

CHEMICAL IMMUNOLOGY

ANTIBODY ENGINEERING

IgG Effector Mechanisms

by

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Introduction

Antibodies, otherwise known as immunoglobulins, are glycoproteins which perform a major role in helping to defend the host against infection. They exist as both membrane bound receptors of B-lymphocytes and also as secreted proteins, making up about 10% to 20% of plasma protein concentration in most mammalian species [1,2]. Immunoglobulins are found in most vertebrates including fishes, amphibians, reptiles, birds and of course mammals. Their general structure has been conserved through evolution, being made up of a combination of "heavy" and "light" chains containing multiple homologous protein domains having a characteristic fold of a series of anti-parallel β -strands rolled up into a globular β -barrel structure stabilised by a conserved disulphide bond between two of the strands [3]. Most immunoglobulins have a basic symmetrical structural subunit of two heavy chains and two light chains giving rise to two identical antigen binding sites [1,2,3,4].

The N-terminal domain is called the Variable or V-region domain and it is somatic changes in and selection of this which gives rise to differences in antibody binding affinity and specificity for antigen. Immunoglobulins in most species also exist in several different classes and sometimes subclasses, characterised by different numbers and types of Constant or C-region domains associated with virtually any of the possible V-regions [1,2,3]. The differences in the constant regions or classes of the antibodies allows for antibodies with different effector functions [1,2,3,4].

Comparative studies between species show that although immunoglobulins exist in nearly all vertebrates there are differences in the types found and in their functional properties. In mammalian species the immunoglobulin classes consist of IgM, IgD, IgG, IgA and IgE with heavy chains designated respectively as μ , δ , γ , α and ϵ [1,2,3,4]. Detailed studies within some species has revealed that sometimes these classes are further subdivided into subclasses, although the precise numbers of subclasses and the gene organisation varies between species indicating that these have arisen as recent evolutionary events [5]. Thus in rabbits IgA exists as thirteen subclasses [6], whilst in humans there are two IgA subclasses and in rats and mice only one [1,2,3]. Many species also have several subclasses of IgG including humans, rats and mice which have four subclasses whilst rabbits only seem to have one IgG [1,2,3,4,5]. In addition there are two commonly occurring types of immunoglobulin light chain consisting of one V-region domain and one C-region domain called kappa and lambda (κ and λ) [1,2,3]. As a result of the processes which regulate somatic rearrangements and expression of these immunoglobulin genes each single B-cell shows isotypic and allotypic exclusion by producing antibodies with a single type and sequence of heavy chain associated with a single type and sequence of light chain [1,2,4]. The usage of κ and λ varies between species and in rats and mice approximately 90 to 95% of immunoglobulins have κ light chains and 5 to 10% λ , whereas in humans the figure is nearer 60% κ and 40% λ .

IgG isotypes

The IgG class is one which is characteristic of mammalian species although both it and the mammalian IgE class seem to share some evolutionary characteristics with the amphibian and avian IgY class [7]. IgG is the major class of immunoglobulin found in mammalian sera and it has

a heavy chain with a V-region domain followed by three C-region domains $C_{\gamma 1}$, $C_{\gamma 2}$, $C_{\gamma 3}$ (see figure 1) [1,2,3,4]. Between the $C_{\gamma 2}$ and $C_{\gamma 3}$ domains there is a less conserved proline rich stretch of amino acids which appears to confer flexibility on the molecule, called the hinge region (see figures 1 and 2a) [. In most IgG isotypes the two heavy chains are covalently joined by disulphide bonds between cysteine residues in the hinge region although the exact number and positions of disulphides varies with subclass and species [1,2,3]. Again depending upon the subclass each light chain is also usually covalently bonded by a single disulphide to a heavy chain, sometimes to a residue in the γ chain hinge region (at position 220 for human IgG1), and at other times to a cysteine residue in the N-terminal half of the $C_{\gamma 1}$ domain at position 131 or 132 [3](see figure 1). Interestingly in species of the camel family, investigators have found that in addition to conventional IgG molecules they also have subclasses of IgG which do not associate with light chain and seem to lack a $C_{\gamma 1}$ domain [15].

A number of available crystal structures (see figure 2) show that in the Fab, the heavy and light chain V-region domains pack closely together and the antigen binding specificity is formed by the close arrangement of three hypervariable loops from each domain [8]. The $C_{\gamma 1}$ domain also has a close packing arrangement with light chain C_{κ} or C_{λ} domain [8]. In the Fc fragment the two $C_{\gamma 3}$ domains also show a tight packing arrangement, but in contrast the two $C_{\gamma 2}$ domains are held apart with the space between being occupied by conserved N-linked carbohydrate on each of the chains [9]. The two heavy chains are again brought together at the N-terminal end of the $C_{\gamma 2}$ domains by the covalent disulphide bonding of the hinge region.

As can be seen from the human, rat and mouse sequences shown in figure 1, the IgG molecule is highly homologous between species. The four human IgG subclasses are very similar in sequence (greater than 90% over the constant region domains $C_{\gamma 1}$, $C_{\gamma 2}$ and $C_{\gamma 3}$) probably as a result of recent gene duplication events within evolution [3,4,16,17]. As will be described below this high homology within human IgG subclasses has enabled the important residues affecting function to be studied [4,16,17,18,19].

