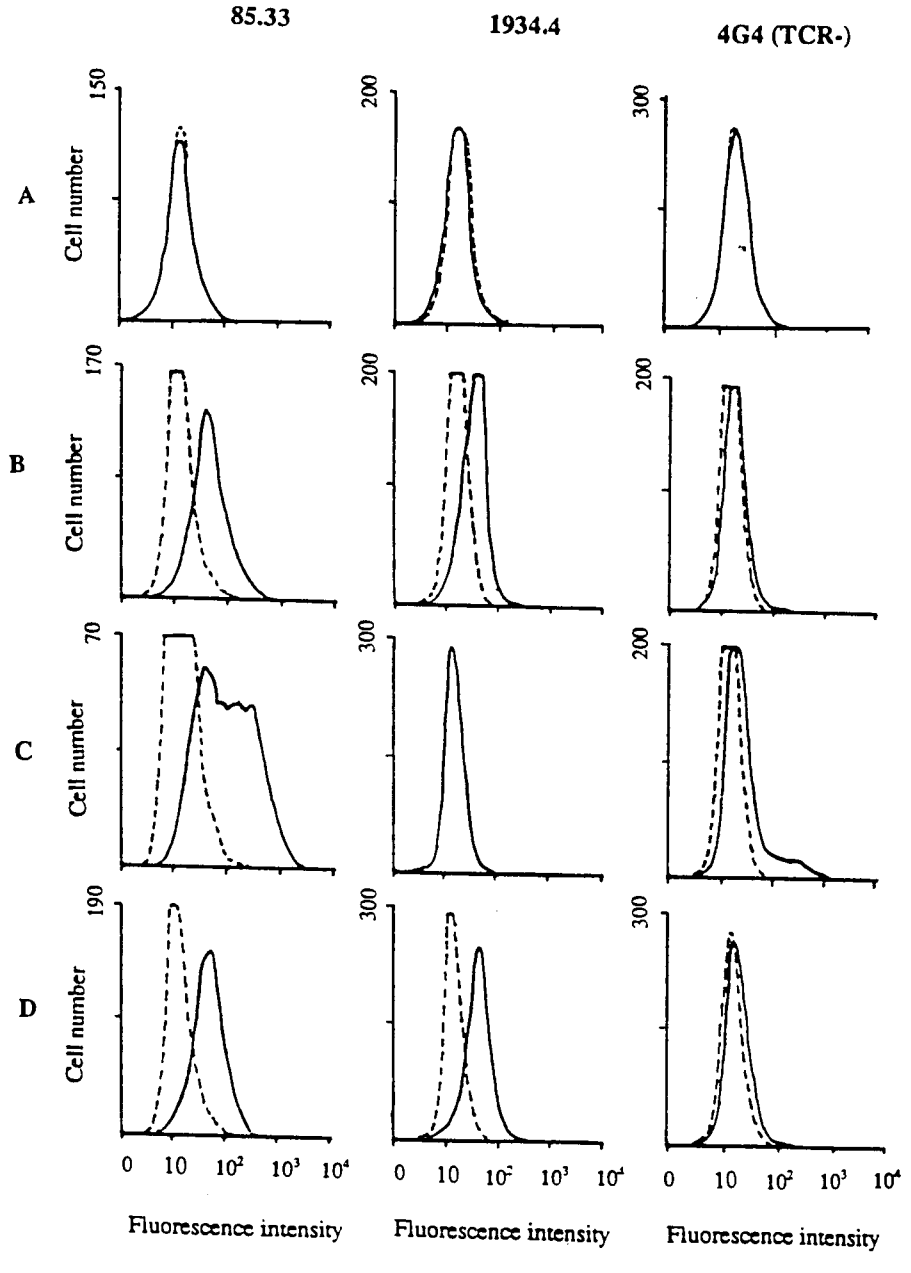

Figure 4. Flow cytometric analyses of scFvs that recognize recombinant Vα85.33. A, scFv 5; B, scFv 34; C, scFv 118 and D, scFv 142. The T cell hybridomas used are 85.33 (106), 1934.4 (105) and the TCR negative cell line 4G4.

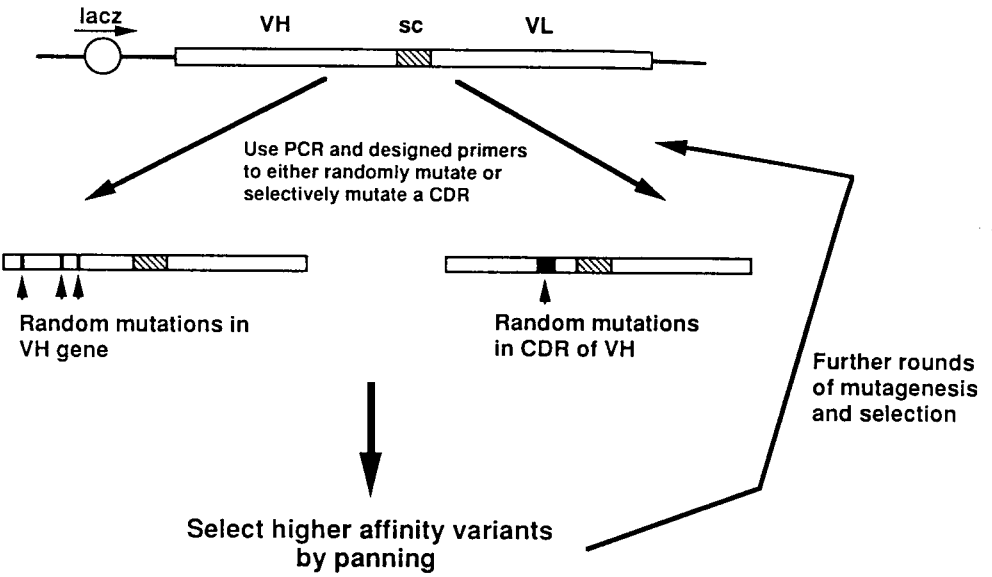

Figure 5. Strategy for improvement of affinity of antibody scFv or Fab fragments. Open circle = lacz promoter, open boxes = VH and VL genes and sc (hatched box) = single chain linker peptide.

cloned for expression as bacteriophage display libraries. Using this approach, the affinity of anti-4-hydroxy-3-nitrophenylacetyl/NIP-caproic acid [53] and anti-progesterone [54] antibody fragments have both been increased by 4- and 30-fold, respectively. An alternative method is to insert random CDRs either as cassettes or using the PCR into the VH and Vκ genes (Figure 5). In some respects, this may be preferable to the error prone PCR route, as only the CDRs (and flanking framework regions) are targeted. Although to date this approach has not been shown to increase the affinity of an existing Fv or Fab, it has been used to isolate antibody fragments with good affinities for both proteins and haptens [55,56,72,74] from naive libraries.

In addition, chain shuffling [108–110] can be used to improve affinities of existing antibodies. This may have advantages over the use of point mutagenesis or CDR mutagenesis, as the in vitro mutagenesis probably produces a reasonable proportion of misfolded scFvs or Fabs. Clearly, the use of VH and Vκ genes from antibody producing cells is less likely to result in such an outcome. A recent extension of this approach is to carry out chain shuffling by expressing a repertoire of Fd (VH-CH1) genes on the surface of phage, and to denature and refold these Fds in the presence of a single light chain of anti-2-phenyloxal-5-one specificity [114]. This Fd shuffling experiment resulted in the production of an Fab with a ten-fold increase in affinity over the original antibody from which the light chain was derived. Thus, this approach provides an alternative to the shuffling of V genes at the genetic level, but its success is clearly dependent upon efficient renaturation of phage bound Fds.

Increasing the Avidity of Antibody Fragments

One obvious way of generating bivalent Fvs or Fabs is to link them to the constant regions of antibodies and to express them as complete IgGs. However, this increases the in vivo half-life of the fragment dramatically, and may not be attractive if rapid clearance is required for use in imaging, for example. A number of different approaches have recently been described for the production of both bivalent Fvs and Fabs and these are as follows:

i) linkage through disulfide bridges. This has been used for both Fvs [115] and Fabs [116,117]. In the case of the Fv, an extra cysteine was linked at the genetic level to the carboxy terminus of the anti-lysozyme VHD1.3 domain [115]. Expression of the Fv followed by linkage to form a dimer using chemical methods resulted in the production of bivalent Fvs. For the Fab, a similar approach was taken [116,117], except that Fabs of two different specificities were linked to produce a bispecific Fab with high efficiency.

ii) linkage through dimerization domains. Dimeric Fvs can be produced from recombinant *E. coli* cells by tagging the Fv with one of several dimerization domains. For example, the dimerization domains can be derived from amphipathic helices from a four-helix bundle design or a leucine zipper ([118,119], reviewed in [120]). These domains are linked to the scFv via the flexible hinge region derived from murine IgG3. Both dimerization domains were effective in promoting scFv dimer formation, but the four helix bundle domain appears to dimerize more stably in dilute solutions and is therefore more effective in increasing avidity [119].

