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The Impact of Glycosylation on the Biological Function and Structure of Human Immunoglobulins

James N. Arnold,^{1,2} Mark R. Wormald,¹
Robert B. Sim,² Pauline M. Rudd,¹
and Raymond A. Dwek¹

¹Oxford Glycobiology Institute, Department of Biochemistry and ²MRC Immunochemistry Unit, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom; email: raymond.dwek@exeter.ox.ac.uk, james.arnold@bioch.ox.ac.uk, mark.wormald@bioch.ox.ac.uk, bob.sim@bioch.ox.ac.uk, pauline.rudd@bioch.ox.ac.uk

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Key Words

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Abstract

Immunoglobulins are the major secretory products of the adaptive immune system. Each is characterized by a distinctive set of glycoforms that reflects the wide variation in the number, type, and location of their oligosaccharides. In a given physiological state, glycoform populations are reproducible; therefore, disease-associated alterations provide diagnostic biomarkers (e.g., for rheumatoid arthritis) and contribute to disease pathogenesis. The oligosaccharides provide important recognition epitopes that engage with lectins, endowing the immunoglobulins with an expanded functional repertoire. The sugars play specific structural roles, maintaining and modulating effector functions that are physiologically relevant and can be manipulated to optimize the properties of therapeutic antibodies. New molecular models of all the immunoglobulins are included to provide a basis for informed and critical discussion. The models were constructed by combining glycan sequencing data with oligosaccharide linkage and dynamics information from the Glycobiology Institute experimental database and protein structural data from “The Protein Data Bank.”

Fc: crystalizable fragment

Glycoform: each glycosylation site on a glycoprotein contains a range of oligosaccharides. A glycoprotein thus consists of a collection of "glycoforms" with the same amino acid sequence but different locations or structures of the attached oligosaccharides

Mannan-binding lectin (MBL): C-type lectin composed of oligomers of a trimeric subunit that contains a collagen-like domain and a carbohydrate recognition domain with specificity for terminal GlcNAc>glucose>mannose>fucose residues

Fab: antigen-binding fragment

FROM IMMUNOLOGY TO GLYCOBIOLOGY

Glycobiology owes much to the late Nobel laureate, Professor Rodney R. Porter, FRS, who recognized the importance of understanding the role of glycosylation on the antibody molecule. He encouraged the development of this emerging field in the 1980s. In the Biochemistry Department in Oxford in 1969, he instigated the use of magnetic resonance to study the combining sites of antibody molecules. This led, in 1975, to the structure of the DNP-binding MOPC 315 antibody combining site (1). One of the main assumptions, which turned out to be the case, was that the immunoglobulin (Ig) fold would be conserved (2)!

In 1980, the site to which C1q bound on the antibody Fc (crystalizable fragment) region was identified, followed by a detailed investigation of the structural and functional roles of the conserved Fc carbohydrates (3). The first glycoprotein crystal structure was an IgG Fc obtained by Diesenhofer (4). It revealed that one of the surfaces of the Fc was a hydrophobic face that was covered by carbohydrates, and so determining the structure of the carbohydrates and assessing what role, if any, they played became important.

In 1985, investigators demonstrated that a population of 32 oligosaccharide structures was associated with the IgG molecule, contrary to the expected one or two. By evaluating more than 1400 oligosaccharide sequences, researchers showed that in rheumatoid arthritis (RA) there is a shift in this population (5). This was an important technical achievement and also provided one of the first glycan diagnostics. This was also the first evidence that glycoproteins generally exist in many glycosylated variants, or glycoforms, in which an ensemble of oligosaccharides is associated with each glycosylation site (3). Subsequently, a role was demonstrated for aggregated agalactosylated IgG (IgG-G0) in the activation of

the lectin pathway of complement through the binding of mannan-binding lectin (MBL) (6).