IgG Functions

Essentially all antibodies are able to act as adaptable linkers to the available effector systems by

virtue of the ability to recognise and bind antigen through the V-regions whilst interacting with conserved effector systems through the C-regions. Having two identical binding sites in a single IgG molecule allows the antibody to bind with higher avidity to antigens with repeating epitopes or to aggregates of antigen [1,2,4]. The flexibility of the IgG molecule, inherent within the elbow bend of the Fab (between V and C domains), and particularly within the hinge region allows for the two binding sites to cope with antigenic epitopes with a range of spacing and orientations (see figure 2)[1,2,4,13,20]. The binding of antigen can result in direct inactivation of infectious agents by blocking of functional sites with enzymic or receptor binding activity. More importantly IgG antibodies aggregated by antigen can interact with other components of the immune system by either activating the complement cascade or by binding to Fc_γ receptors on various cell types [1,2,4]. Both of these processes can assist in the opsonisation of antigen and in the triggering of inflammation and the enhancement of an immune response against an infectious agent [1,2]. In addition to these antigen dependent functions, IgG is also transported specifically to the neonate from the maternal plasma and also IgG antibodies show a reduced catabolic rate (or increased half-life) compared to other Ig classes and plasma proteins [1,2,4,21]. Certain bacteria have exploited conserved sequences on the IgG molecule and have evolved proteins, eg *Staphylococcus aureus* Protein A and *Streptococcal* Protein G, with high affinity for many IgG subclasses from many different species (see figure 2b) [1,2,4,22]. In addition low affinity auto-antibodies termed rheumatoid factors which are often IgM anti-IgG Fc antibodies have been identified in several species. The sites recognised by these antibodies appear to be conserved and many of these rheumatoid factor antibodies also make use of conserved germline V-region sequences [23].

Yet another property reported for IgG subclasses in certain species (principally mouse IgG3 and perhaps the homologous rat IgG2c) is an ability to self aggregate through sites in the C_γ3 region of the Fc [24]. Thus when these IgG antibodies bind to antigen their Fc regions allow large aggregates to form. Monoclonal antibodies of these subclasses also have a tendency to aggregate when purified particularly when stored in the cold (cryoglobulins) or at lower ionic strength (euglobulins).

In species such as the rabbit the single IgG class of antibody seems able to activate complement as well as to bind to Fc_γ receptors, however in several other species gene duplication events have lead

to multiple IgG subclasses (eg human, rat and mouse having 4 IgG subclasses each) and then through changes in sequence some of these subclasses have lost the ability to activate complement or to bind to some Fc γ R types [1,2,3,4,5,16,17,18]. Although IgG antibodies and their associated effector systems are obviously conserved and homologous between species the numbers of IgG subclasses vary and many of the duplications seem to have occurred after speciation [4,5]. This suggests that there may not be direct functional equivalence between subclasses in each species.

Any consideration of the interactions of IgG antibodies with Fc receptors needs to take account of the different classes of receptor. In humans there are three identified classes of Fc receptors for human IgG (Fc γ R). Much is known about the gene organisation and the structure and function of these receptors and they can be readily detected on the cell surface using specific monoclonal antibodies [25,26]. Human Fc γ RI (CD64) can bind monomeric IgG with high affinity and is expressed constitutively on macrophages and monocytes and can be induced on neutrophils and eosinophils. Human Fc γ RII (CD32) binds IgG only in complexed or polymeric forms and is widely expressed on a range of cell types including monocytes, macrophages, basophils, eosinophils, langerhans cells, B-cells and platelets. Human Fc γ RIII (CD16) is also a medium to low affinity receptor and is expressed on macrophages, some large granular lymphocytes (LGL), killer cells (K-cell), some natural killer cells (NK-cells), and neutrophils and can be induced on eosinophils and monocytes [25,26].

However the expression of these three receptor classes on cell types is very complex for several reasons [25,26]. Firstly each of these three classes of receptor are encoded by a cluster of closely related genes, Fc γ RIA, B and C, Fc γ RIIA, B and C and Fc γ RIIIA and B. Most of these genes encode transmembrane receptors which either have cytoplasmic domains capable of signal transduction or which associate with signalling co-receptor complexes [26]. For example the Fc γ RIIIa form is a transmembrane receptor expressed in conjunction with γ , ζ and β chains, and is found on K/NK cells, monocytes and macrophages. This receptor complex is very homologous to the T-cell receptor complex with CD3. In contrast to the other Fc γ receptors the human (but not the mouse) Fc γ RIIB, found on neutrophils and eosinophils is not transmembrane but is instead a glycosyl-phosphatidyl-inositol (GPI) anchored receptor [25,26]. Additional complexity arises because some of these genes then give rise to multiple transcripts eg Fc γ RIIb1, b2 and b3 and

finally some of these genes exist within the population in different polymorphic forms eg. $Fc_{\gamma}RIIa-R131 / Fc_{\gamma}RIIa-H131$ and $Fc_{\gamma}RIIIbNA1 / Fc_{\gamma}RIIIbNA2$ [25].

Recombinant IgG antibodies

The recognition that the specificity resides within the variable domains of the IgG molecule whilst the functions of the different subclasses reside within the constant domains facilitates the ready manipulation of the genes and hence the proteins as recombinant products. Thus variable regions from mouse or rat monoclonal antibodies were co-expressed with human IgG constant regions to create chimeric antibodies with human effector functions [28,29,30,31,32]. Such molecules were recognised to be of potential therapeutic value as well as facilitating studies of the structural basis of antibody functions [31,32,33].

