

Glycosylation as a strategy to improve antibody-based therapeutics

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Abstract | To date, more than 20 recombinant immunoglobulin G (IgG) antibody therapeutics are licensed for the treatment of various diseases. The mechanism of action of recombinant monoclonal antibodies (rMAbs) has been extensively investigated and several distinct pathways have been defined; selective activation of specific pathways may optimize clinical outcomes for different diseases, such as cancer and chronic inflammation. Human IgG is a glycoprotein with oligosaccharides attached at a single site. These are essential to the mode of action of rMAbs, and the antibody efficacy can vary depending on the particular oligosaccharide that is attached. Methods are now becoming available that allow the production of rMAbs bearing pre-selected oligosaccharides — glycoforms — to provide maximum efficacy for a given disease indication. This Review summarizes current knowledge of these methods and avenues for their exploitation in the clinic.

Diantennary oligosaccharide

An oligosaccharide that has two mannose 'arms'; as opposed to triantennary, tetra-antennary and so on.

Recombinant monoclonal antibody (rMAb) therapeutics are exemplars of translational medicine. The rMAbs currently licensed represent a significant success in terms of the clinical benefit delivered and the revenue generated in the biopharmaceutical industry. Additionally, it is estimated that ~30% of new drugs that are likely to be licensed during the next decade will be based on antibody products^{1–3}. High-volume production of these large biological molecules, combined with the maintenance of their structural and functional fidelity, results in high costs, which can limit their availability to patients owing to the strain that it puts on national and private health budgets. Although the hallmark of an antibody is its specificity for the target antigen, the antigen–antibody complex formed must also be able to be removed and destroyed. The humoral antibody response can be mediated by antibodies from nine antibody classes and subclasses, each of which has unique mechanisms of action (known as effector functions). To date, all licensed rMAbs have been of the immunoglobulin G (IgG) class; however, each of the four human IgG subclasses also exhibits unique effector functions. Therefore, when developing an rMAb therapeutic, it is important to select the IgG subclass that is anticipated to have the most potent activity for a given disease indication. The presence of oligosaccharides attached at a single site on each of the IgG heavy chains is essential for the antibody's effector functions, and efficacy can vary depending on the particular oligosaccharide that is attached. Methods that allow the production of rMAbs bearing homogeneous

oligosaccharides (glycoforms) are now becoming available. It is hoped that delivery of rMAbs that are optimized for specificity and effector functions, together with patient profiling, will have a significant impact on the cost of treatment.

Human IgG isolated from normal serum is comprised of multiple glycoforms owing to the addition of diverse complex diantennary oligosaccharides in the IgG Fc (crystallizable fragment) region. The presence or absence of IgG Fc oligosaccharides does not affect antigen binding but has a profound effect on the biological mechanisms that are activated by the immune complexes formed. Basic research has allowed the generation of a series of homogeneous antibody glycoforms and demonstrated significant differences in their effector-function profiles and/or efficacy. Production vehicles, including mammalian cells, yeasts and plants, have been engineered to maximize the production of selected IgG glycoforms. The biopharmaceutical industry now faces the challenge of translating these laboratory protocols into the manufacture of selected homogeneous antibody glycoforms. In contrast to modulation of effector functions by protein engineering, a major benefit of this optimization approach is that production of an rMAb of a single naturally occurring glycoform does not contribute to potential immunogenicity.

The development of antibody therapeutics

The original technology for generating MAbs of defined specificity was developed in the mouse and immediately applied to the generation of MAbs with specificity

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for human proteins⁴. Although early clinical success was reported for the treatment of an acute episode of kidney graft rejection with mouse IgG2a antibodies reactive against the cell-surface protein CD3, exposing humans to mouse antibodies provokes a human anti-mouse antibody (HAMA) response. The application of genetic engineering to the development of chimeric mouse–human rMAbs and, later, ‘humanized’ and/or ‘fully human’ rMAbs promised reduced immunogenicity; however, a proportion of patients develop human anti-chimeric antibodies or human anti-human antibodies, respectively, to these antibodies⁵. Such antibody responses can prejudice treatment if they are neutralizing, if they result in clearance of the therapeutic as immune complexes or if they sensitize the patient for severe reactions on re-exposure^{5–8}. Importantly, these technologies allow the human antibody constant region isotype — which determines the effector functions activated by immune complexes — to be selected based on its perceived suitability for a given disease indication *in vivo*^{9–11}. To date, all approved rMAbs have been of the human IgG isotype, and they have predominantly been of the IgG1 subclass.

The potential for immunogenicity imposes a demand for vehicles that can deliver products with absolute structural fidelity, including appropriate post-translational modifications. However, inherent to the uniqueness of an individual antibody product is an inevitable potential for immunogenicity. The required fidelity cannot be realized using current production vehicles — that is, Chinese hamster ovary (CHO) and mouse (NS0 and Sp2/0) cell lines. Consequently, these production cell lines are being engineered for both product quantity and quality. A particular focus is glycosylation, as it has been shown that biological activities vary between different natural glycoforms and that non-natural glycoforms can be immunogenic.