Rodney Porter played a major role in setting up the Oxford Glycobiology Institute. In 1988, the term glycobiology was introduced in the *Annual Review of Biochemistry* (6a). In 1992, the word glycobiology found its way into the *Oxford English Dictionary*. Glycobiology has now emerged as a distinct discipline, mainly because of the advances in techniques for releasing, labeling, separating, and sequencing oligosaccharides from glycoproteins. The determination of carbohydrate structures can now be considered among the range of techniques available to the well-founded laboratory (7). There are many established structural and recognition functions for the sugars in the immune system and inflammation (8). A particularly important general role is the provision of epitopes that allow glycoproteins to enter the endoplasmic reticulum (ER) lectin quality control pathways (9). Since the analysis of the IgG glycosylation in RA, many papers have dealt with the glycosylation of all classes of antibodies. However, in 2004, there was an unexpected and exciting discovery of a new form of antibody. IgG 2G12, obtained from a patient, is a rare anti-gp120 antibody that neutralizes about 40% of all human immunodeficiency virus (HIV) clades (10). In 2G12 the heavy chains (HCs) of the Fab regions are interlocked in a domain-exchanged configuration generating two variable light (VL)-variable heavy (VH) combining sites and a VH-VH groove. This extended paratope provides the large surface necessary for high-avidity binding of clustered oligomannose sugars on the silent face of the HIV envelope glycoprotein, gp120 (11). The epitope recognized by 2G12 is a template for immunogen design and provides a further challenge for glycobiology. It is fitting that the antibody molecule for which Rodney Porter was so well known has still further mysteries to reveal.

This review is dedicated with gratitude to the memory of Professor Rodney R. Porter, FRS.

IMMUNOGLOBULINS AS GLYCOPROTEINS

IgG are the major secretory products of the adaptive immune system. They are glycoproteins found in all higher vertebrates (12) that provide the host with long-term defense against antigens to which it has been exposed. In humans, five distinct classes have been identified (IgG, IgM, IgA, IgE, and IgD) that share similar structures composed of Ig domains (13). The Igs display considerable diversity in the location and number of the conserved N-linked glycosylation sites that are situated both on the Fc (crystalizable fragment) and Fab (antigen-binding fragment) (**Figure 1**). In IgG, IgA, and IgD, the Fc and Fab are linked by a flexible hinge region that has no defined secondary structure. In IgM and IgE, this flexible linker is replaced by a more rigid Ig domain. The hinge region can contain N- and O-linked glycans (**Figure 1**). Each Ig molecule contains two identical 5077-kDa class-specific HCs (γ , μ , α , ϵ , and δ), joined by one or more disulphide bridges. Each HC is bridged by a disulphide bond to a 25-kDa LC, of which there are two isoforms, κ and λ . HC glycosylation accounts for 2%–3% of the molecular weight of IgG (150 kDa) but ranges up to 12%–14% for the more heavily glycosylated Igs such as IgM, IgD, and IgE. The LCs contain no conserved glycosylation sites.

The oligosaccharides attached to the Igs are large (~2 kDa each) and, when not constrained by their location, are flexible, particularly around the N-glycosidic linkage. The glycans are multifunctional, playing crucial structural roles and participating in binding events, for example to serum lectins such as MBL (6). Other important roles for the Ig glycans include maintaining solubility and con-

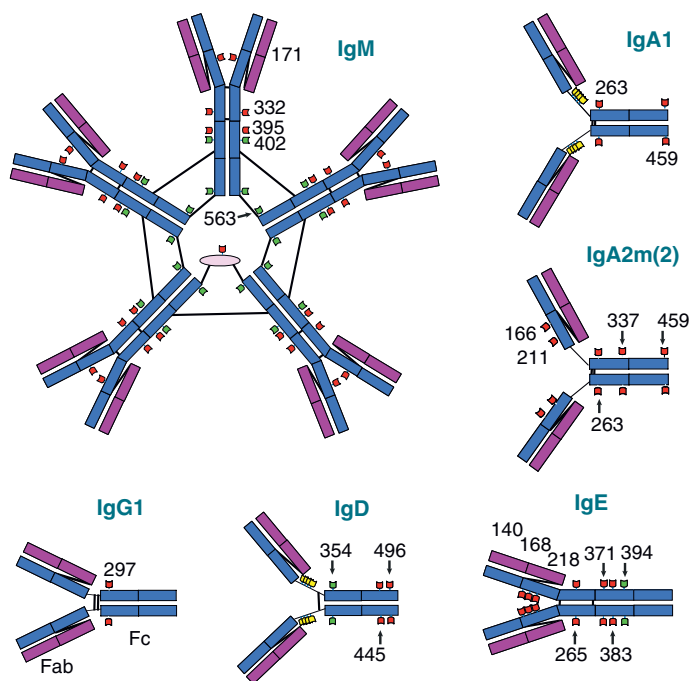


Figure 1

Diagrammatic representations of IgM, IgA1, IgA2m(2), IgG1, IgD, and IgE showing LCs (purple) and HCs (blue), IgM J chain (pink), and interchain disulphide bridges (black). Sites of N-glycosylation are shown with complex (red) or oligomannose (green) glycans according to the glycan analysis. Clusters of O-glycans are yellow. Note: IgA and IgM both contain 18 amino acid-residue tailpieces at the C-termini of their HCs (not shown).