Further improvements in the protein engineering of recombinant antibodies involved manipulating sequences within individual domains in order to investigate the structural basis for IgG effector functions. Two basic approaches have been used. Firstly researchers have compared the functions and sequences of naturally occurring IgG molecules of different subclasses and from different species [3,16,29,30,31,32,34]. These comparisons are used to make predictions about which sequences are involved in determining the functional differences. The predictions can be tested either by mutating residues to an inappropriate type such as to Alanine or alternatively sequences in one IgG subclass can be changed to be similar to another subclass either by site directed mutagenesis or by making chimeric constructs between the two subclasses [35,36,37].

Information on the effector functions of different IgG subclasses was confirmed using a number of matched panels of recombinant antibodies [29,30,31]. A complete matched set of monoclonal antibodies including the human IgG isotypes IgG1 (allotype G1m(za)), IgG2, IgG3 (allotypes G3m(b) and G3m(g)) and IgG4, was constructed with specificity for the hapten NP and its derivative NIP (5-iodo-4-hydroxy-3-nitrophenylacetyl) [30]. The antibodies were tested for their abilities to mediate autologous complement-dependent lysis of human red blood cells labelled with NIP, and for antibody dependent cell-mediated cytotoxicity (ADCC) of a NIP conjugated human lymphoblastoid cell line by activated human mononuclear cells. The IgG1 antibody proved to be the most effective in both complement-dependent and cell-mediated cytotoxicity [30]. The

effector cells in ADCC were inhibited by a CD16 (Fc γ RIII) monoclonal antibody and had the phenotype of K-cells. The two IgG3 antibodies were not quite as good as the IgG1 antibody in either assay and the two allotypes of IgG3 showed differences in complement lysis. Surprisingly this was despite the fact that IgG3 was shown to be fixing many more molecules of C1q than IgG1. Further studies demonstrated that although human IgG1 bound less C1 than human IgG3 there was a much more efficient deposition of C4b on the cell surface which accounted for the more effective cell lysis by IgG1 [38]. The other isotypes, IgG2 and IgG4, did not show any significant functional activity in these assays [30].

Two independent groups have investigated the effect of varying the antigen density as well as the epitope patchiness for complement lysis triggered by the NIP chimerics [39,40]. The IgG1 antibody was most effective when the antigen concentration was higher, whereas the IgG3 antibody was relatively better at lower concentrations. The IgG2 antibody gave good lysis at very high concentrations of antigen but the IgG4 antibody did not give lysis under any conditions. It was also shown that IgG1 and IgG3 activated the classical pathway of complement but not the alternative pathway. At high antigen concentration the IgG2 antibody could also activate the classical pathway but the IgG4 antibodies could not. However the IgG2 antibody, did activate the alternative pathway of complement.

In another series of studies using matched sets of human IgG1, 2, 3 and 4 antibodies to the conventional human cell surface antigen CD52 the IgG1 antibody was again found to be the most effective antibody in complement-mediated lysis [32]. The IgG3 antibody was the next most active (two fold lower in titre) followed by the IgG2 antibody (10 fold lower titre) whilst the IgG4 antibody did not work at all in complement lysis. Comparing these results with the NIP chimerics revealed a large degree of similarity except that the IgG2 antibody to CD52 antigen was more active in complement lysis [30,32]. As described above, this observation may be due to different antigen densities, or clustering of antigen, the CD52 antigen being equivalent to the NIP antigen at high concentrations [39,40].

Residues affecting complement binding

As mentioned above human IgG4 is the only human IgG subclass which does not activate

complement and the subclasses IgG1 and 3 are the most effective [16,30,32,39,40]. For mouse it is the the subclasses IgG2a and IgG2b which are active with IgG1 and possibly IgG3 being inactive whilst in rat all four subclasses are active with IgG2b and IgG1 being the most effective [16,21,34,35]. Using comparisons of the amino acid sequences of these IgG antibodies, the residues which might be responsible for the different functions were identified (see Figures 1 and 2) [16]. This led to a series of experiments in which site directed mutations of such residues were introduced into a complement fixing mouse IgG2b antibody, with specificity for the hapten NIP. A sequence motif in the C_γ2 region involving Glu 318, Lys 320 and Lys 322 was identified as being crucial for the binding of C1q the first component of the complement cascade [35].

From figure 1 it can be seen that this motif is also present in some isotypes which do not bind and activate complement such as human IgG4 and so there must be other features which are crucial to function. It was proposed that the difference between IgG3 and IgG4 was therefore dependent upon the former having a long and very flexible hinge region whilst the latter has a short and more rigid hinge [16,41]. Thus the greater segmental flexibility of the IgG3 might allow greater access to the Fc region of the antibody for complement binding. To answer this question, the hinges of IgG3 and IgG4 were swapped in anti-DNS antibodies but it was found that although the upper hinge length was responsible for the big differences in segmental flexibility, this was not responsible for the differences seen in complement binding [42]. Further experiments with domain swap mutants involving IgG1, 3 and 4 with specificity for the hapten DNS and then domain swaps involving IgG1 and IgG4 with specificity for the lymphocyte antigen CD52 demonstrated that the genetic hinge region only has a marginal effect on complement activation and the crucial differences between IgG4 and IgG1 are in the COOH terminal half of the C_γ2 domain [43,44,45]. Between human IgG1 and IgG4 there are only four residues which differ in this region, (296 Tyr/Phe, 327 Ala/Gly, 330 Ala/Ser, 331 Pro/Ser), in the anti-DNS antibody system and also repeated with a NIP hapten specific antibody it was shown that a substitution of Serine at position 331 in IgG4 for Proline (as in IgG1, 2 and 3) endows IgG4 with the ability to activate complement [45,46].

Recently starting with a human IgG1 antibody specific for human the MHC class-II, HLA-DR antigen, mutations were introduced to attempt to alter complement and Fc_γR binding [47].