An alternative dimerization domain that has been reported is the use of an immunoglobulin Cκ domain linked to a scFv fragment [121]. This domain has also been used to drive and stabilize the association of the extracellular regions of a soluble TCRα:β heterodimer expressed in mammalian cells [90]. The Cκ:Cκ interaction is therefore of sufficient stability to generate dimers of covalently attached proteins such as scFvs or TCRs.

Diabodies or Multivalent Fvs

Several reports have documented the tendency of scFvs to aggregate into dimers and higher order multimers [122–124]. Dimerization can be favored by varying the length of the single chain linker peptide, and the effect of linker length on the multimerization/binding affinity of the scFv has been described in a number of studies [122,123]. Bispecific Fvs can also be produced by linking the VH and VL of two different antibodies A and B to form two different "cross over" chains VHA-VLB and VHB-VLA [122,123]. Dimerization results in the generation of a bispecific scFv comprising the binding specificity of A linked to that of B. To optimize the production of bivalent scFvs for an antibody of interest, the current data suggest that several different linker lengths should be tested. The need for this optimization is due to the fact that for different scFvs, it is likely that the linker can not only affect dimerization but also the binding affinity.

IV. REBUILDING ANTIBODIES FOR THERAPY

Clearly for use in therapy, effector functions need to be added to the recombinant (sc) Fv or Fab fragments. The most obvious way to incorporate these is to use genetic manipulation to link the Fc or constant regions of an IgG antibody to the Fab or Fv, respectively. This raises questions as to which isotype should be used, and a number of studies have demonstrated that for human use, the human IgG1 isotype is most effective in both complement fixation and ADCC [8,9]. More recently, it has emerged that V regions linked to the IgG1 isotype have a higher affinity for binding to surface bound antigen than those linked to the other human isotypes [125,126]. The reason for this latter observation is not clear, but is presumably related to the segmental flexibility of the IgG molecule. To produce the complete antibody in glycosylated form (the latter being necessary for effective binding to FcRs; [127–129]), a variety of expression hosts are available that range from myeloma to insect cells ([130,131], reviewed in [132,133]).

A further advantage of using whole IgGs in therapy is that the Fc region confers long serum persistence on the molecule [134], and in many cases this may override the advantages of using smaller, but rapidly cleared Fvs or Fabs that may be more penetrative [135]. Our data concerning the role of the Fc region in the control of IgG catabolism ("catabolism control") indicate that for optimal serum persistence, the CH2, CH3 domain and hinge region should all be linked to the Fab or Fv fragment (sections on *Evidence that both CH2–CH3 domain interfaces are required for catabolism control and transcytosis* and *The role of hinge disulfide bridge in IgG catabolism* below in §VII). Therefore, proteins that approach the size of the complete IgG molecule should be built if serum persistence is required. In this respect, the addition of the CH2 domain to a CD4–toxin conjugate resulted in an increase in biological half-life to a value that was still less than that of a complete IgG [136]. The following sections review the role of the Fc in controlling IgG catabolism and in transcytosis, and the localization of the site of the Fc that is responsible for these functions.

V. THE BRAMBELL HYPOTHESIS: A COMMON MECHANISM FOR TRANSCYTOSIS AND IgG CATABOLISM?

The hypothesis of Brambell and colleagues postulated that IgG transcytosis and catabolism control are achieved through the same mechanism [137–139]. This hypothesis suggests that both processes, on the one hand controlling IgG transport from mother to fetus or neonate across the epithelial barrier of placenta, yolk sac or gut and, on the other hand, its elimination from the circulation are dependent on a receptor-mediated process. In the Brambell model [137–139] the transport process is initiated by the uptake of IgG by epithelial cells followed by specific binding to a limited number of receptors. These receptors interact with the Fc region of the IgG molecule. The bound molecules are transcytosed to the opposite pole of the cells and delivered intact into the circulation of the fetus (in placental and yolk sac transcytosis) or of the neonatal animal (in intestinal transcytosis), whilst the unbound IgG molecules are destroyed. By analogy, IgG catabolism was postulated to occur by the uptake of circulating IgG by "catabolic" cells, binding to specific "protective" receptors through the Fc region and ultimately returning the bound IgG into the circulation [137–139]. In this model, unbound IgG molecules are degraded.

The existence of such protective receptors has been demonstrated in the epithelial cells of intestine of rats and mice [140–146]. Suckling rats acquire immunity by transport of IgG from milk across the small intestine and into the circulation. Transcytosis is mediated by a membrane bound receptor that binds IgG at the pH of the intestinal lumen (pH 6) and releases it at the pH of the blood (pH 7.4). This receptor (FcRn) is a heterodimer of two polypeptides (Mr 45–53 kDa and 14 kDa), and the 45–53 kDa protein shares homology with the members of class I major histocompatibility complex glycoproteins [142–144]. The 14 kDa component of the heterodimer is β2-microglobulin which extends the similarity of FcRn to class I molecules further. Recombinant soluble FcRn [145,146] binds to the IgG Fc fragment in a ratio of two FcRn to one Fc, indicating two binding sites per Fc. A receptor with similar (if not identical) structure was identified on rat yolk sac cells, but its cellular localization was found to be different [147]; this receptor was identified by electron microscopy in endosomal vesicles of the apical and basolateral cytoplasm and not at the luminal surface of the cell. Since both yolk sac and placenta are bathed on maternal and fetal sides by a milieu with neutral pH (pH 7.4), the binding of IgG molecules to FcRn should occur intracellularly at the acidic pH of endosomes after fluid-phase pinocytosis of the IgG [147].

In contrast to the Fc receptors involved in IgG transcytosis, the receptors involved in the control of the catabolic rates of IgGs have not yet been identified. Moreover, even the cells (and tissues) that are involved in IgG catabolism have yet to be unequivocally characterized [148,149]. Since no single organ has been clearly identified as being the major site of IgG catabolism, the possibility of diffuse catabolism throughout the body has been suggested by many authors [148]. Thus, studies of IgG localization have indicated that organs containing endothelial cells (e.g., liver, muscle, skin) are more active catabolic sites than other tissues [148]. It is therefore possible that endothelial cells from the above mentioned sites and/or other sites are the catabolic cells of IgG. These endothelial cells are constantly exposed to circulating IgG, and are a cellular barrier between the intravascular and extravascular compartments of the body and therefore might be endowed with a role in IgG catabolism. There are some indirect data supporting the concept that similar to transcytosis, the control of IgG catabolism occurs via an Fc receptor mediated process. The rate of IgG catabolism varies in direct proportion to the IgG concentration in plasma, and is accelerated in individuals with increased IgG levels and conversely reduced in subjects with low IgG concentration [148,149]. This concentration–catabolism relation would be consistent with the presence of limiting numbers of protective Fc receptors in the catabolic cells, as suggested by the Brambell model [137–139]. Furthermore, consistent with the Brambell hypothesis, the correlation between the serum concentration and the half-life of IgG calculated using the Brambell equation is in excellent agreement with the data obtained experimentally in animals and patients [148,149].

VI. THE LOCALIZATION OF THE SITE OF IgG INVOLVED IN CATABOLISM AND TRANSCYTOSIS

The Use of Proteolytically Derived IgG Fragments

Prior to the application of molecular biology to the study of proteins, the only way to learn about the intramolecular localization of effector functions of immuno-

globulins was to use enzymatic digestion to generate smaller fragments containing different portions of the molecule. For the IgG molecule, Fab and Fc fragments were obtained by papain digestion [150], and using these fragments, the Fc region was shown to have the same properties as the complete IgG molecule in both transcytosis and catabolism [140,151–156]. Subfragmentation of the Fc region by digestion with trypsin [155], pepsin [157] or plasmin [158] yields the fragments containing the CH2 or CH3 domains in addition to a molecule of IgG devoid of its CH3 domain (Facb). These fragments have been used to characterize further the location of the sites of the Fc that are involved in transcytosis and control of catabolism (designated the "catabolic site").

The study of the catabolic rates (half-lives) of the CH2 and CH3 domains obtained by trypsin digestion of a human IgG Fc fragment [155] has shown that the CH2 domain persists in the circulation of rabbits for almost as long as the intact Fc fragment or IgG molecule, whereas the CH3 domain is rapidly eliminated (Table 2). The rapid elimination of the CH3 domain from the circulation was confirmed in rats by measuring the half-life of this fragment derived from rat IgG [159]. However, enzymatic removal of the carboxyterminal peptide from the rat Fc fragment (approximately 20–25 amino acid residues) resulted in the rapid elimination of the truncated Fc fragment (1.6 hr vs. 52 hr for intact Fc fragment) [159]. This result suggests that the carboxyterminal amino acid residues of the CH3 domain are involved in the control of catabolism of the whole molecule, but *only* when this domain is linked to the CH2 domain. Taken together, these data suggest that both CH2 and CH3 domain residues are involved in the control of catabolism of the IgG molecule.