The basic structure of human IgG antibodies

In humans the IgG antibody class predominates in the blood and equilibrates with the extravascular space; these characteristics are key to the design of antibody therapeutics with appropriate pharmacodynamics. In a natural immune response the pathogen–IgG immune complexes that are formed activate a wide range of effector functions, resulting in the killing, removal and destruction of the pathogen. Four subclasses of human IgG are defined and enumerated according to their relative concentrations in normal serum: IgG1, IgG2, IgG3 and IgG4, which respectively account for approximately 60%, 25%, 10% and 5% of serum IgG. Each IgG subclass exhibits a unique profile of effector functions when evaluated by *in vitro* assays^{9–11}.

In its simplest form an individual IgG molecule is composed of two identical light chains and two identical heavy chains, which are in turn comprised of repeating structural motifs (homology regions) of ~110 amino-acid residues. The tertiary structure of each homology region defines the immunoglobulin fold or domain^{9–11}. Domains of the light and heavy chains pair in covalent and non-covalent association to form three independent

protein moieties connected through a flexible linker (the hinge region) (FIG. 1). Two of these moieties, referred to as Fab (antigen-binding fragment) regions, are of identical structure and each form the same specific antigen-binding site; the third, the Fc, forms interaction sites for ligands that activate clearance and transport mechanisms. These effector ligands include three structurally homologous cellular Fc receptor types (FcγRI, FcγRII and FcγRIII), the C1q component of complement and the neonatal Fc receptor (FcRn)^{9–14}; the latter influences the catabolic half-life of the antibody and its transport into the extravascular space and across the placenta. Activation of Fc receptors and of the C1q component of complement initiates inflammatory cascades that combat and resolve episodes of infection. These activities are crucially dependent on IgG Fc N-linked glycosylation and vary between antibody glycoforms^{9–20}. By contrast, binding to FcRn, and hence the catabolic half-life, is not dependent on antibody glycoform¹⁰; data are not currently available for transport across the human placenta.

The IgG Fc region is a homodimer comprised of covalent inter-heavy chain disulphide-bonded hinge regions and non-covalently paired CH3 domains; the CH2 domains are glycosylated through covalent attachment of oligosaccharide at Asn297. X-ray crystallographic analysis reveals that the structure of the oligosaccharide is defined (as opposed to undefined owing to continuous mobility), is integral to the IgG Fc structure and forms multiple non-covalent interactions with the protein surface of the CH2 domain; thus, the conformation of the IgG Fc glycoprotein moiety results from reciprocal interactions between the protein and the oligosaccharides^{10,20,21}. There is evidence that interaction sites on IgG Fc for FcγRI, FcγRII, FcγRIII and C1q effector ligands are comprised principally of only the protein moiety; however, generation of the interaction sites for these ligands is dependent on IgG Fc protein–oligosaccharide interactions. Thus, effector mechanisms mediated through FcγRI, FcγRII, FcγRIII and C1q are severely compromised or ablated for aglycosylated or deglycosylated forms of IgG^{9–20}.

It is generally reported that IgG Fc oligosaccharides of normal human IgG are predominantly devoid of terminal sialic acid residues^{21–24}; however, recent studies have suggested that IgG Fc sialylated molecules (<10% of total IgG Fc molecules) may have beneficial properties. Small but possibly significant differences in the affinities of sialylated and non-sialylated IgG Fc for FcγR have been reported^{25,26}; for example, in a complex mouse model of inflammation, polyclonal human IgGs bearing Fc sialyl sugar residues were shown to be anti-inflammatory^{27,28}. This anti-inflammatory activity was proposed to be mediated by an FcγR-independent mechanism. It is thought that this model might reflect the situation in human patients in which high doses of polyclonal IgG alleviate the symptoms of inflammatory autoimmune conditions — that is, they exhibit anti-inflammatory activity. We await the results of further studies of this phenomenon in experimental models that are more relevant to humans.