Surprisingly two results with this human IgG1 antibody show a big difference with earlier studies

using mouse IgG2b antibodies specific for NIP [35]. Firstly, a change in the residue 320, previously reported from experiments with mouse IgG2b as being a crucial residue for C1q binding, from Lys to Ala had no effect on complement. In contrast a change in the residue Leu 235 to Glu which had previously been implicated in Fc γ RI binding but not in complement activation using the mouse IgG2b, abolished complement lysis by the human IgG1 [36]. It should be noted that in the three dimensional folding of the β -strands that residues which are distant in the linear chain are brought closer together (cf Figures 1 and 2).

The question of the role of the hinge in IgG is still not settled completely. In the experiments with anti-DNS the hinge length of the IgG3 was shortened by deleting the repeat exons encoding a repeating peptide pattern (see Figure 1). Whilst it was reported that an antibody without a hinge was inactive in complement activation, the shortened hinge versions were all apparently similarly active [42]. However in a system using antibodies specific for the Hapten NIP it was reported that shortening the hinge of IgG3 resulted in an improvement in the complement activation by this isotype [48,49]. Also in this NIP system the hinge could be completely replaced with a single disulphide bond whilst still retaining the ability to activate complement [49,50]. Human IgG1 has a different heavy-light chain disulphide bridge, compared to IgG2, 3 and 4, whereby the light chain is disulphide bonded to the upper hinge region of the antibody at position 220 as opposed to a cysteine normally found at position 131 in the first half of the C γ 1 domain (see Figure 1). In the three dimensional structure these residues are about 0.6 nm apart (see Figure 2). A set of mutated chimeric antibodies specific for the GD2, melanoma surface antigen, in which the arrangement of the light heavy chain bond in IgG1 was altered to be more like the other subclasses, was prepared and compared in complement lysis with human complement [51]. The mutated IgG1 antibodies lost their ability to activate complement indicating that residues in the Fab and upper hinge region are critical [51].

Further evidence for an involvement of residues in the Fab comes from recent studies on mutated human IgG1 antibodies to the CD52 antigen [33]. In human IgG1 there are several naturally occurring allotypes of the heavy chain in the human population which vary in frequency in different racial groups [52,53]. Alternative allotypic residues are recognised at positions 214 in the C γ 1 (proximal to the hinge) and at positions in the C γ 3. A gene encoding the wild type allotype

G1m(1,17) was mutated to give either the alternative natural allotype G1m(3), or artificial allotypes G1m(1,3) or G1m(17) [18,33]. Surprisingly it was found that the ability of the antibodies to cause complement mediated lysis was quantitatively dependent on the residue found at position 214 (see Figures 1 and 2) in the Fab [18,33].

Residues affecting high affinity Fc_γRI (CD64) binding

The same strategies outlined above can be used to determine residues critical for Fc_γ receptor binding and activation. The high affinity binding of monomeric IgG to the Fc_γRI receptor allowed this interaction to be studied. Human IgG1 and IgG3 bind Fc_γRI with the highest affinity (K_d 10⁻⁸ to 10⁻⁹ M) followed by IgG4 which is about 10 fold weaker in its interaction whilst IgG2 does not readily bind to the receptor [16,25]. Rat IgG2b and mouse IgG2a also bind human Fc_γRI readily whilst mouse IgG2b antibodies were found to be poor at binding [54,55]. A sequence comparison of IgG classes indicated that residues in the IgG lower hinge region (encoded by the 5' end of the C_γ2 exon) might be crucial (see figure 1 and 2). Changing the residue Glu 235 to Leu, using site directed mutagenesis, improved the affinity of mouse IgG2b for Fc_γRI by 100 fold [36]. It was shown using reciprocal domain swap mutants between TNP specific IgG1 and IgG2 antibodies that a region spanning 233-238 (Glu Leu Leu Gly Gly Pro in IgG1) was critical for binding and the introduction of the whole of this sequence into IgG2 produced an antibody which bound with higher affinity than IgG1 [56]. This result would indicate that there are of course other critical residues, possibly in the C terminal half of the C_γ2 region but also perhaps the heavy light chain disulphide bonding in the upper hinge region at position 220 (in IgG1) rather than 131 (in IgG2) which are critical. Domain swap mutants between DNS specific IgG2 and IgG3 antibodies also support the critical role of these residues encoded in the C_γ2 regions [57]. Investigation of the residues responsible for the lower binding affinity of IgG4 compared to IgG1 and 3 showed that a change of Phe 234 in IgG4 to Leu improved the affinity [57]. However in addition it was found that in IgG3 the change of Pro 331 for Ser as found in IgG4 decreased the affinity of IgG3 for Fc_γRI [57]. In addition to these domain swap experiments, the introduction of point mutations into NIP specific IgG3 antibodies in which individual residues were changed to Alanine, indicated the critical role of the residues Leu 234, Leu 235 and Gly 237 [58,59].

Residues affecting low affinity Fc_γRII (CD32) binding

Studies of the direct binding of IgG to the low affinity Fc_γRII are more difficult. Most experimental systems employ some form of complexed or aggregated IgG such as the rosetting of IgG sensitised red cells by Fc_γRII bearing leukocytes or alternatively IgG can be aggregated into small multimeric complexes using antigen or F(ab)₂ fragments of anti-light chain specific antibodies mixed in a one to one ratio with the IgG [60,61]. CD3 specific antibodies are to trigger a mitogenic response from T-cells if the antibody Fc aggregates upon binding to Fc receptors on accessory cells and this can also be used to assay binding function to low affinity receptors [55,56]. Generally the observations that IgG1 and IgG3 bound well to Fc_γRII but IgG4 and IgG2 did not again suggested that the sequence differences in the hinge and lower hinge regions might be critical (see figure 2). Using rosette formation between red cells sensitised by point mutated IgG3 antibodies with the cell lines Daudi and K562 has indicated that some of the crucial residues for binding are Leu 234 and Leu 237 [59,60].