Subfragments of the Fc have also been used in transcytosis studies. Thus, Koshland and colleagues [153] have shown that subfragments of mouse Fc containing CH2 or CH3 "homology" regions obtained by procedures similar to those applied to human Fc fragments [155] were not able to inhibit the transport of the parent IgG molecule through the intestine of suckling mice (Table 2). This suggests that the recognition site is not confined, as the catabolic site was claimed to be [155], to a single domain and that both CH2 and CH3 domains contribute to a conformation of the IgG molecule involved in the binding to intestinal Fc receptors. These results were confirmed and extended in a study in which a human placental preparation was shown to bind only intact human Fc fragment but not its CH2 or CH3 domains [160] (Table 2). In contrast, when binding of rabbit IgG fragments to a homologous yolk sac preparation was studied [161], the Facb fragment (IgG minus the CH3 domain) was able to bind to yolk sac even better than the parent IgG molecule while the pFc (CH3 domain) fragment showed no significant binding (Table 2). From these results the authors concluded that the recognition unit for the binding of IgG to yolk sac Fc receptors is localized solely in the CH2 domain. However, neither human pFc fragment nor rabbit Facb fragment showed any ability to inhibit the interaction of human IgG with placental trophoblast [162], indicating that for this tissue the transcytosis site was not localized exclusively in the CH2 domain. In summary, the studies using fragments of IgG obtained by proteolysis did not result in a definitive localization of the catabolic or transcytosis sites but suggested that the CH2 domain, and possibly the CH3 domain, were involved. The obvious limitations of using proteolytically derived fragments hindered a more precise localization of the site of the IgG molecule involved in catabolism and transcytosis.

Table 2. Localization of Transcytosis and Catabolic Sites in the IgG Molecule by Using Proteolytic Fragments

IgG fragments	Transcytosis			Catabolism[d]
	Placenta[a] (pmoles displaced)	Yolk sac[b] (pmoles bound)	Intestinal[c] (% inhibition	Half-life (hr)
IgG	45	95	41	74
Fc	49	ND	66	70
CH_2	0	ND	10	62
CH_3 (pFc)	0	5	5	16
Facb[e]	ND	150	ND	ND
Fab	0	3	4	17

[a] McNabb et al. (160). [b] Johanson et al. (161). [c]Guyer et al. (153). [d]Yasmeen et al. (155). [e]IgG devoid of CH3 (158).

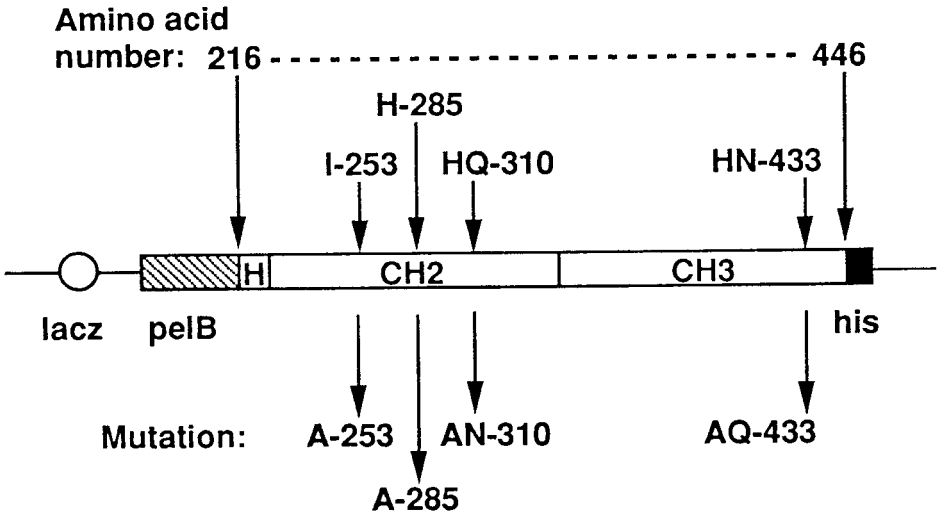

Figure 6. Vector used for the expression of Fc fragments and mutants (see §VII). Open circle = lacz promoter, hatched box = pelB leader sequence, open box = Fc fragment (with no mutations or mutations at positions shown), filled in box = his_6 peptide tag. The numbers (EU numbering, [191]) of the N and C terminal amino acids of the recombinant Fc fragment are shown. The mutants are as follows: i) I-253, ile 253 to ala 253; ii) H-285, his 285 to ala 285; iii) HQ-310, his 310 to ala 310 and gln 311 to asn 311; iv) HN-433, his 433 to ala 433 and asn 434 to gln 434; v) HQ-310 and HN-433 within the same Fc fragment.

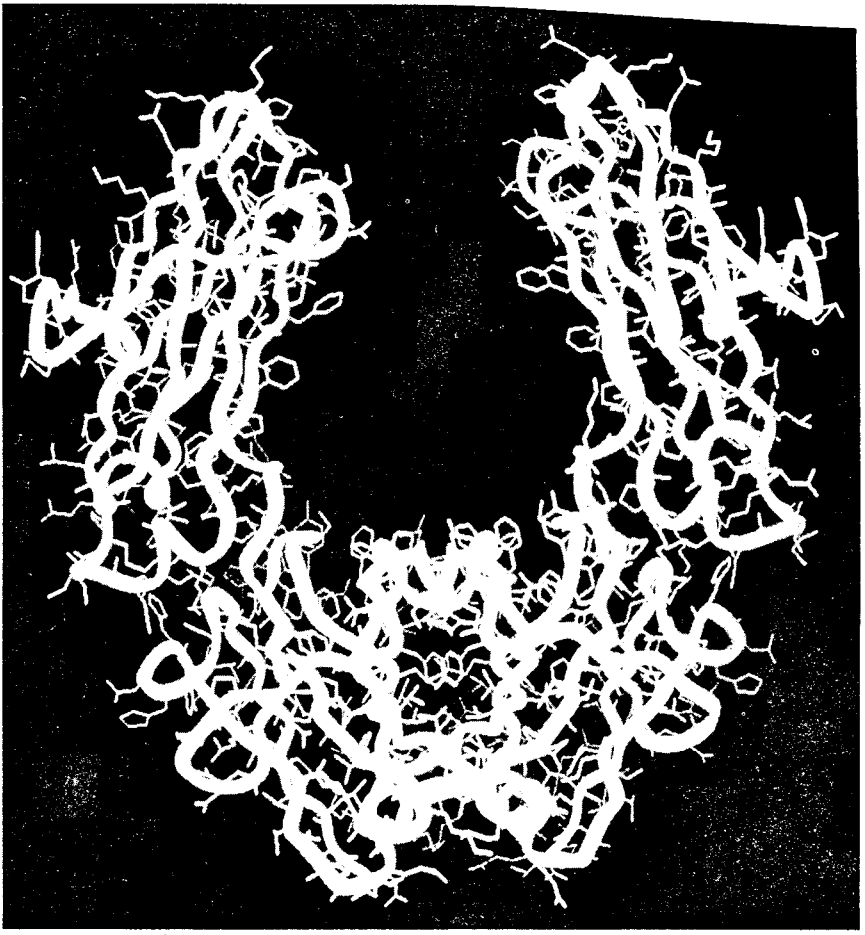

Figure 7 (see COLOR PLATE 5.7). The structure of human Fc (13). The residues of murine IgG1 that were mutated in this study are conserved in both human and murine IgG1 (Table 3) and are shown highlighted in yellow (Ile-253 to Ala-253), blue (His-310 to Ala-310 and Gln-311 to Asn-311) and green (His-433 to Ala-433 and Asn-434 to Gln-434).

IgGs Complexed with Staphylococcal Protein A

Staphylococcal protein A (SpA) binds to the Fc region of IgG of a variety of species with different affinities [163]. Trypsin digestion of this protein yields a monovalent fragment (fragment B) with a molecular mass of 7 kDa [164] which binds to the Fc fragment in a molar ratio of 2:1 [13]. Solution of the X-ray structure of human Fc (Figure 7) complexed with fragment B allowed the identification of amino acid residues involved in the contact between these two proteins [13]. These amino acid residues are clustered in three hydrophobic patches which are remarkably conserved in the IgG molecules of many species (Table 3). The invariance of Ile-253 in the CH2 domain as a highly exposed hydrophobic residue strongly suggests that it should have a functional role beyond binding SpA. Both CH2 and CH3 domains

Table 3. Amino Acid Residues of CH_2–CH_3 Interface of Various IgGs Involved in SpA Binding

Species	Subclass	Amino acid residues from SpA–Fc contacts		
		252-254	308-312	433-436
Human	IgG1	Met-Ile-Ser	Val-Leu-His-Gln-Asn	His-Asn-His-Tyr
	IgG2	Met-Ile-Ser	Val-Val-His-Gln-Asp	His-Asn-His-Tyr
	IgG3 allotype	Met-Ile-Ser	Val-Leu-His-Gln-Asp	His-Asn-Arg-Phe
	IgG4	Met-Ile-Ser	Val-Leu-His-Gln-Asp	His-Asn-His-Tyr
Mouse	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
Rat	IgG1	Thr-Ile-Thr	Ile-Leu-His-Gln-Asp	His-Asn-His-His
	IgG2a	Thr-Ile-Thr	Ile-Val-His-Arg-Asp	His-Asn-His-His
	IgG2b	Leu-Ile-Ser	Ile-Gln-His-Gln-Asp	His-Asn-His-His
	IgG2c	Met-Ile-Ser	Ile-Gln-His-Gln-Asp	His-Asn-His-His
Guinea pig	IgG1	Met-Ile-Ser	Ile-Glx-His-Asx-	Pro-Asn-His-Val
	IgG2	Met-Ile-Ser	Ile-Gln-His-Gln-Asp	His-Asn-His-Val
Rabbit	IgG	Met-Ile-Ser	Ile-Ala-His-Gln-Asp	His-Asn-His-Tyr

and histidine residues are needed for the binding of IgG to SpA as demonstrated by the inability of the Facb fragment and CH3 domain [165] to interact with SpA and by the inhibition of the interaction by covalent modification of the histidine residues of human IgG1 [166]. Further evidence for the involvement of histidines is that an allotype of human IgG3 containing arginine rather than histidine at position 435 does not bind to SpA [166,167].