formation (14); facilitating subcellular transport, secretion, and clearance (15); and maintaining effector functions by ensuring optimal binding of the Fc to Fc receptors (16). In this review we present for the first time models of the complete set of glycosylated Igs to enable a more informed and critical appreciation of the roles of the glycans. We also discuss the significance of the glycans attached to the Igs in health and their aberrant glycosylation in diseases such as RA, IgA nephropathy, and congenital disorders of glycosylation (CDGs). Finally, we focus on anticarbohydrate antibodies, for example against the HIV envelope glycoprotein gp120, and on recent advances in the manipulation of glycosylation pathways for the production of therapeutic antibodies.

N-linked glycan:

an oligosaccharide covalently linked to a polypeptide through the side chain of an Asn residue in the consensus sequence, Asn Xaa Ser/Thr, where Xaa is any amino acid except Pro

O-linked glycan:

a glycan linked to the hydroxyl group in the side chain of a Ser or Thr residue, often in Ser/Thr/Pro-rich domains

CDGs: congenital disorders of glycosylation

IgG-G0, -G1, and -G2: predominant glycoforms of IgG based on the number of galactose residues terminating the Asn-297 glycan

GlcNAc: N-acetylglucosamine

DIVERSE FUNCTIONS FOR N-LINKED GLYCANS IN THE Ig FAMILY

Conserved N-glycans Maintain IgG Fc Effector Functions

Human serum IgG consists of four structural subclasses, IgG₁₋₄, that differ in their γ -chain sequences and disulphide bridging patterns. IgG₁ (Figure 2) is the most abundant serum Ig, circulating at 10–15 mg/ml. All IgG molecules contain a single conserved N-linked glycosylation site (Asn-297) in each of the C γ 2 domains. The glycan helps to maintain the quaternary structure and the stability of the Fc (14, 16). Importantly, the oligosaccharides can also present glycan epitopes for lectin binding (6, 17). X-ray crystallography (4, 18, 19) indicates that, in a single IgG molecule, the two Asn-297 sites may be differently glycosylated. Each Asn-297 site contains one of a family of 32 glycans that can be assigned to three subsets, IgG-G0, -

G1, and -G2 (20–22). The bi-antennary glycans occupying Asn-297 in IgG-G2 glycoforms have both arms terminating in galactose residues [$\sim$ 16% of the total human serum IgG glycan pool (20)]. In IgG-G1, a terminal galactose residue is missing from one or the other arm, exposing a GlcNAc residue [$\sim$ 35% of the glycan pool (20)]. There is a preference for the galactosylation of the α 1,6 arm compared with the α 1,3 arm (21, 22). In IgG-G0, neither arm contains galactose residues, and both terminate with GlcNAc [$\sim$ 35% of the glycan pool (20)]. IgG-G0 levels in the serum are not uniform and increase after the age of 25 years (23). The remaining 14% of the glycan pool contains sialylated G1- and G2-type glycans (20). Diversity also arises from the presence or absence of bisecting GlcNAc residues (on 30% of the glycan pool) and core fucose (on 70% of the glycan pool) (Table 1).