A complication in the interpretation of these results is the observation that two alternative alleles of human Fc_γRIIa exist and that this leads to a functional difference in the ability of the receptor to discriminate between different IgG isotypes. This was originally identified for mitogenic responses with murine CD3 antibodies but has been found to affect binding of rat and human isotypes [54,55]. Thus Fc_γRIIa-R131 binds mouse IgG1 but not human IgG2 whilst Fc_γRIIa-H131 doesn't bind mouse IgG1 but does bind human IgG2. Both forms of the receptor bind human IgG1 but do not bind human IgG4. Only a single amino acid change in the receptor (R->H131) is responsible for this remarkable ability to discriminate between the different isotypes. Further studies have also indicated that the polymorphism affects binding of rat IgG2b [55]. Rat IgG2b behaves in a similar way to human IgG2 and opposite to mouse IgG1, however unlike human IgG2 the rat IgG2b also binds to the high affinity Fc_γRI receptor [55]. Another observation made using mixed rat IgG2b with mouse IgG1 hybrid Fc antibodies was that two identical rat IgG2b heavy chains are required for binding to Fc_γRIIa but one is sufficient for measurable Fc_γRI binding [55].

Recent results suggest that functionally the Fc_γRIIa polymorphism may have important consequences with regard to resistance to certain infections as mediated by IgG2 and the racial

differences in the allele frequencies may in part explain the observed geographical and racial differences in disease incidences such as Haemophilus influenzae infections [61]. In another clinical situation, this polymorphism in the binding of human IgG2 by Fc γ RIIa has been implicated as a risk factor in the development of heparin-induced thrombocytopenia [62].

Residues affecting ADCC through Fc γ RIII (CD16)

As mentioned above the Fc γ RIII receptor exists in two different forms in humans, either as a transmembrane receptor (Fc γ RIIIa) found on K and NK-cells as well as activated macrophages or as a GPI anchored molecule (Fc γ RIIIb) on cells such as neutrophils. Most studies have concentrated on functional assays of antibody-dependent cell-mediated cytotoxicity (ADCC) using effector cells expressing the transmembrane Fc γ RIIIa. The rat IgG2b and the human IgG1 antibodies to NIP and to CD52 were particularly potent at triggering ADCC with human peripheral blood mononuclear cells and activated lymphocytes as the effectors whereas human IgG4 and rat IgG2a were poor [30,32,34,63]. A series of domain swap mutants between human IgG1 and human IgG4 were constructed to identify residues responsible for the observed functional differences [44]. Firstly it was verified that all of the critical differences between these two isotypes lie in the C γ 2 domain and secondly that they were in the COOH terminal end of the C γ 2 domain, a similar result to complement activation as described above and involving four possible amino acid changes (296 Tyr:Phe, 327 Ala:Gly, 330 Ala:Ser, 331 Pro:Ser) with in particular the residue change Pro 331 in IgG1 for Ser in IgG4 prominent (see figure 1 and 2) [44]. However in a different set of experiments using point mutations of residues in the lower hinge region of IgG3, the residues 235 and 237 were identified as critical for ADCC [59]. The importance of this region for ADCC through Fc γ RIIIa was confirmed with the anti-HLA DR IgG1 antibody, where changing the Gly at 237 to Ala or exchanging the whole region 233 to 236 for the sequence found in IgG2 reduced activity [47]. As mentioned above in this same system a change of Leu 235 to Glu not only abolished binding for Fc γ RI but also complement activation but had no effect on ADCC through Fc γ RIIIa [47]. The heavy-light chain disulphide bonding of IgG1 at position 220 in the hinge rather than 131 as in other classes may also be important. It was reported that the mutation of these residues in an IgG1 antibody specific for the melanoma antigen GD2 abolished ADCC by peripheral blood mononuclear cells although it was not formally demonstrated that Fc γ RIIIa was

the receptor involved [51].

The results obtained with the domain swap mutants of IgG1 and IgG4 antibodies were however further complicated. It was found that the results obtained in this system were dependent upon the donor of the lymphocyte effectors [44]. With some donors IgG1 was effective whilst IgG4 was ineffective, and in this case the domain swap mutants implicated the residues in the COOH half of the C_γ2 as critical. For some donors it was surprisingly found that IgG1 and IgG4 were both effective in ADCC and all of the domain swap mutants were indistinguishable. With such donors of effectors it was found that the four isotypes IgG1, IgG2, IgG3 and IgG4 gave very similar levels of activity [44]. Several genetic polymorphisms of the Fc_γRIIIa gene have recently been described but it remains to be demonstrated which if any of these might be responsible for the functional polymorphism seen with the CD52 antibodies [64,65].

As mentioned above, rat IgG2b antibodies were found to mediate ADCC with human K-cells whereas rat IgG2a antibodies did not [63]. In a similar fashion to the results with Fc_γRIIa, studies with hybrid Fc antibodies between rat IgG2b and rat IgG2a also showed that two identical rat IgG2b heavy chains were required in order to see functional ADCC [55,63]. One explanation for these observations is that one IgG antibody needs to bind or interact with two Fc_γRIIa or Fc_γRIIIa receptors to mediate functional binding. Although it could be argued that this is due to an abnormal structure generated in the hinge region when two different isotypes are paired this does not seem to disturb the interaction with Fc_γRI [55].