Binding of SpA or its fragment B to rabbit or mouse IgG has drastic consequences for the catabolic rate of IgG in homologous recipients [168–170] (Table 4). The rapid elimination of IgG with bound fragment B suggested that the residues located at or near the Fc: fragment B interface may be involved in IgG clearance [168–170]. This was the first direct evidence that the catabolic site might be accommodated by both CH2 and CH3 domains and not solely by the CH2 domain as suggested by the experiments with isolated CH2 and CH3 domains [155]. However, as interpreted by the authors at that time it was also possible that the binding of fragment B to the CH2–CH3 interface hindered the catabolic site located exclusively on the CH2 domain. More recently, a similar experiment was carried out in which the ability of fragment B to interfere with the binding of human IgG to recombinant rat FcRn was analyzed [171]. It was shown that fragment B inhibits FcRn binding to IgG, localizing the FcRn binding site of IgG to the CH2–CH3 interface (Table 4). A recombinant CH3 domain did not interact with FcRn [171] suggesting that either it does not participate in the building of the binding site or that it needs the CH2 domain to build the binding site. Taken together these two studies [168,171] strongly suggest that the catabolic and transcytosis sites are overlapping in the CH2–CH3 interface of the Fc region, but again do not unequivocally prove that residues in either CH2 or CH3 domains are involved in these processes.

Table 4. Catabolism and Transcytosis of IgG Complexed With SpA Derivatives

Protein	Catabolism[a] (half-life, hr)		Transcytosis[b] (RUs)
	Rabbit	Mouse	Rat
IgG	153	106	1221
IgG+SpA	28	9	ND
IgG+fragment B	22	8	49

[a]Half-life of IgG complexed with ^{125}I-labeled SpA or fragment B (168). [b]Inhibition of rat FcRn–human IgG interaction by a functional analogue of fragment B of SpA measured by BIA core analysis in relative response units (RUs) (171).

Indirect evidence that amino acid residues in the CH3 domain might be involved in catabolism control is the shorter half-life of human IgG3 (7 days) in comparison to the other subclasses of IgG (21 days) [172]. Since IgG3 does not bind to SpA and since it has an Arg residue instead of His in position 435 [166] it suggests that this position is critical for both SpA binding and catabolism. This position seems to also be important for transcytosis, since when human IgG was bound to its receptor on trophoblast cells it was unable to bind SpA, suggesting that the simultaneous binding of SpA and FcRn to the CH2–CH3 interface is not sterically possible [173].

Binding of fragment B to IgG does not interfere with binding to FcR of lymphocytes (FcRγII) or macrophages (FcRγI) [168], indicating that the catabolic site and FcRn binding site are distinct from that involved in the binding to classical FcRs located in the lower hinge region [174]. This is consistent with the data suggesting that the catabolic and transcytosis sites are at the CH2–CH3 domain interface. It is interesting to observe that the CH2–CH3 domain interface is also the target for rheumatoid factor (RF) binding [175]. Site directed mutagenesis of human gamma chains of Fc was used to demonstrate that His-435 is an essential residue in RF binding to IgG, and residues of the CH2 domain that are involved in binding SpA contribute in a more variable way to the binding of RFs [175]. Taken together, these results strongly suggest that the CH2–CH3 interface plays an important role in controlling two important effector functions of IgG (catabolism and transcytosis) and is also involved in the binding of SpA and RFs. It is therefore tempting to speculate that in patients with high RF levels, IgGs are catabolized more rapidly [176] due to the blocking of the catabolic site. This hypothesis has yet to be rigorously tested.

Transcytosis and Catabolism of Mutant IgG Molecules

The use of mutant IgG molecules that occur either naturally or have been made using genetic manipulation has proved useful in elucidating the site involved in catabolism and transcytosis. These experiments suggest that residues in the CH3 domain might play a role in these two processes, but do not rule out the involvement of CH2 domain residues. The first experiment to be reported using a mutant

IgG was that of Spiegelberg and colleagues [177]. Using a human myeloma IgG with a deleted CH3 domain it was shown that the absence of the CH3 domain from a human IgG results in an accelerated catabolism of this truncated IgG [178]. Furthermore, an abnormal human IgG3 with a deletion in the hinge region and a partially altered CH3 domain had a shorter half-life than normal IgG3 in SCID mice (5 days vs. 7 days) indicating that either the hinge region or some segment of the CH3 domain might be involved in the control of the catabolic rate of this immunoglobulin [177]. The possible participation of the CH3 domain in the building of the catabolic site of IgG was confirmed by a recent investigation concerning the half-life of mouse IgG2b mutants lacking the CH3 domain [179]. The half-life of such a truncated IgG2b molecule was shorter than that of the parent molecule (78 hr vs. 118 hr), but also mutants lacking the CH2 and even CH1 domains behaved similarly [179]. This latter behavior suggests that the truncated IgG2b molecules might have undergone conformational changes, impairing their interaction with the catabolic receptors irrespective of which domain was deleted. Naturally occurring hybrid molecules consisting of CH1, hinge and most of the CH2 domain from IgG2b and the carboxyterminal part of the CH2 domain and all CH3 sequences from IgG2a had the half-life of the IgG2a molecule [179]. From these data the authors suggested that the sequences in the CH3 domain are important in determining the different intravascular half-lives of IgG2a and IgG2b [179]. However, the hybrid contains not only the CH3 domain from IgG2a but also the carboxyterminal portion of CH2 domain and it could therefore be argued that the IgG2a derived CH2 sequences may encompass the catabolic site of the IgG molecule. The participation of the CH2 domain in the control of catabolism was reported independently by other authors claiming that a mouse/human chimeric antibody devoid of CH2 domain was cleared from the blood of tumor bearing mice much more rapidly that the intact antibody with an elimination rate close to that of a human $F(ab')_2$ fragment [180].

VII. THE USE OF SITE-DIRECTED MUTAGENESIS TO IDENTIFY THE CATABOLIC AND TRANCYTOSIS SITES OF IgG

The Role of Residues at the CH2–CH3 Interface in Controlling Catabolism

The studies on the localization of transcytosis and catabolic sites in the IgG molecule by investigating the behavior of proteolytic fragments and various abnormal, mutant or hybrid counterparts have indicated that the sites might be located in both CH2 and CH3 domains at their hydrophobic interface. This concept is further supported by the effect on these two processes of complexing fragment B of SpA with IgG. To test directly that the SpA binding site overlaps with the transcytosis and catabolic sites, the amino acid residues that are both conserved in IgG sequences and involved in SpA binding (Table 3) have been changed by in vitro mutagenesis and the pharmacokinetics of various mutant mouse IgG1 Fc fragments studied [10].

Plasmids encoding wild-type IgG1 Fc-hinge, and mutants I-253, HQ-310, HN-433 and HQ-310/HN-433 were constructed for this study (Figure 6, Table 5). These fragments all contain hinge regions derived from the IgG1 molecule. The residues that have been mutated are in close proximity to the CH2–CH3 domain interface (Figure 7) and are also conserved in most of the IgG isotypes in both mouse and man (Table 3). The wild-type and mutant Fc-hinge fragments were expressed and

Table 5. Recombinant Fc-Hinge Fragments Derived From Murine IgG1 (10)

Designation	Mutation
WT Fc-hinge	Wild-type sequence
I-253	Ile-253 to Ala-253
HQ-310	His-310 to Ala-310 and Gln-311 to Asn-311
HN-433	His-433 to Ala-433 and Asn-434 to Gln-434
HQ-310/HN-433	HQ-310 and HN-433 within same Fc-hinge
H-285	His-285 to Ala-285

purified from recombinant *E. coli* cells. As a control in these experiments, a mutation was made in the Fc-hinge fragment which converted histidine at position 285 to alanine. This histidine is located in a loop on the external surface of the CH2 domain [13] and is distal to the CH2–CH3 domain interface. Analyses using reducing and non-reducing SDS-PAGE indicate that the wild-type and mutant Fc-hinge fragments are expressed as sulfhydryl-linked homodimers due to the presence of the hinge region in their molecule. Furthermore, CD analyses indicated that all these recombinant proteins were correctly folded [10].