There is a hydrophobic patch on each IgG Fc γ -chain that interacts with the Asn-297

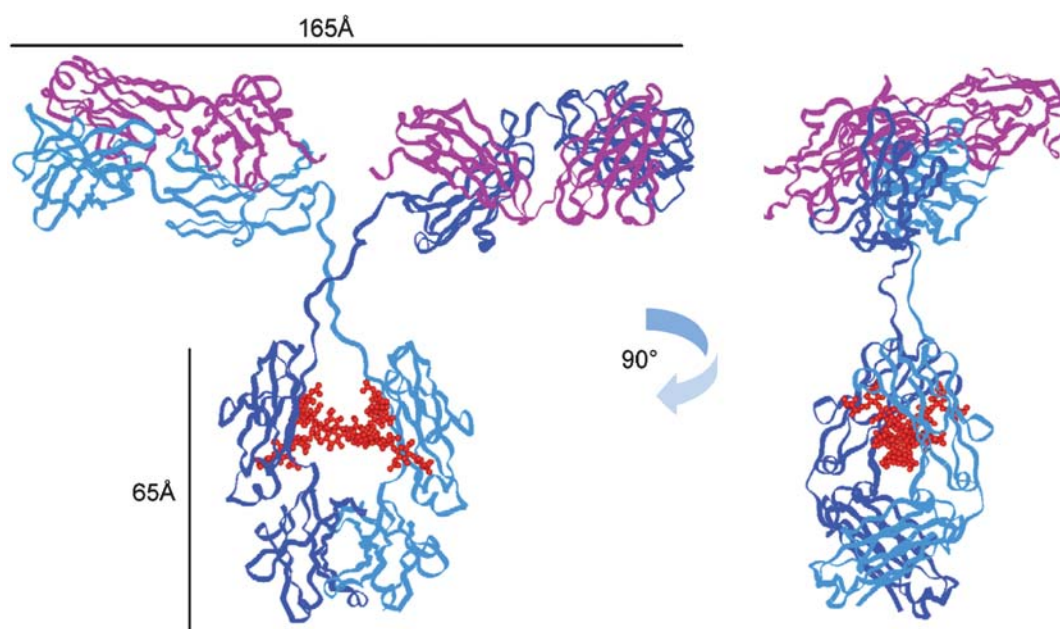


Figure 2

Molecular models of IgG1 based on the crystal structure (4, 137). The glycan structures are based on data in Table 1. See Figure 1 for color code.

Table 1 Analysis of the key features of the N-glycans released from the Igs^a

	Complex glycans						Oligomannose glycans			
	N-linked glycosylation sites	Sialylation (%)		Term. galactose (%)	Term. GlcNAc (%)	Bisecting GlcNAc (%)	Core fucose (%)	Predominant complex glycan ^a	N-linked glycosylation site	Oligomannose glycan structures ^a
		S1	S2							
IgG IgE	Asn-297	11	4	50	35	32	69	FA ₂	Asn-394	Man ₄₋₈ GlcNAc ₂
	Asn-140, Asn-168, Asn-218, Asn-265, Asn-371, Asn-383	45	42	12	1	15	67	FA ₂ G ₂ S ₂		
IgA1 sIgA	Asn-263, Asn-459	49	44	7	0	29	32	A ₂ G ₂ S ₁	unknown (Asn-263 or Asn-459) Asn-354	12% of total glycans Man ₅₋₉ GlcNAc ₂
	Asn-263, Asn-459	11	2	23	52	75	50	FA ₂ B		
IgD	Asn-445, Asn-496	41	34	24	<1	51	34	FA ₂ G ₂ S ₂	Asn-354	Glc ₁ Man ₈₋₉ GlcNAc ₂ and Man ₅₋₉ GlcNAc ₂
IgM	Asn-171, Asn-332, Asn-395	71	10	16	4	52	85	FA ₂ BG ₂ S ₁	Asn-402, Asn-563	Man ₅₋₉ GlcNAc ₂ (Asn-563 17% occupied)

^aAbbreviations: A₂, bi-antennary glycan containing two antennae linked to the trimannosyl core; F, core fucose α1,6-linked to the inner core GlcNAc residue; G(χ), S(χ), number (χ) of galactose and sialic acid residues respectively attached to antennae; B, bisecting GlcNAc-linked β1,4 to the mannose residue attached to chitobiose core; Manχ, number (χ) of mannose residues attached to the chitobiose core; Glc(χ), number (χ) of glucose residues attached to mannose. For further details see **Figure 7**. Data taken from Arnold et al. (31, 66), Mattu et al. (38), Royle et al. (44), and Butler et al. (20).