Glycosylation and effector functions

IgG molecules have a highly conserved N-linked glycosylation site within the C_γ2 domain at Asn 297 (see figures 1 and 2) which has been found to be critical for complement mediated lysis as well as binding to and activation of all three Fc_γR classes of receptor [1,2,3,4]. Antibodies produced without carbohydrate, either through use of metabolic inhibitors, endoglycosidases by site directed mutation of the attachment site, or produced in bacterial expression systems all show greatly reduced biological functions [16,17,18,66,67]. An aglycosylated human IgG antibody with specificity for the mouse CD8 antigen was found not to deplete mouse CD8 lymphocytes in-vivo whereas the glycosylated human IgG subclasses were very effective at depleting cells [68]. This

property has been exploited in the production of an aglycosylated form of the humanised IgG1 CD3 antibody which can block T-cell functions without depleting the cells or triggering cytokine release thus eliminating some of the severe side effects of CD3 antibody therapy [69]. Whilst glycosylation of antibody is important for function, the precise structures attached to the IgG are complex and can vary from one cell line to another depending upon the glycosyl transferases present [67,70]. There is some suggestion that the precise carbohydrate structure present on an antibody might have some influence over the biological activity of the antibody in complement activation and Fc_γR binding although further investigation is revealing an important role for the precise oligosaccharide sequences and their interactions with the protein sequences found in each isotype [67,71,72]. Two functions of IgG antibody which do not seem to be strongly dependent upon glycosylation are neonatal transport and secondly the catabolic rate of IgG [21,73].

Neonatal Transport and the role of FcRn

An interesting aspect of the IgG class in mammals which have been studied is the acquisition of maternal IgG by the neonate [1,2]. This is thought to provide a level of protection against infection early in life before the infants own adaptive immune response has had a chance to develop a significant repertoire of it's own. However there are significant differences in the way that species obtain this IgG. In some species, for example rats, mice, horses and pigs the IgG appears to be transferred to the infant during the first few hours of life in the colostrum and the IgG is then absorbed from the gut. In other species, humans being a key example, the IgG is transferred across the placenta to the neonate in utero.

An Fc receptor called FcRn was identified in rats and mice which seemed to be responsible for the transport of IgG across the gut during the first 24 hours after birth. The interesting feature of this receptor is that it was found to be associated with β_2 -microglobulin and had homology with MHC-class I molecules [74]. Studies of β_2 -microglobulin knockout transgenic mice have confirmed that it is required for the acquisition of maternal IgG by neonates but that IgG levels in maternal colostrum are unaltered [75]. The rat FcRn was co-crystallized with IgG and a full structure of the complex determined. The receptor has a very similar structure to MHC Class-I but does not appear to bind peptides in the groove between the two α -helices (see figure 2b) [76]. Instead one end of the two α -helices seem to form a contact with the interface between the C_{γ2} and C_{γ3}

domains of the IgG Fc [12]. Interestingly this is a very similar region to a binding site in the Fc identified from a crystal structure of *Staphylococcus aureus* Protein A and human IgG1 (see figure 2b) [11]. Protein A binding shows a remarkable degree of conservation across species and between many IgG subclasses and perhaps the reason is that this same part of the IgG molecule is conserved through a requirement for interaction with FcRn like receptors.

The receptor exhibits only weak binding with IgG at neutral pH but shows increased affinity at low pH as might be encountered in the gut or in intracellular vesicles [77,78]. It is thought that this pH dependence is involved in the recycling of the receptor and the reversible binding of FcRn to IgG. Using a soluble form of the FcRn receptor and recombinant IgG antibodies in plasmon resonance binding assays the pH dependence was shown to be dependent upon titrating histidine residues in the Fc of the IgG molecule (residues 310 in the C_γ2 and 433 in the C_γ3 domain) and also in the FcRn heavy chain molecule itself (residues 250 and 251) (see figures 1 and 2) [78]. Mutations in the C_γ2 residues 253, 310, 311, and the C_γ3 residues 433 and 434 of a mouse IgG1 antibody have been shown to reduce the transmission from the intestinal lumen of neonatal mice [21,79]. These studies also investigated the effect of mutating one heavy chain only in the Fc pair and demonstrated that two functional heavy chains were required for efficient transport and this suggests that transport involves two FcRn molecules interacting with one IgG [21,79].

For many years there has been a search for the human Fc receptor(s) responsible for the trans-placental transport of maternal IgG. Some workers have argued in favour of the receptors Fc_γRI, Fc_γRII and or Fc_γRIII as being involved in this transport. All four human IgG subclasses appear to be transported so this observation is not readily reconciled with the affinities of the four subclasses for the classical Fc_γ receptors. A human homologue with high homology to the rat FcRn has been identified and sequenced, the molecule has a an MHC Class-I like α -chain associated with β_2 -microglobulin [80]. The transfected and expressed molecule shows a similar pH dependence in binding to IgG as compared to rat FcRn. Also a recently published study using confocal fluorescent microscopy has identified β_2 -microglobulin and IgG as being co-localized to intracellular apical granules in human placental syncytiotrophoblasts [81]. The evidence thus now seems compelling that a homologous MHC Class-I like molecule is involved in maternal-fetal transport of IgG, the transport being across the gut in some animals and across the placenta in

others.