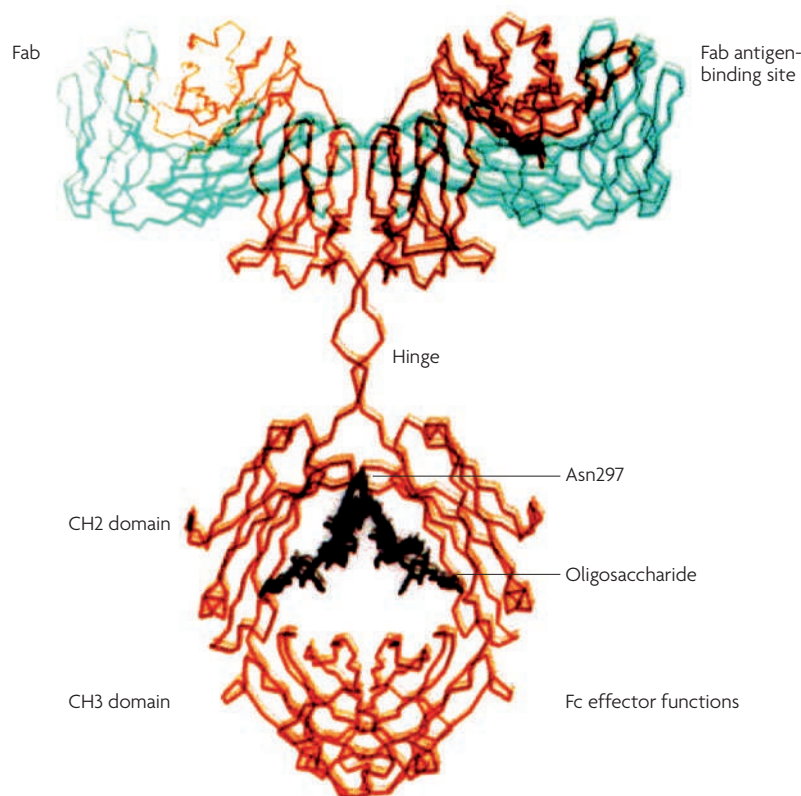


Figure 1 | The α -carbon backbone structure of the immunoglobulin G (IgG) molecule. This structural representation illustrates the sequence of antiparallel β -pleated sheet domains that constitute the immunoglobulin fold. The oligosaccharide is integral to the protein structure and has a defined conformation. CH, constant heavy; Fab, antigen-binding fragment; Fc, crystallizable fragment.

Glycoform profiles of serum-derived IgG

The oligosaccharides present in the IgG Fc of normal polyclonal IgG, attached at Asn297, are of the complex diantennary type and are comprised of a core heptasaccharide with variable addition of fucose, galactose, bisecting *N*-acetylglucosamine and sialic acid (FIG. 2A); sialylation is modest, with <10% of structures being monosialylated or disialylated^{11,21–24}. The relative yields and structures of the neutral oligosaccharides that may be released from normal IgG Fc by incubation with the glycosidase PNGase F are respectively shown in FIG. 2B,C. Each heavy chain may bear one of a total of 32 unique oligosaccharides, and random pairing of heavy-chain glycoforms could generate ~500 glycoforms. Given the paucity of sialylation, neutral oligosaccharides predominate, but they alone allow for the generation of 128 unique glycoforms; the possible number of combinations (16×16) is divided by 2 because of the symmetry of the molecule.

A shorthand system of nomenclature for the oligosaccharides uses G0, G1 and G2 for oligosaccharides bearing zero, one or two galactose residues, respectively. When fucose is present G0F, G1F or G2F is used; when bisecting *N*-acetylglucosamine is present a B is added — for example, G0B or G0BF. The glycoform of the whole molecule is the oligosaccharides present on each heavy chain and may be represented as (G0–G0), (G0–G0F), (G0F–G0F), (G0–G1), (G0F–G1) and so on.

The glycoform profile of normal polyclonal IgG is subject to variation with age, over the term of pregnancy and in a number of inflammatory conditions^{11,22–24}. The major change reported is the extent of galactosylation, usually reported as the percentage of [G0 + G0F] oligosaccharides released. Thus, the levels of [G0 + G0F] reported for young children and elderly adults exceed those reported for adults between 20 and 50 years of age. Increased levels of [G0 + G0F] are reported in a number of inflammatory diseases that have an autoimmune component — for example, rheumatoid arthritis, Crohn's disease and vasculitis. Although they do not equate to the aetiology of the disease, these changes may represent 'acute-phase reactants' that reflect inflammation and may contribute to an exacerbation of inflammation (see below). Analysis of monoclonal human IgG proteins isolated from the sera of patients with multiple myeloma revealed individual (and unique) glycoform profiles, including major differences in the levels of galactosylation, fucosylation and the addition of bisecting *N*-acetylglucosamine residues^{29,30}. It may be anticipated, therefore, that the IgG response to an individual antigen (pathogen) may be comprised of predominant glycoforms.

Approximately 30% of polyclonal human IgG molecules bear *N*-linked oligosaccharides in the IgG Fab region, in addition to those attached at the conserved glycosylation site at Asn297 in the IgG Fc^{11,31–33}. When present they are attached to the variable regions of the kappa (V κ) or lambda (V λ) light chains, to the heavy (VH) chains or to both. In the immunoglobulin sequence database ~20% of the IgG variable regions have *N*-linked glycosylation consensus sequences (Asn-X-Thr/Ser, where X can be any amino acid except proline). Interestingly, these consensus sequences are mostly not encoded in the germ line; rather, they mostly result from somatic mutation — suggesting positive selection for improved antigen binding. Analysis of polyclonal human IgG Fab reveals the presence of diantennary oligosaccharides that are extensively galactosylated and substantially sialylated, in contrast to the oligosaccharides released from IgG Fc^{11,31–33}. The functional significance of IgG Fab glycosylation of polyclonal IgG has not been fully evaluated, but data emerging for monoclonal antibodies suggest that V κ , V λ or VH glycosylation can have a neutral, positive or negative influence on antigen binding¹¹.