Variable region/domain: the Fab variable region/domain of an Ig that binds to antigen. The variable region is unique to each individual Ig-producing B cell clone

GTase: galactosyltransferase

Complex glycans: bi-, tri-, or tetra-antennary glycans that contain combinations of GlcNAc, galactose, fucose, and sialic acid residues attached to the chitobiose core

glycan (4). This hydrophobic patch is similar to those in Ig Fab variable regions that interact with carbohydrate antigens (24). Each Asn-297 glycan takes part in ~70 noncovalent interactions with the protein surface that restrict the movement of the glycan. The crystal structure of IgG Fc (4) predicts a total of 38 noncovalent interactions between the hexasaccharide unit, GlcNAc₂Man₃GlcNAc, and the Fc C γ 2 domain. There are 13 further interactions for the additional outer arm GlcNAc, 27 for galactose on the α 1,6 arm, and 7 for the core fucose (25). The glycan-protein interactions stabilize the Fc and help to maintain the structure (14, 16, 26). The Man α 1,3 branch of the sugar does not contact the protein surface and protrudes into the space between the Fc domains (4) (**Figure 2**).

Proteins exert some control over the processing of their glycans, and in IgG the addition of terminal sialic acid to galactose on the α 1,6 arm of the sugar at Asn-297 is inefficient. The location of the sugars in the interstitial space between the C γ 2 domains, the orientation of the oligosaccharide chain, which is constrained by the protein, and the interaction of the galactose residue with a hydrophobic pocket all provide a rationale for the finding that the galactose is a poor substrate for the sialyltransferase (4, 22). Consistent with this rationale, alanine scanning to replace amino acids 241, 243, 263, 265, or 301, all of which contact the oligosaccharide, results in increased galactosylation and sialylation of the Asn-297 glycans (27). A possible explanation is that these mutations have altered the local protein 3D structure of the Fc and/or the orientation of the sugar residues, favoring the attachment of galactose and sialic acid to the α 1,3 arm by β 1,4 galactosyltransferase (GTase) and the α 2,6 sialyltransferase, respectively.

N-glycans Cross-Link IgE and Fc ϵ RI by Binding Galectin-3

IgE (**Figure 3**) is the least abundant Ig in serum (<1 μ g/ml) and the most heavily glyco-

sylated [$\sim$ 12% w/w carbohydrate (28)]. There are seven N-linked glycosylation sites in the ϵ -chain (28) (**Figure 1**). Asn-394 is occupied exclusively by oligomannose structures (28–30), predominantly Man₅GlcNAc₂ (31). Complex glycans occupy the remaining sites (**Table 1**). Sialic acid residues are attached to galactose in α 2,6- (and not α 2,3-) linkages (31). In Igs in general, sialic acid is α 2,6-linked to galactose, and α 2,3-linked sialic acid is present in only minor constituents of the glycan pools. α 2,3-sialyltransferase is expressed in B cells; however, either lower expression levels or steric constraints render it less efficient than the α 2,6-sialyltransferase. Terminal galactose residues are targets for soluble S-type lectins such as galectin-3, also known as IgE-binding protein (32). Galectin-3 is a 31-kDa lectin that is expressed on the surfaces of cells such as macrophages and is also secreted. Galectin-3 is dimeric and cross-links IgE and Fc ϵ RI through their N-glycans, mediating the inflammatory response.

The N-linked glycans significantly reduce the flexibility of IgE, particularly in the Fab where the oligosaccharides in the C ϵ 1 domain form a tripod around the C-terminus (**Figure 3**). In contrast to the flexible hinge regions of IgG, IgA, and IgD, IgE has an additional C ϵ hinge domain, which also restricts the range of motion of the Fab relative to the Fc (**Figure 3**). These restrictions, imposed by both the glycosylation and the IgE protein structure, may serve to control the antigen-binding capability of IgE, reducing the possibility of inappropriate engagement that could evoke severe and potentially fatal immune responses.

IgA N-glycans Bind Pathogens and Mediate Clearance

There are two subclasses of IgA, IgA1 and IgA2 (**Figures 1** and **4**), that have arisen through gene duplication and that share considerable sequence similarity (33). Both contain two conserved N-linked sites in each α -chain (**Figure 1**), located in the C α 2

domain and on the 18 amino acid tailpiece. IgA2 is further divided into two subsets that contain an extra two or three N-linked glycosylation sites. IgA is the major antibody found in external secretions such as colostrum, and it plays a major role in the protection of mucosal surfaces (33, 34). There are significant differences between the glycosylation of serum IgA and secretory IgA (sIgA), as discussed below.