The recombinant wild-type and mutant Fc-hinge fragments were radiolabeled with ^{125}I and injected into groups of three-four mice and the serum radioactivity monitored as a function of time [10]. For comparative purposes, the complete IgG1 molecule produced by the RFB4 hybridoma was also radiolabeled and used in clearance studies. For each protein, the elimination curves in different mice were almost identical and Figure 8 shows representative curves for one mouse from within each group. HPLC analyses of serum samples indicated that the wild-type and mutant Fc-hinge fragments persist in the serum as intact homodimeric molecules, and are not associated with other serum proteins. The half-lives of the glycosylated IgG molecule and the recombinant wild-type/mutant Fc-hinge fragments are shown in Table 6. The α phase half-lives of the complete IgG1 molecule and the wild-type Fc-hinge fragment are different, and this is probably due to their size difference (160 kDa for complete IgG1 and 55 kDa for Fc-hinge fragment). In contrast to the α phase, the β phase half-life of the wild-type Fc-hinge fragment is the same, within the bounds of experimental error, as that of the glycosylated IgG1 molecule. The data clearly demonstrate that the mutations have significant effects on the half-lives of the α and β phases of the IgG1 Fc-hinge fragment. Mutations in the CH2 domain (I-253 and HQ-310) have a more marked effect than those in the CH3 domain (HN-433), and the HQ-310/HN-433 mutant is cleared slightly more rapidly than either of the two mutants from which it is derived. The half-life of the H-285 mutant demonstrates that mutation of a histidine residue in the Fc-hinge that is distal to the CH2–CH3 domain interface does not result in a decrease in β phase half-life. Significantly, this indicates that mutation of surface exposed histidines per se is not the cause of the more rapid clearance of the HQ-310/HN-433 mutant.

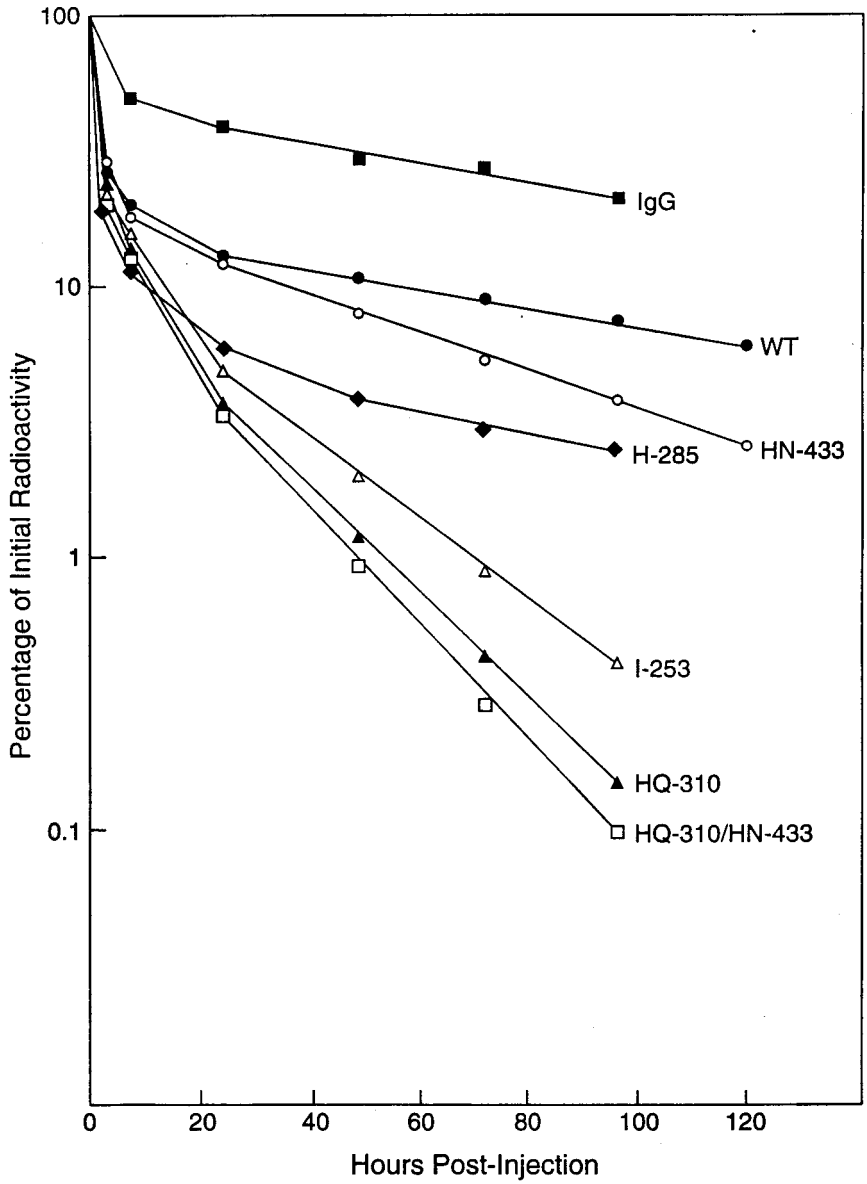

Figure 8. Clearance curves of wild-type and mutant Fc fragments. IgG = complete glycosylated IgG1 molecule and WT = wild-type Fc-hinge fragment. Mutants are as described in the text.

The effects of the mutations on binding of the Fc-hinge fragments to SpA were analyzed in direct binding studies (Table 7). The results of these experiments indicate that all of the mutants are impaired in SpA binding, with the HQ-310/HN-433 mutant having the lowest binding activity. These data are in agreement with the X-ray structure of human IgG1 complexed with SpA [13]. There is a good correlation between the binding to SpA of a particular Fc-hinge fragment and its half-life

Table 6. Half-Lives of the WT and Mutant Fc-Hinge Fragments (10)

Fc-hinge fragment	α phase $t_{1/2}$ (hr)	β phase $t_{1/2}$ (hr)
IgG1	20.1 ± 0.4	85.3 ± 4.3
Wild-type	10.5 ± 0.8	82.9 ± 10.0
I-253	6.7 ± 0.2	20.0 ± 0.6
HQ-310	6.0 ± 0.6	17.5 ± 1.6
HN-433	10.3 ± 1.2	50.3 ± 2.9
HQ-310/HN-433	5.8 ± 0.2	15.6 ± 0.8
H-285	8.7 ± 1.1	76.0 ± 14.6

Table 7. Correlation Between SpA Binding Activities, Catabolism and Transcytosis of Recombinant Wild-Type and Mutant Fc-Hinge Fragments (10, 46)

Fragment	SpA-binding % of wild-type Fc-hinge binding activity	Catabolism (β-phase, hr)	Transcytosis (% inhibition of IgG transmission)
Wild-type Fc-hinge	100	82.9	55.3
I-253	22.6	20.0	20.2
HQ-310	12.0	17.5	8.9
HN-433	24.9	50.3	44.4
HQ-310/HN-433	9.7	15.6	5.4

(Spearman coefficient 0.9), which suggests that the SpA-binding site overlaps with the catabolic site. This is consistent with earlier data that showed that SpA (fragment B)–IgG complexes are cleared more rapidly than uncomplexed IgG (Table 4).

Intestinal Transcytosis of Wild-Type and Mutated Fc-Hinge Fragments

The study involving catabolism of WT and mutant Fc-hinge fragments has been extended to transcytosis [46]. Intestinal transfer of radiolabeled IgG1 and Fc fragments from the proximal intestine to the circulation was analyzed in vivo by feeding neonates with nanomolar quantities of the appropriate protein. Approximately 45% of the radioactivity associated with the IgG1 molecule and Fc fragment produced by papain digestion (designated Fc-papain) could be detected in the plasma (Figure 9). The WT Fc-hinge fragment behaved similarly in these studies, with 40% of the radioactivity being transferred into the circulation (Figure 9). In

