

Review

Isotype and glycoform selection for antibody therapeutics

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ABSTRACT

We live in a hostile environment but are protected by the innate and adaptive immune system. A major component of the latter is mediated by antibody molecules that bind to pathogens, with exquisite specificity, and the immune complex formed activates cellular mechanisms leading to the removal and destruction of the complex. Five classes of antibody are identified; however, the IgG class predominates in serum and a majority of monoclonal antibody (mAb) therapeutics are based on the IgG format. Selection within the antibody repertoire allows the generation of (mAb) having specificity for any selected target, including human antigens. This review focuses on the structure and function of the Fc region of IgG molecules that mediates biologic functions, within immune complexes, by interactions with cellular Fc receptors (FcγR) and/or the C1q component of complement. A property of IgG that is suited to its use as a therapeutic is the long catabolic half life of ~21 days, mediated through the structurally distinct neonatal Fc receptor (FcRn). Our understanding of structure/function relationships is such that we can contemplate engineering the IgG–Fc to enhance or eliminate biologic activities to generate therapeutics considered optimal for a given disease indication. There are four subclasses of human IgG that exhibit high sequence homology but a unique profile of biologic activities. The FcγR and the C1q binding functions are dependent on glycosylation of the IgG–Fc. Normal human serum IgG is comprised of multiple glycoforms and biologic activities, other than catabolism, varies between glycoforms.

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Introduction

We live in a hostile environment and are constantly exposed to potential pathogens; however, we are mostly unaware of these insults due to protective innate and adaptive immune responses [1,2]. An intact immune system is essential to the integrity of the individual. The adaptive immune system has many components but the focus in this review is the role of antibodies. Initial contact with antigen provokes a primary response and the production of antibodies of the IgM class. Further stimulation by antigen may lead to a secondary response characterized by the production of antibody of the IgG, IgA, IgE and/or IgD class; a process referred to as class or isotype switching. There are four subclasses of IgG (IgG1, IgG2, IgG3 and IgG4) and two subclasses of IgA (IgA1 and IgA2), giving a total of nine antibody isotypes in humans [3–6]. In addition there is a significant polymorphism within Ig genes [7]. Secondary immune responses also establish memory that provides for a rapid and amplified response to subsequent contact with the same pathogen. Each antibody isotype has unique structural and functional properties. Protective antibody responses are extremely heterogeneous due to the production of antibodies of unique specificity for individual epitopes expressed on the antigen. It is not possible, therefore, to unequivocally define the mechanism

of action that results in immune protection, i.e. neutralization, removal and destruction of pathogens. Quantitatively IgG is the predominant antibody isotype present in normal human serum and, to date, all licensed intact mAb¹ therapeutics are based on the IgG format and are produced by culture of transfected mammalian cells, e.g. Chinese hamster ovary (CHO), mouse NSO or mouse Sp2/0. The quality of the antibody therapeutic product will depend on the ability of the chosen cell type and individual cell line to effect post-translational modifications similar or identical to those of human plasma cells.

Overview of the quaternary structure of human IgG

We have detailed X-ray crystal structures for a number of antibodies and many more of component Fab and Fc fragments; however, they present a rather static model and it is important to have an image of a structure in which the functional “hinge” region allows for independent mobility for the Fab and Fc regions (Fig. 1). This permits functional divalency for antigen binding by the Fab regions and provides for the Fc region to engage ligands that initiate effector pathways. The functional hinge is comprised

¹ Abbreviations used: mAb, monoclonal antibody; FcR, Fc receptors; CHO, Chinese hamster ovary; SpA, Staphylococcal protein A; SpG, Streptococcal protein G; RF, rheumatoid factor.

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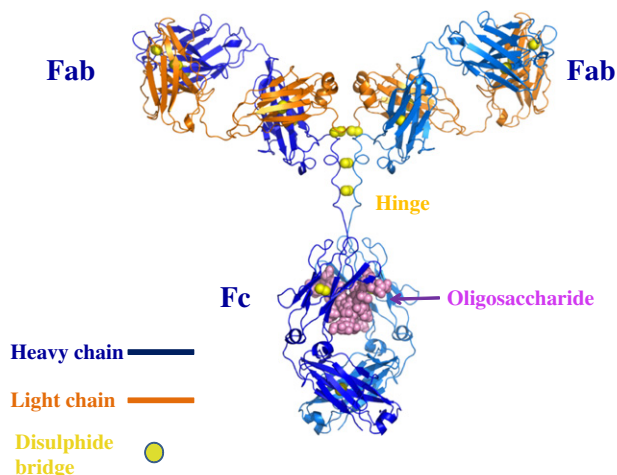


Fig. 1. Alpha carbon backbone structure of an IgG1 molecule. Digestion within the hinge region, by papain, releases the Fab (Fragment antigen binding) and Fc (Fragment crystallisable) fragments. Structure generated by Peter Artymiuk University of Sheffield UK) using PyMOL (<http://pymol.sourceforge.net>).

of three structural regions; mobile upper and lower sequences and a rigid core comprised of cystine and proline residues that form a semi-rigid helical structure within which inter-heavy chain disulphide bridges are formed. The length of the hinge region and the number of inter-heavy chain disulphide bonds differs significantly between the human IgG subclasses and influences mobility and average solution conformation of the Fab and Fc moieties, with respect to each other [3–6]. This may include the ability to assume a “dislocated” form that provides access to effector ligand binding sites localised to the hinge proximal region of the CH₂ domain. The extended hinge region of IgG3 molecules has been engineered to generate hinge regions of different length but revealed no direct relationship between hinge length and the ability to bind and activate the C1 component of complement; however, at least one inter-heavy chain disulphide bridge is required [8].