Serum IgA glycans bind ASGP-R. Serum IgA consists mainly of IgA1 and circulates at around 1 mg/ml, and, although it is predominantly monomeric (**Figure 4**), it also forms dimers and higher polymers (35, 36). The glycosylation of IgA has been widely reported (37–40) (**Table 1**). The galactose-terminating glycans are ligands for the asialoglycoprotein receptor (ASGP-R), which mediates the clearance of IgA from the serum (41, 42). Thirty percent of serum IgA contains N-linked glycans attached to glycosylation sites in the Fab variable regions of the α - and LCs (38). Some of the glycans in the Fab, for example tri-antennary structures, are not present on the Fc (38), presumably because they are more accessible to GlcNAc transferase IV than are the Fc glycans, which remain bi-antennary. The location of the glycans within the tertiary structure of the protein strongly influences the processing because it directly affects the access of the processing enzymes to the developing glycan chain.

Secreted forms of IgA, J chain, and secretory component are heavily glycosylated. sIgA1 and sIgA2 (**Figure 4**) are composed of two identical IgA monomers, covalently linked via disulphide bridges to a glycosylated 16-kDa J chain (43, 44), which is also found in pentameric forms of IgM (45) (**Figure 1**). The sIgA dimer is complexed to a glycosylated polypeptide chain, secretory component (SC). SC is derived from the polymeric Ig receptor (pIgR), which is expressed on the surface of epithelial cells. sIgA complexed with pIgR is secreted through the epithelia onto the mucosal lining of the gastrointestinal and respi-

GLYCAN STRUCTURES

Immunoglobulins are glycoproteins. We have constructed molecular models to visualize the family of antibodies complete with their N- and O-linked glycans (**Figures 2–5** and Supplemental Figures 1–3; for the supplemental figures, follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org>). The different locations (**Figure 1**) of the glycans are determined by the primary amino acid sequence (AsnXaaSer/Thr for N-glycans, Ser/Thr for O-glycans) and the 3D structure of each Ig. Glycan processing is cell and protein specific, and the 3D structure around the glycosylation site also controls the processing of the glycans at each site (86, 141). The models demonstrate dramatically that a large surface area of each protein is shielded by the sugars by virtue of their large size and flexibility. Two N-linked glycans occupy a conformational space comparable to an Ig domain mainly because the sugars are flexible at the N-glycosidic linkage (**Figure 3**). Thus, sugars can preclude or modulate interactions of the Igs with their receptors (**Figure 6**). Specific glycans (**Figure 3**) interact with mammalian lectins and pathogens, and Igs can recognize non-self glycans on pathogens (**Figure 8**), initiating functional responses. The models have been constructed from glycan sequencing data and linkage conformational information combined with protein structural data. Where complete protein structures were unavailable, models were generated using structures of homologous domains.

ratory tract and into tears, saliva, and milk (46). sIgA is subsequently released through proteolytic cleavage of the pIgR to leave the SC wrapped around the IgA dimer. The N-linked glycans attached to the α -chains of sIgA1 and sIgA2 are almost identical. This is expected because the local protein structure around the N-linked glycan sites is identical, and the result is consistent with the well-established influence of the local protein structure on glycan processing (44) (**Table 1**). However, considering their accessibility without the presence of SC, which is associated extracellularly (**Figure 4**), the sugars are surprisingly unprocessed by the B cell glycosylation machinery. The total glycan pool consists of 52% GlcNAc-terminating glycans