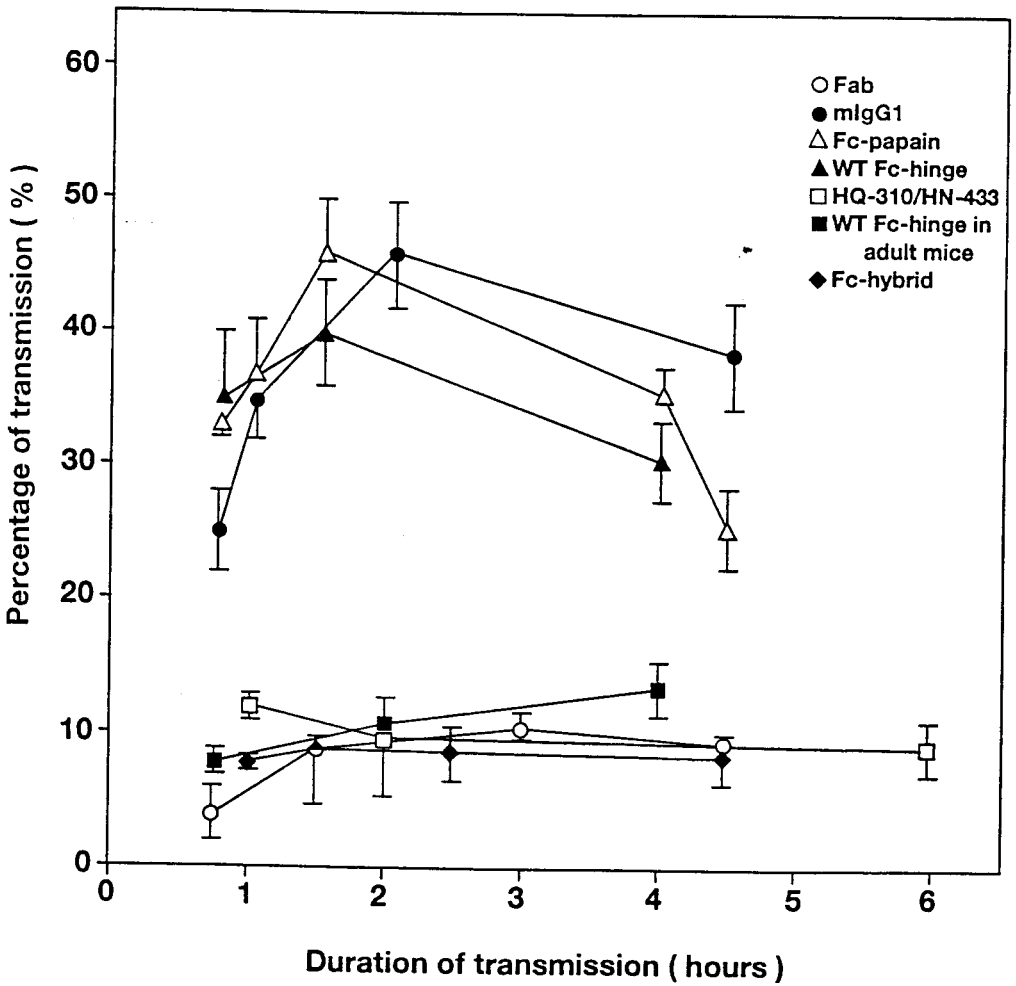

Figure 9. Intestinal transfer of murine IgG1 (mIgG1), Fab, Fc-papain and recombinant Fc hinge fragments.

contrast, for the HQ-310/HN-433 mutant, only 10% transfer was observed. This value is close to that of the radiolabeled Fab fragment used as a negative control, and therefore probably represents transfer by a nonspecific mechanism. In adult mice, the same low level of transfer of the WT Fc-hinge was observed, consistent with the report that FcRn is expressed only in the intestine of neonatal mice [141,144].

The HQ-310/HN-433 mutant is cleared from the serum at a much higher rate than the WT Fc-hinge fragment (Table 6), and it is therefore conceivable that the lower level of radioactivity in the serum of the neonatal mice might be due to more rapid clearance rather than lower transmission. To demonstrate that the mutant is deficient in transfer, experiments to assess the ability of the mutant Fc-hinge fragment to inhibit transfer of radiolabeled IgG1 have therefore been carried out. The results from these experiments would be expected to be independent of clear-

Table 8. Inhibition of Intestinal Transmission of Radiolabeled Murine IgG1 by Recombinant Fc-Hinge Derivatives (46)

Competitor (unlabeled)	Transmission (%)	% inhibition of transmission[a]
PBS	54.1 ± 0.7	–
IgG1	26.3 ± 5.7	51.4
Fc-papain	20.3 ± 7.1	62.5
WT Fc-hinge	24.2 ± 4.3	55.3
HQ-310/HN-433	51.2 ± 8.3	5.4
HQ-310	49.3 ± 7.3	8.9
I-253	43.2 ± 5.3	20.2
HN-433	30.1 ± 4.4	44.4
BSA	49.3 ± 5.1	8.9

[a]Relative to transmission of radiolabeled IgG1 in the presence of PBS.

ance rate. Consistent with the direct transfer data, the inhibition experiments show that the WT Fc-hinge and Fc-papain have similar inhibitory capacities to unlabeled IgG1, whereas the mutant Fc-hinge fragments inhibit transfer at much lower levels (Table 8). Mutations in the CH2 domain have a greater effect on the inhibition than those in the CH3 domain. There is an excellent correlation between the inhibition of transcytosis of Fc derivatives and their half-life (Spearman coefficient 1) on one hand and their ability to bind SpA on the other (Spearman coefficient 0.9) (Table 7). These results indicate that the transcytosis site located at the interface of the CH2 and CH3 domain overlaps with the site that is involved in catabolism and in SpA binding.

To confirm and extend the in vivo results, the binding of IgG1, WT Fc-hinge and the HQ-310/HN-433 mutant to isolated neonatal brush border membranes has been analyzed [46]. The ability of IgG1, WT Fc and the HQ-310/HN-433 mutant to inhibit the binding of radiolabeled IgG1 was assessed in competition binding studies. The results show that at up to a 2000-molar excess the HQ-310/HN-433 mutant does not significantly inhibit the binding of IgG1, in contrast to the WT Fc hinge which inhibits in a similar way to the unlabeled IgG1 molecule (Figure 10). These experiments show unequivocally that the mutations at the CH2–CH3 domain interface affect transfer at the first step in trancytosis, namely binding to FcRn. The structure of FcRn has also been modeled on the basis of the sequence similarities that it shares with structurally solved class I MHC molecules [171,181]. The region of FcRn which interacts with IgG appears to be located on the α_3 domain [171]. It has been suggested that stability differences of this receptor as the pH is varied from 6 to 8 may account for FcRn binding to IgG at pH 6.0–6.5 and release at pH 7.5 [145,146]. Identification of surface accessible histidine residues by molecular modeling, and the pKa values in the range of 6–7 of the side chains of histidine suggested that these residues might be involved in interacting with IgG. Consistent

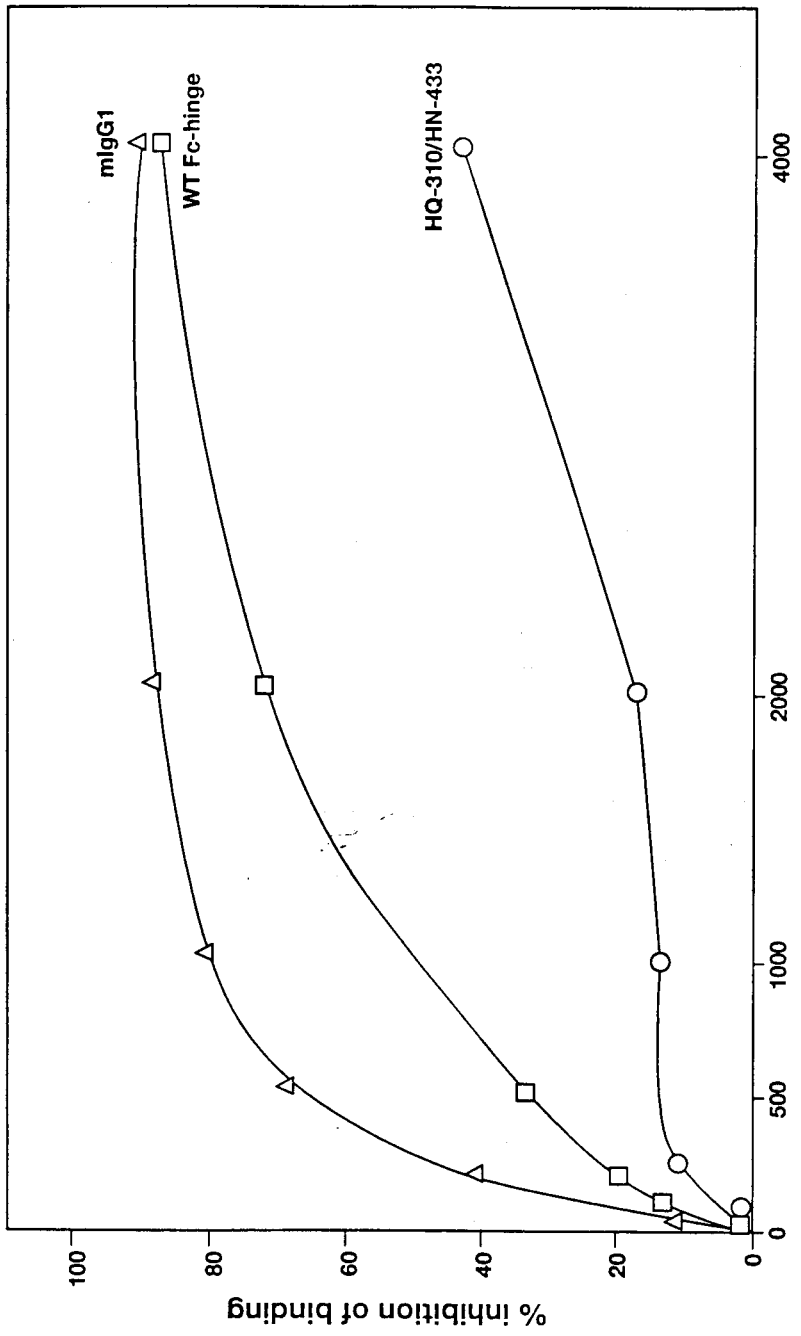

Figure 10. Inhibition of binding of ^{125}[I]-labeled IgG1 to isolated brush borders by unlabeled IgG1 WT and HQ-310/HN-433 mutant Fc hinge fragments.