It is generally observed that the oligosaccharide present in glycoproteins contributes to their solubility and stability. The influence of glycosylation on the thermal stability of human IgG1 Fc was demonstrated for a series of truncated glycoforms using differential scanning micro-calorimetry^{20,34}. It is possible, therefore, that IgG Fab glycosylation may be beneficial when formulating IgG therapeutics at concentrations of 100–150 mg per ml. Such high-concentration formulations allow the development of self-administration protocols, and the high serum concentrations that are achieved can reduce dosing intervals, resulting in reduced cost of treatment. Controlling glycoform fidelity at two sites (Fc and Fab) offers a further challenge to the biopharmaceutical industry.

Differential scanning micro-calorimetry

A thermoanalytic technique in which the difference in the amount of heat required to increase the temperature of a sample and a reference is measured as a function of temperature.

Glycoforms of recombinant IgG antibodies

As Fc receptor and complement binding, and activation, are essentially ablated for aglycosylated IgG Fc, recombinant antibody therapeutics should have fully occupied glycosylation sites. The most widely used production cell lines are CHO, NS0 and Sp2/0, in various engineered forms^{35,36}. These cell lines produce IgG Fc glycoforms bearing the top eight oligosaccharides shown in FIG. 2C; however, the proportion of galactosylated and non-fucosylated IgG Fc is low relative to normal IgG Fc (FIG. 2B). Although these cell lines do not add oligosaccharides containing bisecting *N*-acetylglucosamine residues, they do add sugars that are not present in normal serum-derived IgG, and these can be immunogenic. A particular concern is the addition, by NS0 and Sp2/0 cells, of galactose in $\alpha(1-3)$ linkage to galactose linked $\beta(1-4)$ to the *N*-acetylglucosamine residues (gal $\alpha(1-3)$ gal). Humans and higher primates do not have a functional gene encoding the transferase that adds galactose in $\alpha(1-3)$ linkage; however, owing to continual environmental exposure, humans have an IgG antibody that is specific for this antigen³⁷. Similarly, CHO, NS0 and Sp2/0 cells can add an $\alpha(2-3)$ -linked *N*-glycylneuraminic acid that is not present in humans and that might also be immunogenic³⁸, and CHO cells can add *N*-acetylneuraminic acid in $\alpha(2-3)$ linkage rather than the human $\alpha(2-6)$ linkage.

The licensed antibody therapeutic cetuximab (Erbix) bears an *N*-linked oligosaccharide at Asn88 of the VH region; interestingly there is also a glycosylation motif at Asn41 of the VL region, but it is not occupied³⁹. Analysis of the IgG Fc and IgG Fab oligosaccharides of cetuximab, which is produced by Sp2/0 cells, revealed highly significant differences in composition. Whereas the IgG Fc oligosaccharides are typical — that is, they are predominantly fucosylated non-galactosylated diantennary oligosaccharides — the IgG Fab oligosaccharides are extremely heterogeneous and include complex diantennary and hybrid oligosaccharides as well as galactose in $\alpha(1-3)$ linkage to galactose and *N*-glycylneuraminic acid residues.

A recent study reported that 25 of 76 patients treated with cetuximab had hypersensitivity reactions to the drug, and IgE antibodies against gal $\alpha(1-3)$ gal were detected in 17 pre-treatment samples from these patients⁴⁰. Interestingly, environmental factors seem to influence the development of IgE gal $\alpha(1-3)$ gal responses, as the incidence varied significantly between treatment centres and predominant infectious agents present in local environments.

A detailed analysis of the glycoforms of a humanized IgG rMAb, which is also expressed in Sp2/0 cells, bearing oligosaccharides at Asn56 of the VH and Asn297 revealed the expected IgG Fc profile of predominantly G0F oligosaccharides. However, 11 oligosaccharides were released from the IgG Fab, including diantennary and triantennary oligosaccharides bearing gal $\alpha(1-3)$ gal, *N*-glycylneuraminic acid and *N*-acetylgalactosamine residues⁴¹. The consistent observation of higher levels of galactosylation and sialylation for IgG Fab *N*-linked oligosaccharides than for IgG Fc *N*-linked oligosaccharides is thought to reflect increased exposure and/or accessibility

of the glycosylation sites on Fab fragments. Given the findings of these studies and the potential for NS0 and Sp2/0 cells to add gal $\alpha(1-3)$ gal residues, it seems likely that these cells will cease to be developed for therapeutic antibody/glycoprotein production, unless they are engineered to inactivate the gal $\alpha(1-3)$ and *N*-glycylneuraminic acid transferases.

The double challenge of producing rMAbs with appropriately glycosylated IgG Fc and IgG Fab sites has led to some companies engineering out VH or VL glycosylation motifs when present in candidate rMAbs⁴²; however, present reports suggest that CHO cells can glycosylate VH and/or VL motifs in a manner similar to that observed for normal polyclonal IgG⁴³.