sIgA: secretory IgA

ASGP-R: asialoglycoprotein receptor

SC: secretory component

pIgR: polymeric Ig receptor

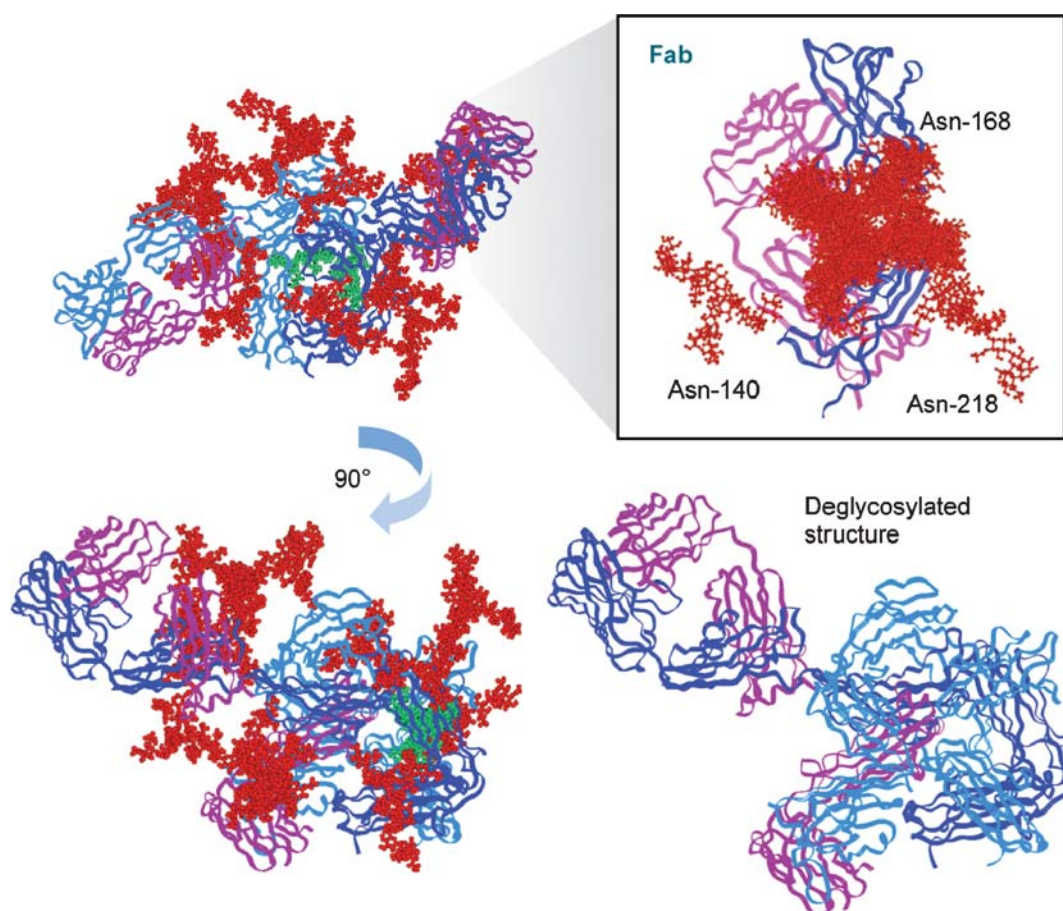


Figure 3

Molecular models of IgE (*left*) based on the crystal structures of the Fc and the Cε2 hinge region (138) and the Fab domains (137). The disulphide bridging was modeled according to the amino acid sequence. The glycan structures are based on data in **Table 1**. Deglycosylated IgE is shown for comparison (*bottom right*). An IgE Fab is highlighted (*top right*), showing Asn-140 and Asn-218 occupied by oligosaccharides in a single orientation. However, in vivo, glycans are very flexible; therefore, the sugar at Asn-168 is represented by a series of plausible conformational overlays to indicate the large surface area of the protein that can be shielded. See **Figure 1** for color code.

Oligomannose glycans: N-linked glycans that contain from 5–9 mannose residues attached to the chitobiose core.

and 12% oligomannose glycans. In contrast, serum IgA1 contains fully processed glycans (**Table 1**). B cells secrete sIgA before the SC binds, suggesting that the lack of glycan processing of sIgA is a direct result of low β1,4 GTase activity within the B cells rather than a lack of accessibility. However, the J chain is processed within the same B cell and yet has a fully processed sialylated glycan (44), suggesting that other currently unknown fac-

tors may control the processing of glycans attached to Ig α-chains. The high incidence of GlcNAc- and mannose-terminating glycans on the IgA α-chain may be physiologically important, perhaps as a binding epitope to decoy pathogens.

sIgA binds microorganisms through its O-glycans. sIgA binds microorganisms through its O-glycans, thus preventing the