Although acquisition of maternal IgG by the infant is normally thought to be protective there are diseases and pathologies which result from the transport of inappropriate specificities. For example haemolytic disease of the new born occurs when anti-paternal allotype blood group IgG antibodies (eg against Rh-D antigens in humans) are acquired by the infant resulting in the destruction of red blood cells by an Fc dependent process [1,2]. Other diseases are associated with maternal antibodies specific for platelet alloantigens (neonatal alloimmune thrombocytopenia) or for example autoantibodies against neurotransmitter receptors[82,83]. Interestingly haemolytic disease of the newborn is also observed in several domestic species, such as horses and pigs, but in line with the absence of placental transfer in these species the pathology only occurs when the infants suckle the colostrum.

Is IgG Catabolism related to binding to FcRn?

It has for many years been realised that the biological half life of IgG molecules is abnormally long (3 to 4 weeks) when compared to other plasma proteins, including other immunoglobulin classes such as IgM and IgA (3-7 days) [1,2,21,84]. The other property which has been noted is that the half-life is also dependent upon the total concentration of IgG in the plasma. Thus if the concentration of IgG is raised as in conditions such as myeloma the half life is greatly reduced whilst if the IgG concentration is lowered as in agammaglobulinaemia the half-life of administered immunoglobulin is extended. This suggested a saturable receptor driven mechanism for the control of the catabolic rate of IgG [85]. Thus a relatively constant plasma concentration of IgG can be maintained over a fairly wide range of synthetic rates. It was proposed that the IgG molecule might be endocytosed and then bound to a receptor which protected it from lysosomal degradation and then recycled it back to the plasma. There is currently strong evidence which indicates that the Fc_γRc (catabolism) receptor is similar or identical to the neonatal Fc receptor FcRn [19,21].

Firstly the half life of IgG is largely a function of the Fc and in particular requires both the C_γ2 and C_γ3 domains [86]. Unlike the effector functions mediated through the receptors Fc_γRI, II and III the half-life is not drastically dependent upon glycosylation of the C_γ2 region, or upon amino acids implicated in binding to these three classes of receptors or to complement [21,73]. Secondly

conserved residues present within IgGs from most species and overlapping with the identified Protein A binding site were identified as being important in controlling catabolic rate (residues 253, 310, 311, 433 and 434) [21,87]. This is the same site to which the FcRn receptor binds as identified within the crystal structure[12]. In particular the same histidine residues which have been identified as being important for the pH dependence of FcRn binding to IgG also are important for controlling catabolic rate [77,78,87]. This pH dependence of binding of FcRn would also fit with the recycling receptor hypothesis mentioned above [85]. Finally the in-vivo half-life of mouse IgG1 appears to require two heavy chains in the Fc which both have wild type sequences just as for transport via the neonatal FcRn [21,86].

So is FcRn or a closely related receptor, FcRc, responsible for the decreased catabolism of IgG molecules? A number of observations reported in the literature seem to implicate FcRn although the authors of these papers do not always make this a conclusion. Thus studies have shown (a) that the rat FcRn is expressed in adult hepatocytes and (b) in probing human tissues by Northern blot with the human homologue, expression of RNA was identified in a range of tissues including liver, kidney and pancreas [87,88]. (c) It had been observed that the IgG concentration of the plasma of transgenic β_2m deficient mice is lower than normal litter mates although the studies did not look at catabolic rates in these animals [75]. This definitive experiment has recently been published by Ghetie et al and they found that in β_2m deficient mice the half-life of IgG1 was very short suggesting that the one receptor FcRn is responsible for both neonatal transport and plasma IgG homeostasis [89].

There are some interesting predictions which might also relate to the role of the FcRn receptor in controlling catabolic rates. Human IgG3 tends to have a shorter half-life than the other human IgG subclasses the most common allotypic form has one of the histidine changes (His 435) in the C_{γ3} such that it does not bind to Protein A [1,2,4,16,17,21]. This might also affect the binding to the catabolic receptor and increase it's catabolic rate. However there is another allotype of IgG3 found mainly within the Japanese population which does have this Histidine (435) and which also binds to Protein A [4,52]. An interesting question is whether these two allotypes would bind with different affinities and have different catabolic rates in-vivo?

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Figure 1

Sequences for human, rat and mouse IgG subclasses derived from the Kabat database [3] are shown aligned for sequence homology. A "*" indicates complete homology between sequences with a "." representing conservative changes, with spaces "_" to allow better alignment. Residues mentioned in the text are indicated using the EU numbering system [3].

Figure 1c, IgG C_H2 Sequences

	233	252	296	318	327
	235	253		320	330
	238	254	297	322	331
Human IGG1	APELLGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Human IGG2	APPVAGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Human IGG3	APELLGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Human IGG4	APEFLGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Mouse Igg1	VPEVSSVFIFPPKPKDITLTPKVT	CVVVDISKDDPEVQ	PSWFYDDVEVHT	TAQTPREEQ	FNSYR
Mouse Igg2a ^b	APDILGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Mouse Igg2a ^a	APNLLGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Mouse Igg2b	APNLEGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Mouse Igg3	AGNILLGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Rat Igg1	GSEVSSVFIFPPKPKDITLTPKVT	CVVVDISKDDPEVQ	PSWFYDDVEVHT	TAQTPREEQ	FNSYR
Rat Igg2a	GSEVSSVFIFPPKPKDITLTPKVT	CVVVDISKDDPEVQ	PSWFYDDVEVHT	TAQTPREEQ	FNSYR
Rat Igg2b	VPELLGGPSVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD
Rat Igg2c	DDNLGRFSPVFLFPKPKDITLMSRTP	EVTKPEVFNWYDGV	EVHNAKTKPREEQ	YNSYR	VSVLTVLHQD