Crystals of 10 intact IgG molecules have been reported [9,10]; however resolution of the Fab and Fc structure was obtained for only two; one was essentially a rheumatoid factor like autoantibody in which the Fab had specificity for a Fc epitope; the other resulted from stabilisation of structure due to the close proximity (contact!) between the Fab and the Fc of a neighbouring molecule [10].

Quaternary structure of IgG–Fc: the protein moiety

An early insight into the structure of IgG–Fc was obtained from the crystal structure of a human IgG1–Fc resolved to 2.9-Å [9]. Similar structures have been reported for human and mouse IgG–Fc and chicken IgY–Fc fragments [3–6]; whilst providing further insights due to progressively higher resolution. The first IgG–Fc structure derived by Deisenhofer [9] was generated by cleavage of polyclonal IgG at the Lys₂₂₂–Thr₂₂₃ peptide bond, within the hinge region, and extending to C-terminus residue 446. It was reported that interpretable electron density was not obtained for residues 223–237 that comprise most of the core and lower hinge region or the C-terminal residues 444–446; it was not known at this time that the CH₃ exon codes for a C-terminal 447 lysine residue that is removed by endogenous carboxypeptidase B. The α carbon IgG structure shown in Fig. 1 is a composite of the Deisenhofer [9] IgG–Fc structure for residues 238–443 with the addition of a computer generated structure for the hinge sequence [12] and a Fab structure [13]. The common structural features reported are:

- (i) The CH₃ domains are well defined due to non-covalent pairing, involving $\sim 2000 \text{ \AA}^2$ of accessible surface area in the (CH₃)₂ module.
- (ii) The area of non-covalent contact between the CH₂ and CH₃ domains is $\sim 800 \text{ \AA}^2$. This suggests that the CH₂–CH₃ contact contributes to the relative stability observed for the C-terminal proximal region of CH₂ domains; as opposed to the “softness” of those parts of the CH₂ domain remote from the CH₂–CH₃ interface.
- (iii) The CH₂ domains do not pair and the hydrophobic surface of each CH₂ domain is “overlaid” by the carbohydrate. Hydrophobic and polar interactions occupy $\sim 500 \text{ \AA}^2/\text{CH}_2$ domain and substitutes for domain pairing.
- (iv) One CH₂ domain was more ordered than the other, due to its crystal contact with a neighbouring CH₂ domain.
- (v) The intrinsic stability of the immunoglobulin fold is reflected in higher structural resolution for the β -sheets regions than for β -bends.

These interpretations and conclusions are confirmed in other X-ray structures obtained for human IgG–Fc alone [9,11,13–15] and in complex with Staphylococcal protein A (SpA) [9], Streptococcal protein G (SpG) [16], rheumatoid factor (RF) [17–19], human sFc γ R1IIa [20], sFc γ R1IIb [21,22] and sFc γ R1IIa [23,24]. The observed internal mobility of the lower hinge and hinge proximal regions of the CH₂ domains allows for an equilibrium of high order conformers to be formed that may differentially bind unique ligands, e.g. the three homologous Fc γ receptor types. Previous proposals that different ligands may bind through “overlapping non-identical sites” may suggest too rigid a structure [25] and may be modified to suggest that each Fc γ R binds to a unique IgG–Fc conformer present within an equilibrium of transient protein structures; however, common amino acid residue side chains and/or main chain atoms may be involved [25,26]. The binding sites for sFc γ R1IIa, sFc γ R1IIb and sFc γ R1IIc are asymmetric with both heavy chains being engaged such that monomeric IgG is univalent for Fc γ receptors and the C1 component of complement; this obviate continuous activation of inflammatory cascades by circulating endogenous IgG *in vivo*; IgG–antigen immune complexes are multivalent and able to activate cellular receptors. Residues of the lower hinge region that are disordered in the Fc crystals are ordered in the Fc–Fc γ R complexes and directly involved in binding the receptor [21,22]. By contrast IgG–Fc is functionally divalent for ligands binding at the CH₂–CH₃ interface e.g. the neonatal Fc receptor (FcRn), RF, SpA, SpG [11,13–19], Fig. 2. Due to the symmetry of

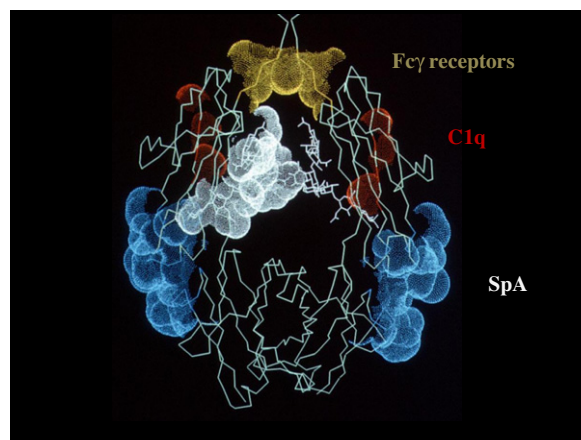


Fig. 2. Expanded view of the IgG–Fc showing the van der Waals radii of interaction sites for Staphylococcal protein A (blue), Fc receptors (beige) and the complement C1q component (red).

the IgG–Fc the two interaction sites are opposed at $\sim 180^\circ$ and each is accessible to bind macromolecular ligands to form multimeric complexes.