IgG Fc glycoforms: structure and function

Structure. Protein engineering, using alanine scanning, has been used to map amino-acid residues deemed to be crucial for Fc γ R and C1q binding. These studies map the binding site for all four of these ligands to the hinge-proximal and lower hinge region of the CH2 domain^{14,16,44–48}. X-ray crystallographic analysis suggests that the lower hinge region of IgG Fc is mobile and without defined structure²¹, which might seem incompatible with the suggestion that this region is directly involved in generating structurally distinct interaction sites for the Fc γ R and C1q ligands. However, the suggestion may be rationalized by the proposal that this region is comprised of multiple conformers in equilibrium, with individual conformers being compatible with specific ligand recognition^{11,14}. X-ray crystallographic analysis of IgG Fc in complex with a soluble recombinant form of the Fc γ RIIIb receptor provides proof of the direct involvement of the CH2 lower hinge regions and hinge-proximal regions in ligand binding^{45,46}. Residues in the hinge region that do not form part of a discrete structure in the 'free' IgG Fc are shown to interface with the receptor and assume discrete conformations with ligand bound. Both heavy chains are involved in forming an asymmetric binding site. Consequently, monomeric IgG is univalent for the Fc γ R; if monomeric IgG were divalent for Fc γ R it could cross-link cellular receptors and hence constantly activate inflammatory reactions.

Study of a series of truncated IgG Fc glycoforms revealed that non-covalent interactions between sugar residues of the $\alpha(1-6)$ arm of the IgG Fc oligosaccharide and the inner protein surface of the CH2 domain determine the overall conformation of the protein–oligosaccharide complex. If the oligosaccharide moiety is progressively truncated a closing of the 'horseshoe-like' structure is observed^{20,34,48}. Reduced structural integrity and functionality was demonstrated when the oligosaccharide was truncated to only the initial trisaccharide^{20,34}, and functionality was completely ablated when the oligosaccharide was further truncated to only the initial covalently bound *N*-acetylglucosamine residue⁴⁸.

Function: ADCC. The effectiveness of rMAbs in oncology depends on sensitizing target cells for subsequent killing by the mechanisms of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity

Alanine scanning

A genetic manipulation that sequentially replaces wild-type amino acids with alanine to determine the impact on the protein's structure and function.

Conformer

A discrete, defined conformation in three-dimensional space.

Antibody-dependent cellular cytotoxicity (ADCC)

Cell death that results when the Fc fragment of an antibody bound to a target cell interacts with Fc receptors on monocytes, macrophages or natural killer cells that are consequently activated to kill the target.

(CDC) and/or the induction of apoptosis. It is unequivocally established that ADCC and CDC are dependent on appropriate glycosylation of the rMAb^{11,16–20}, whereas induction of apoptosis may require only cross-linking of cell-surface antigens. Glycosylation has been a focus

of interest for the biopharmaceutical industry for the past several years, and cell lines have been engineered in efforts to optimize antibody products for ADCC and CDC by the differential addition of fucose, galactose, bisecting *N*-acetylglucosamine and sialic acid.