Figure 1d, IgG C_H3 Sequences

	433	435	436
Human IGG1	GQPREPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Human IGG2	GQPREPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Human IGG3	GQPREPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Human IGG4	GQPREPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Mouse Igg1	GRPKAPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Mouse Igg2a ^b	GFVRAPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Mouse Igg2a ^a	GSVRAPOVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Mouse Igg2b	GLVRAPOVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Mouse Igg3	GRAQTPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Rat Igg1	GRTQVPHVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Rat Igg2a	GTRFGPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Rat Igg2b	GLVRAPOVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN
Rat Igg2c	GKARTPQVYVTLPPSRDELTKNQVSLTCLVKGFYPSDIAVWESNGQPE	NNTYKTPPPVLDSDG	SFFFLYSKLTVDKSRWQOGN

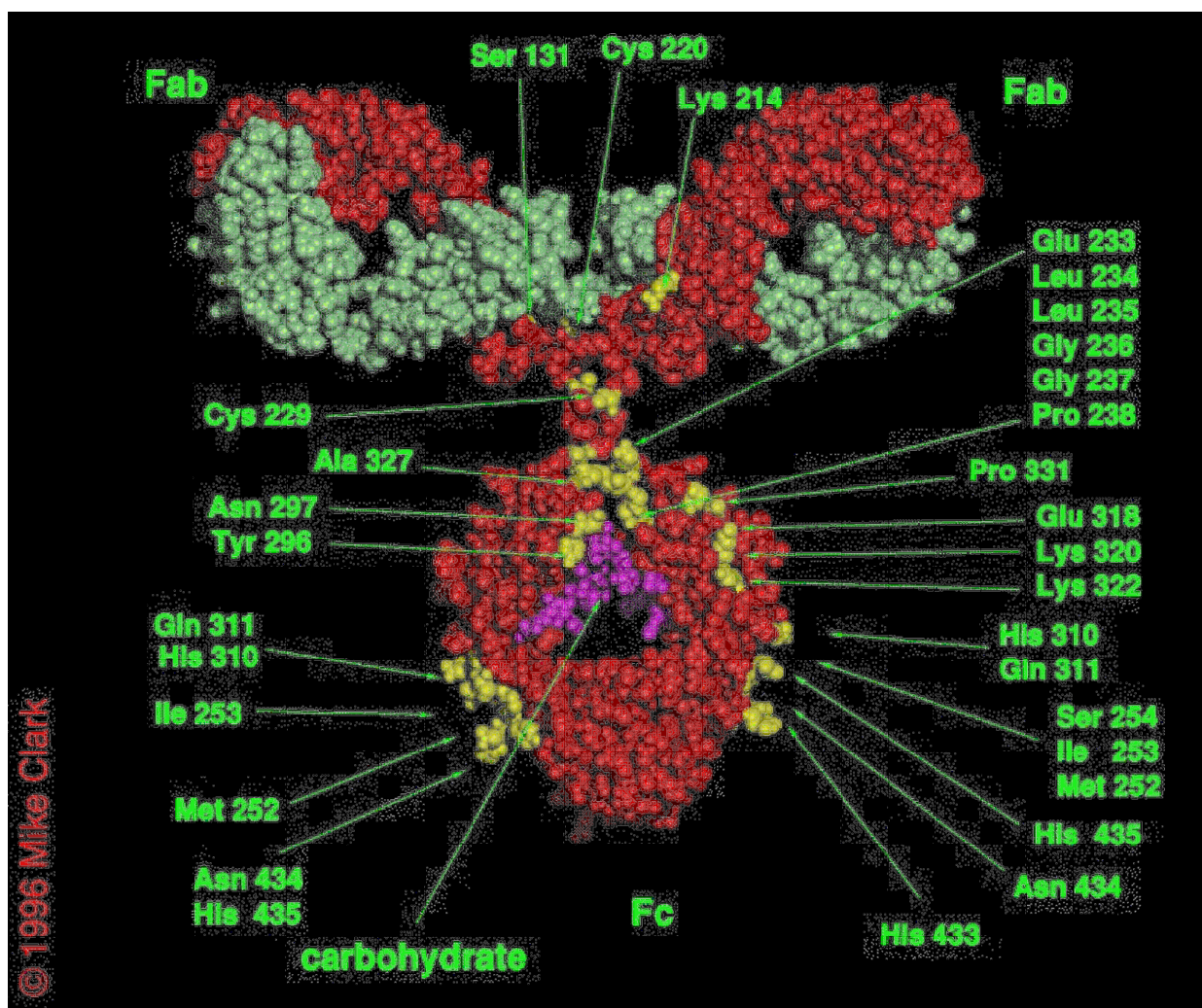


Figure 2

Shown are raster space filling models of a human IgG1 antibody (figure 2a) and the Fc of an IgG antibody interacting with *Staphylococcus aureus* Protein A on the left and FcRn on the right (figure 2b). The models were created from PDB structure files in the Brookhaven Database [8] for a human Fab [9], a human IgG1 Fc [10], a human IgG1 Fc -Protein A complex [11], and a human IgG1-rat FcRn complex [12]. Certain residues, in particular parts of the hinge, are missing from the crystal structures although attempts have been made to computer model these features [13,14]. For the models shown here a peptide for the missing residues in the lower hinge region was created and inserted between the Fab and Fc structures. The final models were then aligned, superimposed and energy minimilised for the protein backbones using the computer software packages Quanta and Charmm, from Biosym Technologies Inc. USA, running on a Silicon Graphics Iris Indigo workstation.