The IgG–Fc oligosaccharide moiety

It is established that human IgGs contain a consensus Fc glycosylation sequon Asn₂₉₇–Ser–Thr (Eu sequence [27] and that the presence of a core diantennary heptasaccharide at this position is essential for optimal activation of Fc γ receptors and the C1 component of complement. The oligosaccharide of normal polyclonal IgG–Fc is heterogeneous and essentially comprised of the core heptasaccharide with variable addition of fucose, galactose, bisecting *N*-acetylglucosamine and sialic acid. Fig. 3; sialylation is modest with <10% of structures being monosialylated or disialylated [28–32]; recent analyses employing high sensitivity mass spectrometry reports the presence of further minor oligosaccharides, e.g. high mannose and hybrid glycoforms [32].

A shorthand nomenclature has been adopted for oligosaccharides released from normal polyclonal IgG–Fc. The core heptasaccharide is designated G0 (zero galactose), Fig. 3, the core bearing one or two galactose residues are designated G1 and G2, respectively. The core + fucose is designated G0F, the core + fucose + galactose G1F, G2F, etc.; when bisecting *N*-acetylglucosamine is present a *b* is added, e.g. G0b, G0bF, G1bF, etc.; sialylation at the galactose residues are designated as G1FSA, G2FBSA, etc. The approximate composition of oligosaccharides released from normal polyclonal human IgG–Fc is G0, 3%; (G1), 3%; G2, 6%; G0F, 23%; G1F, 30%; G2F, 24%; G0bF, 3%; G1bF, 4%; G2bF, 7% [31–34]. It is important to define the glycoform of the whole IgG molecule, e.g. [G0/G1F]; [G1F/G2bF], etc. since it has been shown that symmetrical and asymmetrical pairing of heavy chain glycoforms occurs [35,36] and enhanced Fc γ RIIIa mediated ADCC may be observed for IgG in which one heavy chain only bears oligosaccharide devoid of fucose [37,38]; thus the [G0/G0F] glycoform would be as potent in ADCC as the [G0/G0] glycoform.

Given the paucity of sialylation one of 16 unique neutral heavy chain glycoforms may be attached to give a total of 128 IgG neutral glycoforms [(16 $\times$ 16)/2]: the heavy chains are equivalent due to the symmetry of the IgG molecule. The presence of minor oligosaccharide structures may be significant since each could be the predominant form present on an antibody secreted from a single plasma cell; analysis of monoclonal human IgG, isolated from the sera of patient having plasma cell cancer (multiple myeloma), has shown that the IgG–Fc glycoform profile of the paraprotein is essentially unique for each protein analysed [28,39]. The glycoform profile of the paraprotein was shown to vary at different stages of

the disease whilst that of the normal polyclonal component remained stable [39]. Subtle differences in oligosaccharide processing was observed with a preference for addition of galactose to the $\alpha(1-6)$ arm of IgG1–Fc and the $\alpha(1-3)$ arm of IgG2–Fc; for the IgG3 subclass the arm preference correlated with allotype [28,29,39]. These data suggest a critical balance between the conformation of the IgG–Fc and the steric requirements for glycosyltransferase enzyme activities that may be subject to niche environments within the Golgi apparatus.

The glycoform profile of total polyclonal IgG can vary significantly in health and disease, particularly autoimmune and inflammatory diseases [40–50]. Methods have been developed that allow the glycoform profile of antigen specific polyclonal IgG autoantibodies to be analysed and significant differences in the glycoform profiles of IgG autoantibodies and the bulk IgG have been reported [42,51]. The oligosaccharide profiles of recombinant IgG proteins produced in mammalian cells are significantly influenced by the cell type, the culture method and conditions employed, however, the [G0F/G0F] glycoform predominates (see below). Under conditions of stress, e.g. nutrient depletion, acid pH, etc., deviant glycosylation may be observed e.g. the presence of high mannose forms and/or incomplete occupancy [52].

IgG–Fc protein/oligosaccharide interactions

Uniquely, amongst glycoproteins, X-ray crystallographic analysis of IgG–Fc has yielded electron density for the oligosaccharide [11,13–23]. In each case the oligosaccharide is seen to be integral with the protein structure and to make multiple hydrophobic and polar non-covalent interactions with inner face of the CH₂ domain. The structure of an apparent G1F glycoform, with galactose on the $\alpha(1-6)$ arm, has been resolved; however, galactose present on the $\alpha(1-3)$ arm may be mobile and not resolved. A potential for 72 protein/oligosaccharide interactions have been proposed, including six CH₂ protein/oligosaccharide hydrogen bonds and six hydrogen bonds within each oligosaccharide moiety [11,31,53]. The $\alpha(1-3)$ –Man–GlcNAc arms are orientated towards the internal space between the CH₂ domains and weak lateral interactions between sugar residues have been suggested for some structures but not others [11,54].

In spite of the close association between the oligosaccharide and the protein structure microorganisms have evolved endoglycosidases that cleave oligosaccharides from native IgG–Fc with the loss of effector functions. Thus, *Streptococcus pyogenes* produces endoglycosidase S (EndoS) that cleaves between the primary and secondary *N*-acetylglucosamine residues [55,56]; Peptide: *N*-Glycosidase F (PNGase F), isolated from culture filtrate of *Flavobacterium meningosepticum*, cleaves the peptide/oligosaccharide bond to generate aglycosylated IgG [28,29,49–128].