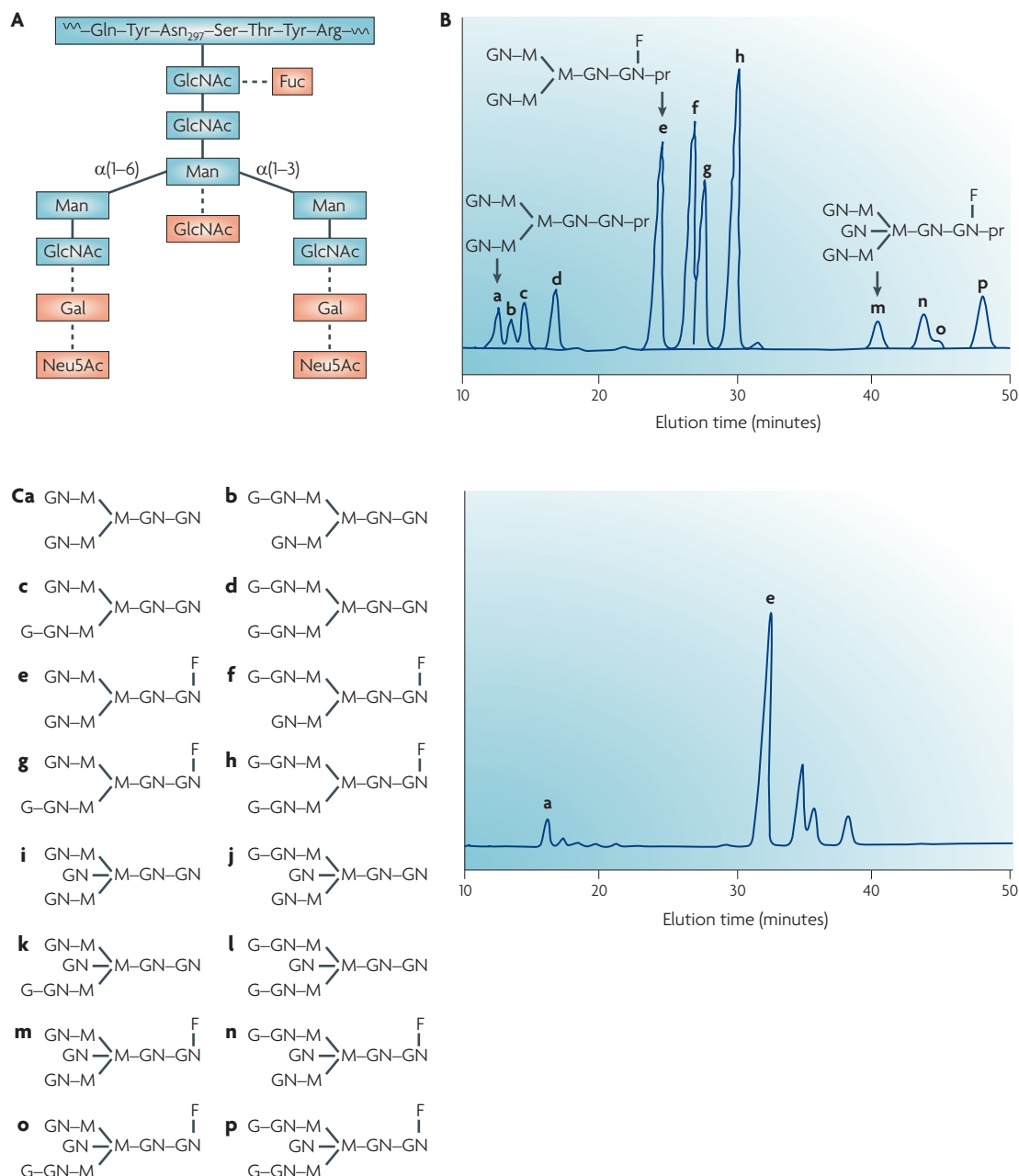


Figure 2 | IgG Fc diantennary-complex oligosaccharide composition. **A** | The oligosaccharides present in the immunoglobulin G (IgG) Fc (crystallizable fragment) region of normal polyclonal IgG attach at Asn297. They form a diantennary complex comprised of a core heptasaccharide (sugars in blue boxes) and outer arms (sugars in red boxes) constructed by variable addition of fucose (Fuc), galactose (Gal), bisecting *N*-acetylglucosamine (GlcNAc), sialic acid and *N*-acetylneuraminic acid (Neu5Ac). **B** | A high-performance liquid chromatography profile of the complex diantennary oligosaccharide structures released from normal human IgG Fc²⁴ (top) and from recombinant IgG produced in Chinese hamster ovary cells (bottom). **C** | The potential library of all neutral complex diantennary oligosaccharides that can be released from normal human IgG Fc. The oligosaccharides are labelled a–p, corresponding with the peaks in part B. Oligosaccharides i–l are collectively <3% of the total amount and therefore cannot be identified in the profile for human serum IgG. F, fucose; G, galactose; GN, *N*-acetylglucosamine; M, mannose; Man, mannose; Pr, protein.

Complement-dependent cytotoxicity (CDC). Cell death that results when the IgG Fc regions of an antibody bound to a target cell activate the C1 component of complement, initiating a cascade of reactions that lead to the formation of a complex that disrupts the cell membrane.

The first rMAb to be licensed for the treatment of cancer was rituximab (Rituxan), a chimeric mouse–human IgG1 antibody against CD20 produced in CHO cells. It is approved for the treatment of non-Hodgkin's lymphoma, in which the target is neoplastic CD20-expressing B lymphocytes^{49,50}. Rituximab was shown to sensitize B cells for killing by ADCC, CDC and the induction of apoptosis *in vitro*. Parameters that influence killing by ADCC and CDC include epitope specificity and the level of expression of the CD20 antigen and the decay-acceleration factors CD55 and CD59. Rituximab proved ineffective in the treatment of other B-cell malignancies in which the level of expression of the CD20 antigen is low — for example, chronic lymphocytic leukaemia. This rMAb has been subject to studies designed to develop glycoforms with enhanced effector functions.

Extensive investigation of the mechanism(s) by which rituximab can kill CD20-positive lymphocytes *in vitro* and *in vivo* has established that recruitment and activation of FcγRIIIa-expressing cells — for example, natural killer (NK) cells — is a dominant pathway^{49,50}. Thus, target CD20-positive tumour cells that are sensitized by bound rituximab engage FcγRIIIa receptors expressed on NK cells, with subsequent activation and release of granzymes. The efficacy of NK cell recruitment and activation seems to be directly determined by the affinity of the IgG Fc for FcγRIIIa which, in turn, varies with the presence or absence of fucose on the oligosaccharide of the IgG Fc. Present rMAb production vehicles (CHO, NS0 and Sp2/0 cells) generate >90% fucosylated forms of IgG Fc. The CHO cell line has been engineered to produce non-fucosylated IgG Fc, and new glycoforms of several licensed rMAbs are nearing the clinic.