Table 9. Catabolism and Transcytosis of Recombinant Hybrid Fc Fragment vs. Parent Molecules (182)

Fc derivatives	Catabolism (half-life, hr)	Transcytosis (% inhibition of IgG1 transmission)
Hybrid Fc[a]	37.9 ± 7.1	21.5
WT Fc-hinge	82.9 ± 10.6	55.3
HQ-310/HN-433	15.6 ± 0.8	5.4

[a]Comprises one WT Fc-hinge polypeptide associated through the hinge disulfide with HQ-310/HN-433 mutant Fc-hinge.

with this concept, alteration of the His-250/His-251 pair in the α_3 domain of recombinant FcRn resulted in a reduced affinity for the FcRn-Fc interaction [171]. The presence of His-310, His-433 [46] and possibly His-435 in the transcytosis site of IgG suggests that these histidines, in addition to those from FcRn [171], might be involved in the pH dependent interaction of IgG with FcRn.

The coincidence of the SpA [13] and FcRn binding sites at the CH2–CH3 domain interface raises questions as to the extent of overlap. In this respect, the pH dependency of FcRn and SpA binding to the IgG1 Fc fragment are different. IgG1 or IgG1-derived Fc fragments bind to SpA at neutral or basic pH (7–9) and dissociate from it at acidic pH (less than 6), whereas for FcRn, binding occurs at acidic pH (6–6.5) and dissociation at pH 7.5–8. This suggests that although some residues of the Fc are involved in interacting with both SpA and FcRn [10], the nature of the interactions in terms of the numbers of residues and surface areas involved probably differ for the two ligands. The extent and nature of the overlap awaits the determination of the X-ray crystallographic structure of FcRn complexed with Fc [146]. It is also conceivable that the CH2–CH3 domain interface is recognized by FcRn on yolk sac and placental trophoblast membranes, extending the role of residues in this region of IgG further.

Evidence That Both CH2–CH3 Domain Interfaces Are Required for Catabolism Control and Transcytosis

To analyze whether one or two functional sites per Fc-hinge dimer are necessary for catabolism and serum persistence in vivo, a recombinant hybrid Fc heterodimer was made which comprises one WT Fc-hinge polypeptide (tagged with a carboxyterminal His6 peptide tag) associated with one HQ-310/HN-433 mutant Fc-hinge polypeptide (tagged with a carboxyterminal C-myc peptide tag). Heterodimers were purified using Ni^{2+}–NTA–agarose followed by anti-myc-Sepharose [182].

The analysis of the hybrid Fc-hinge heterodimer in catabolism and transcytosis studies indicates that it has a significantly shorter half-life and a lower inhibitory capacity of IgG1 intestinal transfer than that of the WT Fc-hinge fragment (Table 9) [182]. Bivalency of IgG therefore appears to be necessary for serum persistence and intestinal transfer (Figure 9 and Table 9), indicating that the binding to FcRn (or to

the putative catabolic receptor) and subsequent transfer is enhanced by the presence of two receptor-binding sites per Fc fragment. This is consistent with a study in which it was shown that two recombinant FcRn molecules are bound by one rat Fc molecule [146]. The Fc-hybrid has a half-life of 37.9 hours, and this is significantly less than that of 82.9 hours for the WT Fc-hinge homodimer but longer than that of the HQ-310/HN-433 mutant. This indicates that two functional catabolic sites located at the CH2–CH3 domain interface of the dimeric Fc are necessary for an Fc/IgG molecule to be maintained in the circulation with pharmacokinetics that are similar to those of a WT Fc-hinge homodimer. However, the longer half-life of the hybrid relative to that of the HQ-310/HN-433 mutant suggests that the hybrid retains some of the pharmacokinetic characteristics of the WT Fc-hinge homodimer.

The possibility that two catabolic sites per Fc are needed for the control of catabolism of IgGs was suggested by others who showed that the SpA-B fragment complexed with Fc in a stoichiometry of one SpA-B fragment molecule per Fc were cleared rapidly from the circulation [169], but at this time the catabolic site had not been localized to specific amino acid residues. The need for two functional sites per molecule has been confirmed by the analysis of a CH2-hinge domain as both monomer and dimer [182]. The dimer has a longer β-phase half-life (61.6 hr) than the monomer (29.1 hr). The half-life of CH2-hinge dimer is significantly less than that of the WT Fc-hinge fragment (82.9 hr) but greater than that of CH3 (21.3 hr), and this suggests that sequences additional to those in the CH2 domain are necessary to constitute the catabolic control site of the IgG1 molecule. This is consistent with the data obtained using site directed mutagenesis ([10] and the first subsection of §VII on *The role of residues at the CH2–CH3 interface in controlling catabolism* above).

The Role of Hinge Disulfide Bridge(s) in IgG Catabolism

The CH3–CH2 contact interface is a flexible point in the Fc region of the IgG molecule [183]. Bending in this region was observed after reduction of hinge disulfides indicating that the cleavage of the S–S group(s) allowed the CH2 domain to assume an altered position relative to the more rigid CH3 domain [184,185]. The conformational change at the CH2–CH3 domain interface may alter accessibility of the amino acid residues that are involved in catabolism control. To investigate this possibility the pharmacokinetics of various murine Fc fragments which differ in the presence/absence of hinge disulfides and hinge sequences was analyzed and compared with the susceptibility of these derivatives to limited proteolysis with pepsin [186]. Pepsin was chosen for these studies as at low pH it is known to cleave at a site close to the CH2–CH3 domain interface [157]. The Fc derivatives used in this study are listed in Table 10. All fragments have molecular masses of about 55 kDa as determined by HPLC at neutral pH. Consistent with the Fc-hinge and Fc-cys fragments being disulfide linked homodimers these two proteins migrated as 55 kDa proteins when analyzed using SDS-PAGE under nonreducing conditions and as 25–30 kDa proteins when reduced [186]. In contrast Fc and Fc-pap migrated as 25–30 kDa proteins under both reducing and nonreducing conditions, indicating that the 55 kDa dimers (observed by HPLC) are non-covalently linked [186]. The glycosylated Fc produced by papain digestion (Fc-pap) does not have an interheavy chain disulfide bridge as papain cleaved the heavy chain at a position C-terminal to the hinge.

Table 10. Half-Lives of the Fc Derivatives With and Without Hinge

Fragment	Hinge sequence	α phase $t_{1/2}$ (hr)	β phase $t_{1/2}$ (hr)
Fc-hinge	YPRDCGCKPCI[a]	9.8 ± 0.8	79.8 ± 9.5
Fc	None	11.0 ± 1.1	48.7 ± 7.8
Fc-cys	GCGGAS	12.6 ± 0.5	72.0 ± 7.1
Fc-pap	None	13.1 ± 0.9	56.1 ± 5.8

[a]Cysteines which form —S—S— bridges are indicated by underlining.

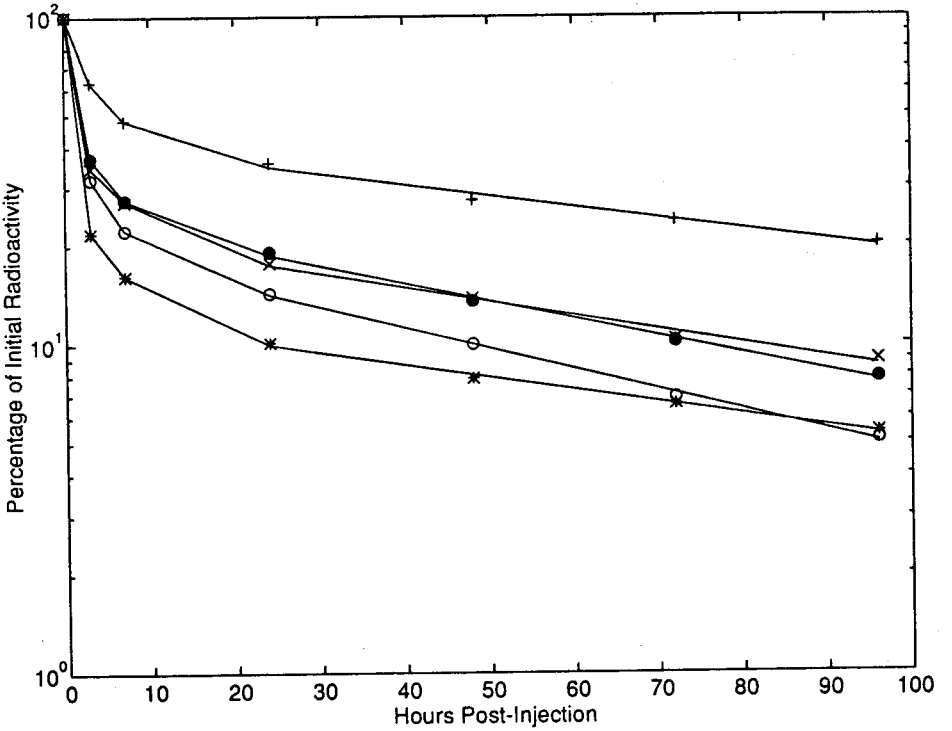

Figure 11. Clearance curves of WT Fc-hinge and derivatives with/without hinge. * = Fc-hinge, o = Fc, × = Fc-cys, closed circles = Fc-pap, and + = mIgG1.