The properties of a series of normal, truncated and aglycosylated glycoforms of IgG1–Fc, generated *in vitro*, were subjected to X-ray crystallographic analysis, differential scanning microcalorimetry (DSC) and Fc γ receptor binding (microcalorimetry) [58,59]. DSC of (G2)₂ and (G0)₂ glycoforms exhibited two transition temperatures, Tm1 and Tm2 of 71.4° and 82.2°, representing the unfolding of the CH₂ and CH₃ domains, respectively. These data suggest that whilst the galactose residue on the $\alpha(1-6)$ arm has substantial contacts with the protein structure it does not impact CH₂ domain stability. Sequential removal of the terminal GlcNAc and the two arm mannose residues, generating a [GlcNAc2Man]/[GlcNAc2Mn] glycoform, resulted in destabilisation of the CH₂ domain and a lowering of Tm1 to 67.7°, Tm2 beings unchanged. The thermodynamic parameters describing CH₂ thermal denaturation of all IgG–Fc glycoforms was consistent with a cooperative unfolding whilst the unfolding of the CH₂ domain of aglycosylated

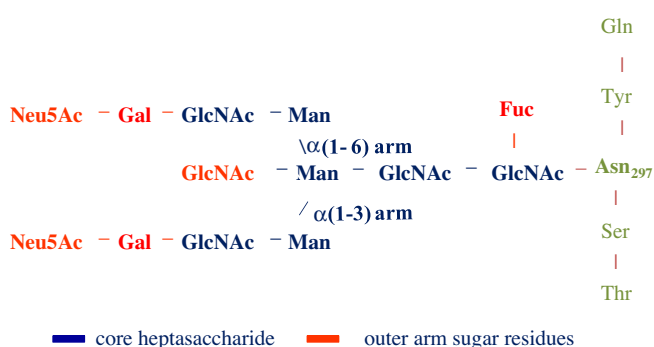


Fig. 3. Potential heterogeneity of diantennary oligosaccharide structures. The core G0 heptasaccharide is shown in blue, additional sugar residues that may be present shown in red. GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Man, mannose; Gal, galactose; Neu5Ac, *N*-acetyl neuraminic acid.

IgG1–Fc was non-cooperative, involving at least one intermediate [58]. It was proposed that this intermediate is a partially unfolded CH₂ domain pair possessing hinge proximal disordered/unfolded loops that may account for the compromised functional activities of deglycosylated IgG and IgG–Fc [58–61]. DSC has proved to be a valuable tool for probing the contributions of buffers and Fab sequence to stability and solubility of intact IgG and antibody fragments [62–66].

The truncated IgG–Fc glycoforms showed progressive increases in the temperature factors for the CH₂ domain, as evidence of progressive structural disorder (destabilisation) [61,58]. Minimal (weak) FcγRI and C1 binding and activation was observed for the [GlcNAc2Man]/[GlcNAc2Mn] glycoform that has the potential to form 31 non-covalent contacts with the protein, including at least three hydrogen bonds [53]. Truncation of the sugar residues results in the mutual approach of CH₂ domains with the generation of a “closed” conformation; in contrast to the “open” conformation observed for the fully galactosylated IgG–Fc [61]. The dramatic loss of FcγR and C1 binding and activation for aglycosylated IgG–Fc contrasts with consistent reports of minimal structural change within the protein structure.

An extensive NMR study of a series of truncated glycoforms showed that trimming of the oligosaccharide was accompanied by concomitant increase in the number of amino acid residues perturbed within the CH₂ domains [67]. Cleavage between the primary and secondary GlcNAc sugar residues induced conformational changes within the lower hinge region, at sites having no direct contact with the carbohydrate moieties, but that form the major FcγR-binding site. Conformation at the CH₂/CH₃ interface, that forms the FcRn and protein A binding sites, was minimally perturbed. A dynamic model was also proposed from an NMR study of differentially galactosylated and sialylated IgG–Fc glycoforms [68]. It was proposed that sugar residues of the α(1–6) arm may be bound or unbound to the CH₂ domain protein surface and that in the latter state are accessible to glycosyltransferases.

IgG–Fc ligand binding, activation and modulation

Cellular IgG–Fc receptors

Two distinct functions for IgG–Fc receptors may be distinguished: (i) to bind antibody/antigen complexes and initiate effector functions leading to their removal and destruction, (ii) to mediate transport across epithelial membranes, i.e. transcytosis.

IgG–Fc receptors (FcγR) mediating antigen clearance

Three types/classes of membrane bound human FcγR (FcγRI [CD64], FcγRII [CD32], FcγRIII [CD16]) and six sub-types [FcγRI, FcγRIIA, FcγRIIB1, FcγRIIB2, FcγRIIC, FcγRIIIA, FcγRIIIB] have been defined by immunochemical, biochemical and gene sequencing studies [69,70]. These receptors may be constitutively expressed on a wide range of leucocytes and expression may be up regulated and/or induced by cytokines generated and released within an inflammatory response. The effector mechanisms activated are diverse and include inactivation and removal (e.g. phagocytosis, respiratory burst and cytolysis), accessory functions such as the enhancement of antigen presentation by dendritic cells, and the down-regulation of growth and differentiation of lymphocytes. It is evident, therefore, that FcγRs play an important role in the induction, establishment and resolution of protective immune responses.