An early report compared the ability of alemtuzumab (Campath-1H), a chimeric IgG1 rMAb against CD52, produced from CHO, NS0 and YB2/0 cells to mediate ADCC, and concluded that alemtuzumab produced in the rat YB2/0 cell line had the highest efficacy. The only structural difference between the products was the presence of complex oligosaccharides bearing bisecting *N*-acetylglucosamine residues⁵¹ in the YB2/0 antibody. Subsequently, two groups engineered the CHO cell line to express the GnTIII enzyme that adds bisecting *N*-acetylglucosamine residues, and demonstrated that ADCC improved by two or more orders of magnitude for a rituximab-like product^{52,53}. Enhanced ADCC was also reported for rituximab produced from CHO cells in which the α(1–6) fucosyl transferase enzyme had been knocked out^{18,36,54}. It is now appreciated that the addition of the *N*-acetylglucosamine sugar residue is a relatively early event in glycoprotein processing in the Golgi apparatus, and the presence of this residue inhibits the later addition of fucose to the primary *N*-acetylglucosamine residue¹⁹. The absence of fucose from the primary *N*-acetylglucosamine results in the IgG1 antibody having increased binding affinity for the FcγRIIIa receptor, with consequent increased efficacy of NK cell-mediated ADCC⁵⁵. Thus, it seems that it is the absence of fucose, not the presence of bisecting *N*-acetylglucosamine *per se*, that results in enhanced ADCC¹⁷. The enhanced ADCC observed for afucosylated IgG Fc results, in part,

from the increased affinity for FcγRIIIa — this allows the afucosylated IgG Fc to overcome the competition from high concentrations of fucosylated IgG in normal serum^{17,54–57}. The findings reported for rituximab have recently been extended with the demonstration of improved ADCC for afucosylated trastuzumab (Herceptin) in a whole-blood assay⁵⁸. The presence or absence of fucose has been shown to have minimal impact on effector functions mediated through other FcγRs or complement.

Non-fucosylated forms of therapeutic antibodies such as rituximab and trastuzumab may be anticipated to have clinical advantage over the fucosylated forms owing to the increased sensitization of target cells to NK cell-mediated ADCC; however, it is important to approach this goal with caution. These reagents should be treated as new therapeutics, as their increased potency could result in enhanced cell killing at first dose with consequent increased release of cytokines and possible adverse reactions. In addition, the presumed lesser need for target sensitization could result in increased killing of normal cells that express the target antigen.

The industry is beginning to respond to the perceived advantages of non-fucosylated antibodies for cancer therapies, but a recent report suggests that there are contrary indications⁵⁹. Two preparations of an antibody specific for epidermal growth factor receptor (EGFR) that differed in their level of fucosylation were shown to be equivalent in a whole-blood assay. However, when ADCC mediated by isolated peripheral blood mononuclear cells (PBMCs) and polymorphonuclear neutrophils (PMNs) were compared, it was shown that low levels of fucosylation favoured PBMC-mediated ADCC whereas high levels of fucosylation favoured PMN-mediated ADCC⁵⁹. It was further claimed that the non-fucosylated antibody favoured activation of FcγRIII whereas the more highly fucosylated antibody favoured FcγRIIa-mediated ADCC. It will be of particular interest to see the outcome of subsequent studies in which homogeneous fucosylated and non-fucosylated antibodies are compared.

A rationale for the increased affinity of non-fucosylated IgG Fc for FcγRIIIa may be the reduction or absence of steric inhibition at the receptor–ligand interface. The natural membrane-bound form of FcγRIIIa is highly glycosylated, and one oligosaccharide moiety is attached to an Asn (Asn162) at the receptor–ligand interface. It seems that the presence of fucose on IgG Fc fails to accommodate this receptor-bound oligosaccharide: FcγRIIIa that is not glycosylated at Asn162 has the same binding affinity for fucosylated as non-fucosylated IgG Fc⁶⁰.

Function: CDC. Removal of terminal galactose residues from the chimeric mouse–human IgG1 antibody alemtuzumab was shown to reduce CDC but to be without effect on FcγR-mediated functions⁶¹. Similarly, the (G1F–G1F) glycoform of rituximab triggered a CDC response twice as large as that triggered by the (G0F–G0F) glycoform⁶². The product that gained licensing approval was comprised of complex diantennary oligosaccharides

Granzyme

An apoptosis-inducing serine protease released from cytoplasmic granules by cytotoxic T cells and NK cells.

with a G1F content of ~25%; regulatory authorities required, therefore, that galactosylation of the manufactured product be controlled to within a few per cent of this value.

The main glycoform produced by CHO, NS0 and Sp2/0 cells is the (G0F–G0F) glycoform — that is, oligosaccharides with terminal *N*-acetylglucosamine sugar residues. The serum protein mannose-binding lectin (MBL) can recognize and bind arrays of *N*-acetylglucosamine^{63–68}. It is possible, therefore, that immune complexes comprised of (G0F–G0F) IgG may engage and activate these lectin molecules. MBL is a structural homologue of the C1q molecule, and it forms a complex with MASP1, MASP2 and MASP3, which are structural homologues of C1s and C1r. The MBL–MASP complex circulates in the blood and when activated triggers the complement cascade by binding and cleaving C4, as for the C1 complex. A degalactosylated glycoform of IgG1 was shown to bind and activate the MBL/MASP complex and thus to initiate this cascade⁶³. Similarly, a (G0F–G0F) glycoform of IgG4 Fc was also shown to bind MBL⁶⁴. These findings suggest that, in inflammatory diseases characterized by increased levels of (G0F–G0F), IgG glycoforms may contribute to inflammation by activating the lectin pathway^{66–68}.