The clearance curves of the Fc fragments are biphasic (Figure 11) and show that Fc-hinge and Fc-cys have similar half-lives whereas the half-lives for hingeless Fc-pap and Fc are significantly shorter (Table 10). The kinetics of the digestion of each fragment was determined by measuring the amount of released material that was non-precipitable by TCA as function of time (Figure 12). From these data it is clear that the Fc-hinge and Fc-cys are more sensitive to pepsin attack than Fc and

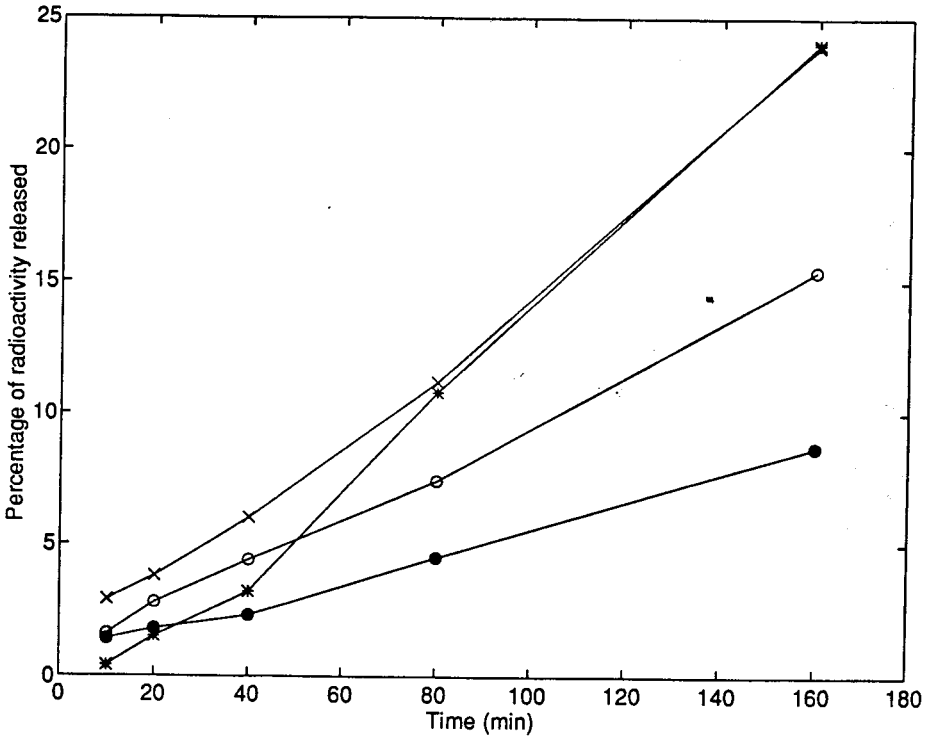

Figure 12. Kinetics of pepsin digestion of various Fc derivatives expressed as % of radiolabeled material that was non-precipitable by trichloroacetic acid as a function of time after pepsin addition. * = Fc-hinge, o = Fc, × = Fc-cys and closed circles = Fc-pap.

Fc-pap, indicating that by constraining the N-termini of the CH2 domains by one or more S–S bridges, the Fc fragment is more susceptible to pepsin proteolysis. The sequence of the hinge itself plays no role in enhanced susceptibility as the synthetic hinge (sequence: gly-cys-gly-gly-ala-ser) can be used to replace the "natural" IgG1 hinge to produce an Fc with indistinguishable properties. The increased clearance rates of non-covalently linked Fc fragments suggests that the flexibility of the CH2–CH3 joining region results in decreased functional activity of the catabolic site residues and a more compact conformation of the Fc fragments with increased resistance to pepsin attack. The loss of functional activity is presumably due to an alteration in the relative orientation of the CH2 and CH3 domains which results in decreased binding to the putative Fc receptors that protect IgG against degradation [137–139]. Consistent with this concept is the observation that reduced and alkylated IgG is also defective in intestinal transcytosis [160,187].

These results suggest that the hinge disulfide plays a role in constraining the movement of the CH2 domain relative to the CH3 domain and splitting of the —S—S— bonds results in increased mobility of the CH2 domain. This in turn changes the conformation of the CH2–CH3 domain interface which is manifested by an increased clearance rate (due to alteration of the catabolic site) and a de-

creased susceptibility to pepsin digestion [186]. Significantly, the requirement for an intact hinge disulfide in serum persistence of the Fc fragments indicates that for genetic manipulation of antibodies or other therapeutic proteins directed towards increasing their half-lives, the hinge region should be included with both CH2 and CH3 domains.

VIII. CATABOLISM VS. TRANSCYTOSIS

The close correlation between the effects of the mutations of the Fc-hinge fragments on intestinal transfer and pharmacokinetics is consistent with the earlier hypothesis of Brambell suggesting that both processes involve the interaction of cell bound Fc receptors [137–139]. In the case of the control of IgG catabolism, these receptors were postulated to bind and protect the circulating IgG from degradation and to release them back into the circulation [137–139]. In contrast to intestinal transcytosis for which FcRn has been shown to be involved [143], the Fc receptors and cell types involved in catabolism control have yet to be identified. If, by analogy with intestinal transcytosis the protective receptors involved in the control of catabolism bind to IgG in a pH-dependent manner, it is tempting to speculate that binding of IgG to these receptors does not occur on the surface of the cells involved in catabolism but in an intracellular acidic compartment from which the internalized IgG could be recycled and released at the cell surface. This putative model for IgG catabolism is similar to that proposed by Rodewald and colleagues [147] for the function of FcRn in placenta and yolk sac.

The observation that the WT Fc-hinge fragment is catabolized or trancytosed in a similar way to intact glycosylated IgG1 suggests that the conclusion concerning the location of catabolic and transcytosis sites in murine IgG1, drawn from experiments using aglycosylated recombinant Fc derivatives, can be extended to glycosylated IgG1. Furthermore, as murine and rat IgG subclasses can cross-inhibit intestinal transfer of each other [188], this indicates that the region of the molecule that is involved in transcytosis may be shared by all subclasses and across species. Consistent with this, the amino acid residues that have been demonstrated to be important for catabolism and transcytosis are highly conserved in murine isotypes and IgG isotypes of other species (Table 3). This suggests that the conclusions drawn from the investigation of the catabolism and intestinal transcytosis of the murine IgG1 molecule can be extended to all mammalian IgGs.

IX. IMPLICATIONS OF THE STUDY OF IgG CATABOLISM FOR THE ENGINEERING OF THERAPEUTIC ANTIBODIES

The identification of residues that control the pharmacokinetics of murine IgG1 has implications for the modulation of the clearance rates of antibodies of therapeutic interest. The high degree of conservation of Ile253, His310, Gln311, His433 and Asn 434 in human IgG isotypes ([189], and Table 3) indicates that mutagenesis of these residues could affect the pharmacokinetics. However, from our work with the murine IgG1 isotype, it is likely that mutation of these residues would result in more rapid clearance. Although this would have uses in generating antibodies for imaging, bivalent Fabs or Fvs might be preferable for this purpose. A longer term goal would be to increase the serum persistence of IgGs, thereby reducing the

necessary dose. Towards this aim, an improved knowledge of the Fc receptors involved in controlling catabolism is warranted.

CONCLUDING REMARKS

Recent developments in genetic engineering now allow the isolation of antibody Fab or Fv fragments that recognize an almost unlimited number of different antigens. These Fvs or Fabs can be linked to constant regions of the isotype of choice. Thus, it is now possible to generate designer antibodies of the desired specificity. These antibodies have the optimal effector functions for their particular use. Recent work in the area of IgG catabolism has resulted in the identification of the site of the IgG molecule that regulates serum persistence, and this opens up new opportunities for modulating the clearance rates of therapeutic antibodies.

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