Multiple parameters determine the structure and biological activities of immune complexes: (i) valency of the antibody, (ii) average affinity/avidity of the antibody population, (iii) isotype of the antibody, (iv) valency or epitope density of the antigen, (v)

valency of individual effector ligands, (v) cumulative valency when multiple ligands are engaged, e.g. FcγR and complement receptors, etc., (vii) proportions of each antibody isotype within a polyclonal response [71].

The FcγRI receptor is constitutively expressed on mononuclear phagocytes and dendritic cells; however, expression can be up-regulated and/or induced by the action of cytokines. FcγRIIIa is the most widely expressed FcγR and is found on most hemopoietic cells. Polymorphic variants of FcγRIIIa are identified by the presence of histidine (FcγRIIIa-131H) or arginine (FcγRIIIa-131R) at amino acid residue 131. The higher affinity of the FcγRIIIa-131H form for IgG2 results in differing cellular responses to engagement by IgG2 immune complexes [69–73]; higher phagocytic capacity for *Streptococcus pneumoniae* opsonised with IgG2 antibody was observed for neutrophils of donors homozygous for FcγRIIIa-131H than for FcγRIIIa-131R. The FcγRIIb receptor is expressed on B-lymphocytes and monocytes and ligation of this receptor results in growth and differentiation inhibition [69,70].

Initially the FcγRIIIa receptor was reported to bind and be activated by IgG1 and IgG3 only; however, recognition of a polymorphism in the receptor and the differential influence of IgG glycoforms have radically changed our understanding, with important clinical consequences. The avidity of binding of IgG differs between the FcγRIIIa-158V and FcγRIIIa-158F polymorphic variants [74]. It was demonstrated, *in vitro*, that IgG1 antibody is more efficient at mediating ADCC through homozygous FcγRIIIa-158V bearing cells than homozygous FcγRIIIa-158F or heterozygous FcγRIIIa-158V/FcγRIIIa-158F cells [75]. It appears that similar differences in ADCC efficacy might pertain *in vivo*, since amongst patients diagnosed with systemic lupus erythematosus and treated with Rituxan those homozygous for FcγRIIIa-158V experienced the more favourable responses [76]. Similarly, FcγRIIIa polymorphisms were shown to influence the response of Crohn's disease patients to infliximab [77] and red blood cell clearance by anti-D antibody [78].

All FcγR are trans-membrane molecules, except FcγRIIb that is glycosylphosphatidylinositol (GPI)-anchored within the membrane of neutrophils. FcγRI and FcγRIIIa are members of the multi-chain immune recognition receptor (MIRR) family and are present in the membrane as hetero-oligomeric complexes: an IgG/antigen complex binding α chain and a signalling γ chain; the FcγRIIIa α chain of NK cells is also associated with a signalling ζ chain. FcγRIIIa and FcγRIIb molecules are comprised of an α chain only [69,70]. The FcγR alpha chains show a high degree of sequence homology in their extra cellular domains (70–98%) but differ significantly in their cytoplasmic domains. The cytoplasmic domains of γ chains and the FcγRIIIa α chain express the immunoreceptor tyrosine-based activation motif (ITAM) that is involved in the early stages of intracellular signal generation. By contrast the FcγRIIb receptor α chain expresses an immunoreceptor tyrosine-based inhibition motif (ITIM) [3,4,69,70]. Cellular activation may be dependent on the balance between the relative levels of expression of these two isoforms and hence the balance of signals generated through the ITAM and ITIM motifs [69,70].

FcR binding sites on IgG

The crystal structure of IgG–Fc in complex with soluble recombinant FcγRIIb and FcγRIIIa reveal direct involvement of the lower hinge and hinge proximal CH₂ domain residues [21–24]. One structure suggested a possible contribution of the primary N-acetylglucosamine residue to binding [21] whilst another primary publication held that there is no direct contact [22]; in a subsequent review the authors of the latter publication stated that the oligosaccharides contributes ~100 Å² to the contact interface [79]; this conclusion resulted from refinement of the crystal data (P. Sun – personal communication). These investigators also

demonstrated that whilst the binding of deglycosylated IgG–Fc to the *Escherichia coli* derived aglycosylated FcγRIIIb was undetectable the binding of deglycosylated whole IgG was only decreased, 10–15-fold [80]. This serves to remind us to exercise caution when tempted to extrapolate from *in vitro* experimental data to *in vivo* biological outcomes. Analysis of the complex formed between IgG–Fc and recombinant glycosylated FcγRIIIa has confirmed the general features of the interaction but has also revealed a critical role for glycosylation of FcγRIIIa [23,24] – see below. Thus the IgG–Fc/FcγRIIIa interaction is significantly influenced by the glycoform of each component. The involvement of both heavy chains in the formation of an asymmetric binding site provides a structural explanation for an essential requirement – that the IgG should be univalent for the FcγR; if monomeric IgG were divalent it could cross link cellular receptors and hence constantly activate inflammatory reactions.