Immunogenicity. The mannose receptor is a C-type lectin, expressed at the surface of macrophages, endothelial cells and dendritic cells, that recognizes arrays of mannose and *N*-acetylglucosamine residues⁶⁹. Thus, immune complexes formed with (G0F–G0F) antibody glycoforms may engage this receptor. This is of particular interest, or concern, for the potentiation of immunogenicity, as dendritic cells are ‘professional’ antigen-presenting cells⁷⁰. Fully galactosylated IgG glycoforms may undergo minimal uptake by dendritic cells.

Clearance. Normal serum IgG has a long half-life: 20–25 days for the IgG1, IgG2 and IgG4 subclasses and ~7 days for IgG3. The long half-life is dependent on the FcRn region⁷¹ and, as stated above, is independent of the natural glycoform; however, this may not be the case for unnatural glycoforms of recombinant antibody molecules. Recombinant antibodies produced in CHO, NS0 or Sp2/0 cells usually have small quantities of unnatural glycoforms of IgG, and concern has been expressed for the possible impact of these glycoforms on the antibody half-life and clearance mechanisms. Particular attention has been paid to high-mannose glycoforms as it was thought that they might be targeted to mannose receptor-bearing cells. However, other studies showed that such glycoforms are cleared from the circulation at the same rate as the natural glycoforms of IgG Fc⁷².

The long half-life of IgG Fc can be exploited to extend the serum half-life of other protein molecules by generating IgG Fc fusion proteins; however, the nature of the fusion protein and particularly its glycoforms may influence its half-life. The IgG Fc fusion protein lenercept (Tenefuse) is comprised of the 55 kDa tumour necrosis factor receptor (TNFR55) fused with IgG1 Fc. The TNFR55 component has three glycosylation sites, and

glycoforms with exposed *N*-acetylglucosamine residues were shown to be cleared through the mannose receptor and to have a shorter half-life than IgG Fc^{73,74}.

Production of selected IgG glycoforms

The data for homogenous IgG glycoforms included in this Review have been produced, at the laboratory scale, by enzymatic modifications of recombinant IgGs *in vitro* or by engineered cells *ex vivo*. They suggest that selected homogeneous antibody glycoforms of IgG could be more efficacious than heterogeneous glycoforms as *in vivo* therapeutics, and biopharmaceutical companies are developing ‘in-house’ production vehicles for the manufacture of homogeneous antibody glycoforms. For the production of non-fucosylated antibodies, Roche acquired the Glycart technology, Lonza have announced a reciprocal agreement with Biowa for a fucosyl transferase-knockout CHO-K1 cell line, and Merck acquired the company GlycoFi, which generated knockout and knock-in variants of *Pichia pastoris* that allow the selective production of IgGs with homogeneous glycoforms bearing complex oligosaccharides that are either devoid of galactose, fully galactosylated or fully sialylated. Similarly, Biolex and Ingenion have developed variants of *Lemna* and moss, respectively, that can produce homogeneous non-galactosylated or fully galactosylated protein glycoforms.

Conclusions and future directions

Understanding the significance of the heterogeneity of IgG antibody glycoforms has been a major challenge for the manufacture of natural antibody glycoform products. As is usually the case, confronting and resolving this challenge has opened up new opportunities for tailoring antibody therapeutics to maximize their efficacy. The dramatic increase in ADCC activity observed for non-fucosylated rituximab and trastuzumab in *in vitro* whole-blood assays suggests that they may be similarly efficacious *in vivo*; such increased efficacy could have a downside in that increased and/or additional unwanted side effects may occur — for example, release of inflammatory mediators and cytokines. It would seem, therefore, that these products should be treated as new or follow-on biologics and be subject to rigorous pre-clinical and clinical evaluation.

There is new evidence that the presence or absence of fucose on IgG Fc may differentially influence activation of FcγRIIa and FcγRIIIa expressed on mononuclear and polymorphonuclear cells, respectively⁵⁹. In addition, there is some evidence that FcγRs may themselves be differentially glycosylated depending on the tissue or cell type in which they are produced *in vivo*⁷⁵. It may be anticipated therefore that a combination of protein and glycosylation engineering may be able to selectively target individual populations of effector cells.

Opportunities are also opening up for the generation of synthetic IgG Fc glycoforms that might allow the development of probes to further dissect the complexities of inflammatory responses mediated by the humoral immune system^{11,20,76,77}. Advances in this exciting field are eagerly awaited.

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Competing interests statement

The authors declare [competing financial interests](#): see web version for details.

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