FcRn: catabolism and transcytosis

Transcytosis

The FcRn receptor was first identified from studies of the transport of IgG across the gut of new-born rats and designated the neonatal Fc receptor, hence FcRn; subsequently the human homologue was identified in human placenta and was presumed to effect transport IgG from mother to foetus [80–82]. The interaction site on IgG–Fc is at the CH₂/CH₃ interface and the CH₃ sequence -H-N-H-Y-H- (Eu: 433–436) is of functional significance since titration of these histidine residues accounts for the observed binding of IgG to FcγRn at pH 6.0–6.5 and its release at pH 7.0–7.5 [79–81]. The interaction of IgG–Fc with FcRn appears not to be influenced by the natural IgG–Fc glycoform profile, or indeed the presence or absence of oligosaccharides. On the contrary the *N*-glycans in FcRn contribute to the steady-state membrane distribution and direction of IgG transport [83].

Each of the four human IgG subclasses are transferred across the placenta; however, with differing facility. Cord blood levels of IgG1 may be higher than in matched maternal blood whilst IgG3 and IgG4 levels are equivalent. The level of IgG2 is ~80% of the concentration in maternal blood [84]. It is of interest to note that IgG3 is transferred with equal efficacy to IgG1 although it has a shorter half-life; suggesting that its interaction with FcRn in the environment of the placenta may be different from that in endosomes in the catabolic pathway. During pregnancy the level of galactosylation of maternal IgG increases and there is preferential transport of galactosylated IgG across the placenta [45–47]. This provides circumstantial evidence to suggest that the affinity of IgG for FcγRn may differ between glycoforms, under conditions operative at the interface between the mother and the placenta.

Potential protein therapeutics that have short half-lives, e.g. cytokines, are being generated as fusion proteins with IgG–Fc to extend the half-lives. A further development opens a new route for administration since it has been shown that FcRn is expressed in the central and upper airways of the lung and that drug-IgG–Fc fusion proteins delivered to these sites can be transported, by transcytosis, to the systemic circulation. This is an exciting development with considerable promise and significance [85,86].

Catabolism

The catabolic pathway of human IgG antibodies is also mediated by FcRn that is expressed by many tissues [4,80–86]. FcRn expressing cells take up IgG within pinocytotic vesicles with the formation of vacuoles, subsequent lowering of pH results in saturation binding to FcRn and protection of IgG from cleavage by enzymes present in the vacuole; unbound IgG being degraded. When the membrane of the vacuole is re-cycled to the cellular membrane the IgG/FcRn complex is exposed to pH 7.2 and released

into the extra vascular fluid. The catabolic half-life of human IgG1, 2 and 4 is ~21 days whilst for IgG3 it is ~7 days; IgG3 data has been obtained for IgG3 molecules of G3 m(b) and G3 m(g) allotype in which the histidine 435 residue, present in IgG1, IgG2 and IgG4, is replaced with arginine [7], it has been reported (United States Patent Application 20110003336) replacement of this arginine residue by histidine increases the affinity for FcRn. The IgG allotype G3 m(s,t), present within Mongoloid populations has a histidine residue at position 435 and, presumably, higher binding affinity for FcRn [7]. The catabolic half-life of IgG mediated through FcRn does not appear to be influenced by IgG–Fc glycoform [80–86]; however, it should be emphasised that only glycoforms of IgG bearing neutral oligosaccharides have been evaluated. It may be anticipated that sialylation could influence IgG–FcRn interactions since they would introduce a negative charge in the vicinity of the histidine residues involved in FcRn binding. Protein engineering has been successfully applied to increase the affinity of IgG–Fc binding to FcRn and hence to increase the catabolic half-life [87–89]. Prolongation of the half-life of an IgG therapeutic could translate into reduced frequency of dosing and attendance at clinic; thus reducing the cost of treatment (CoT). There is evidence that glycoproteins expressing terminal *N*-acetylglucosamine residues may be cleared through the mannose receptor and accounts for the enhanced clearance of the IgG–Fc/TNF receptor fusion protein therapeutic (Lenercept) having exposed terminal *N*-acetylglucosamine residues [90,91].

Complement activation

Classical pathway: C1q/C1 binding and activation

The classical pathway is a cascade of enzymatic reactions with amplification at each step, from C1 to C9. Activation of C1q/C1 initiates a cascade of enzymatic reactions with cleavage of downstream complement components to generate fragments that bind to the immune complex whilst liberating other fragments that recruit leucocytes to augment the inflammatory response. In addition to expressing FcγR leucocytes may also express receptors for complement fragments bound to the complex and further opsonising it [3–5,92–96]. The complement cascade also results in the formation of a multimeric “membrane attack complex” that inserts into cellular and bacteria membranes to generate a “pore” that allows the ingress of water and consequent lysis [92–96].

Glycosylation of IgG–Fc is essential for C1 binding and activation and activity can be modulated by glyco-engineering [5,94–97]. Immune complexes incorporating IgG1 and/or IgG3 antibody are highly active whilst for IgG2 only complexes formed in antigen excess may be active; there is a consensus that IgG4 does not activate the classical pathway [98,99]. A hybrid IgG1/IgG3 molecule has been shown to exhibit enhanced activity, relative to either IgG1 or IgG3 alone [92]. This antibody format is available commercially as Complegent (<http://www.biowa.com/technologies/complegent>) Protein engineering studies suggest that the human IgG1 interaction site for C1q/C1 is localised to the hinge proximal region of the CH₂ domain [97,100]. This proposal is supported by the demonstration that replacement of the Pro 331 residue of IgG4 by serine converts it to a molecule that can activate C1; proline 331 is localised at the junction of the b6 bend and the fy3 β-strand and is topographically proximal to histidine 268 [97]. It is likely that replacement of proline by serine has a significant effect on local secondary/tertiary structure in the hinge proximal region. Extensive studies of mutant chimeric human IgG3 proteins have established that the efficiency of C1 activation is not directly determined by the length of the hinge region but that at least one inter-heavy chain disulphide bridge is required [93].

Role of IgG glycoforms in recognition by cellular FcγRs

Since glycosylation of IgG–Fc is essential to recognition and activation of effector ligands quantitative and/or qualitative functional differences between glycoforms might be anticipated. Studies employing recombinant monoclonal antibodies have established that individual glycoforms impact functional activity [31,101–103]. This leads one to speculate whether the immune system responds not only by production of an optimal isotype but also an optimal glycoform. Analytical protocols employing mass spectrometry are now providing the tools to determine the glycoform profile of specific antibody populations and individual glycoform profiles have been reported for anti-platelet auto-antibodies and anti-citrullinated peptide antibodies [43]; it remains to be seen whether these differences are related to disease activity.

The influence of fucose and bisecting N-acetylglucosamine on IgG–Fc activities

Increased ADCC was reported for antibodies produced in Lec 13 CHO cells that lack the presence of fucose [104] and CHO cells transfected with the human β 1,4-N-acetylglucosaminyltransferase III (GnTIII) gene with the addition of bisecting N-acetylglucosamine residues [105,106]. It was later demonstrated that the addition of bisecting N-acetylglucosamine inhibits the endogenous α (1,6)-fucosyltransferase and the addition of fucose [39]. This glycoform, bearing bisecting GlcNAc with the absence of fucose, is a minor component (<3%) of oligosaccharides released from normal polyclonal human IgG–Fc; however, it may be a predominant glycoform produced by an individual plasma cell clone.

Studies of ADCC mediated through FcγRIIIa have revealed that glycosylation at Asn 162 of this receptor has a critical influence on IgG–Fc binding [107,108]. The influence of sFcγRIIIa glycosylation has been further elucidated from the crystal structure of recombinant sFcγRIIIa, bearing complex oligosaccharides at asparagine residues 45 and 162, and non-fucosylated IgG–Fc [23,24]. The overall structure is similar to previously reported crystal structures showing that the lower hinge region and both CH₂ domains are involved in binding to the D2 domain of sFcγRIIIa and the IgG–Fc undergoes a conformational change to a slightly more open structure. The contact surface area is 1218 Å², which is larger than reported for fucosylated IgG–Fc bound to aglycosylated sFcγRIIIb. Interestingly, the N-glycan at 162 interacts with the IgG–Fc N-glycan through hydrogen bonds and van der Waals contacts. This carbohydrate–carbohydrate interaction occupies ~12% (145 Å²) of the total interface area.

The foregoing discussion was confined to the functionality of peripheral blood mononuclear leucocytes; however, the impact of fucosylation is reported to differ for polymorphonuclear cells, e.g. neutrophils [109]. A study employing two batches of a monoclonal one with a high fucose content and the other lower fucose content reported that higher fucose content resulted in more active neutrophil mediated ADCC. A contrary result is reported for neutrophil mediated phagocytosis of killed CD20⁺ lymphoma B cells with exposure to non-fucosylated rituximab resulting in enhanced phagocytosis and apoptosis, in comparison with the fucosylated form [101,110]. It should be noted that *in vitro* binding studies may be compromised since the glycoforms produced by CHO and HEK293 cells are radically different [108] <http://www.biowa.com/technologies/potelligent>.

The influence of galactosylation on IgG–Fc activities

Galactosylation levels are a major source of IgG–Fc glycoform heterogeneity. Lower levels of IgG–Fc galactosylation are observed for older adults and there is also a small but significant gender difference [43–45]. An increase in IgG–Fc galactosylation occurs over the course of normal pregnancy with levels returning to the adult

norm following parturition [44]. Hypogalactosylation of IgG–Fc is reported for a number of inflammatory states associated with autoimmune diseases [46,51–57]. The extent of IgG–Fc galactosylation observed for monoclonal human myeloma IgG proteins is highly variable, indicating that the level of IgG–Fc galactosylation is a clonal property [31,111,112]. The antibody products of CHO, Sp2/0 and NS0 cell lines used in commercial production of recombinant antibody are generally highly fucosylated but hypogalactosylated, relative to normal polyclonal human IgG [111,112]; it is necessary therefore, to consider the possible impact of differential IgG–Fc galactosylation on functional activity.

Several studies have probed the influence of the presence or absence of galactose residues on IgG–Fc structure and function. An NMR study of galactosylated (G2F) and agalactosylated (G0F) glycoforms of IgG–Fc reported the mobility of the glycan to be comparable to that of the backbone polypeptide chain, with the exception of the galactose residue on the α (1,3) arm, which was highly mobile; it was concluded that agalactosylation does not induce any significant change in glycan mobility or protein conformation [67]. This report is consistent with FcγR binding and stability studies showing minimal differences between (G0F)₂ and (G2F)₂ glycoforms [60–63] and crystal structures of a series of truncated glycoforms of IgG–Fc [61]. The NMR study also probed changes in local environments on the binding of soluble recombinant FcγRIII to (G2F)₂ and (G0F)₂ glycoforms of IgG1–Fc and reported chemical shift differences >0.2 ppm for Lys248 and Val308 residues [67]; this is a very localised change distant from the interaction site for the FcγRIIIa moiety. The finding of a changed environment for these residues is interesting since, from the crystal structure, they were not predicted to make contacts with the α (1–6) arm galactose residue; small perturbations for the oligosaccharide contact residues Lys246, Asp249, Thr256 were also observed. A comparison of hydrogen/deuterium exchange for (G2F)₂ and (G0F)₂ IgG–Fc glycoforms revealed altered conformation within the peptide sequence 242–254 [113].

Recombinant IgG antibody therapeutics secreted by mammalian cell lines are hypogalactosylated, relative to normal polyclonal IgG–Fc. The possible consequences for *in vivo* activity are extrapolated from *in vitro* assays and animal experiments. Removal of terminal galactose residues from Campath-1H was shown to reduce classical complement activation but to be without effect on FcγR mediated functions [114]. Similarly, the ability of rituximab to kill tumour cells by the classical complement route was shown to be maximal for the (G2F)₂ glycoform, in comparison to the (G0F)₂ glycoform [115]. The product that gained licensing approval was comprised of ~25% of the G1F oligosaccharide, therefore, regulatory authorities required that galactosylation of the manufactured product be controlled to within a few % of this value. In the absence of galactose the terminal sugar residue is N-acetylglucosamine that may be accessible to both the mannose receptor and/or mannan binding lectin.

Sialylation of IgG–Fc oligosaccharides

A minority of oligosaccharides released from polyclonal IgG–Fc are sialylated whilst ~70% bear one or two galactose residues [28–32]. The paucity of sialylation is presumed to reflect the intimate integration of the oligosaccharides within the IgG–Fc structure restricting access of the α (2,6) sialyltransferase enzyme to terminal galactose residues, rather than being due to any deficit in the sialylation machinery. This conclusion is supported by the finding that when both IgG–Fc and IgG–Fab are glycosylated the latter bears highly galactosylated and sialylated structures, demonstrating that the glycosylation machinery is fully functional [36,50,116]. The presence or absence of terminal galactose and/or sialic acid residues does not influence catabolism since IgG is not catabolised in the liver, via the asialoglycoprotein receptor (ASGPR), but in

multiple cell types that express the FcRn receptor. The balance between structure and accessibility is well illustrated for a panel of IgG–Fc in which individual amino acid residues making contacts with the oligosaccharide were replaced by alanine. In each case hypergalactosylated and highly sialylated glycoforms resulted, suggesting some relaxation of structure allowing access to glycosyl transferases [117].

Recent studies suggest that sialylated human IgG–Fc may inhibit activation of inflammatory cascades. Following binding to the lectin receptor SIGN-R1, in the mouse, or DC-SIGN, in humans, expression of the inhibitory receptor FcγRIIb on inflammatory cells is upregulated, attenuating autoantibody-initiated inflammation [118]. However, it has been asserted that caution should be exercised, when extrapolating from mouse models to humans, since the tissue distribution of SIGN-1 and DC-SIGN differ and that anti-inflammatory activity could be demonstrated for intact IgG and F(ab')₂ fragments [119,120].

Human antibody isotypes other than IgG

Since this review is focused on antibody therapeutics detailed structural and functional discussion is restricted to the IgG class; however, our understanding of the structure and function of the IgA [5,121,122], IgM [5,123,124] and IgE [5,125] isotypes are progressing and therapeutic applications are on the horizon or in the pipeline, if not licensed [126–128].

Concluding remarks

It is salutary to contemplate the finesse of structural changes induced by conservative amino acid replacements (glycine/alanine, 235; phenylalanine/alanine, 243) and/or the presence or absence of a fucose sugar residue can have on the functional activity of the human IgG–Fc. It is also salutary to contemplate the response to evolutionary pressures that has resulted in an IgG–Fc that can engage and activate multiple ligands – to the benefit or detriment of the individual. The search for an understanding of the interactions between structure and function has called for innovative and original thinking, at the bench and *in silico*. We now have the tools to evaluate many activities *in vitro* but the challenge remains to establish models and/or modalities for determining mechanisms activated *in vivo*. This review has focused on the impact of glycosylation on antibody function; however, our understanding is far from complete. It is interesting to note that disparate ligands may bind to the Fc through common amino acid residue contacts; within the hinge proximal region for FcγR and C1q and the CH₂/CH₃ interface for FcRn, SpA, SpG, RFs and IgG–Fc receptors encoded within the genomes of some viruses; the addition of sialic acid might further influence Fc/ligand interactions at this interface. A rationalisation for the topography of ligand binding sites may be the functional necessity for circulating IgG to be monovalent for FcγRs and C1q, to prevent continuous cellular activation whilst providing opportunity for divalency at the CH₂/CH₃ interface. The influence of the IgG–Fc glycoform on functional activity may be exploited to generate homogeneous glycoforms selected for a predetermined functional profile considered optimal for a given disease indication. It is important to note that each glycoform is represented within normal polyclonal IgG–Fc; therefore, they do not have potential to be immunogenic. Many innovative studies have explored engineering of the protein moiety to selectively enhance biologic activities [120]; however, these are mutant forms of IgG, i.e. non-self, that may enhance immunogenicity. This may not be an issue when treating patients for cancer, since they may be immuno-suppressed; however, it is a concern for long treatment for chronic diseases.

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