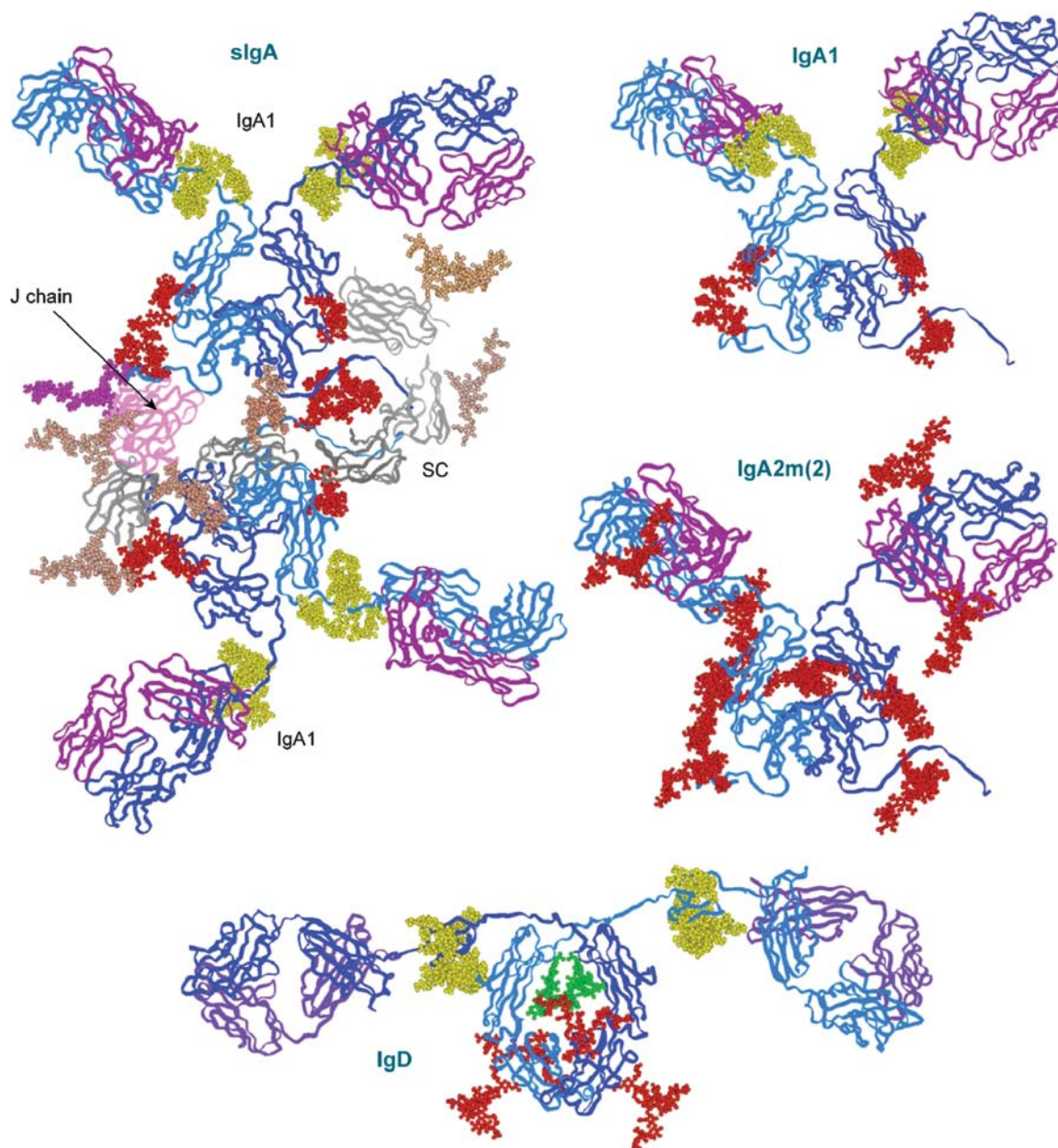


Figure 4

Molecular models of sIgA1, IgA2m(2), IgA, and IgD. sIgA (*top left*), IgA1 (*top right*), and IgA2m(2) (*right*) are based on Reference 44, as are the J chain (*pink*) and secretory component (SC) (*gray with beige complex glycans attached*). The complex glycan attached to the J chain is dark purple. IgD (*bottom*) is adapted from Reference 31. The hinge was constructed from the amino acid sequence and constrained to fit the solution structure (56). The disulphide bridging was modeled according to the amino acid sequence of each Ig. The glycan structures are based on data in **Table 1**. See **Figure 1** for color code.

attachment of pathogens to the gut epithelium and facilitating their excretion in the feces. sIgA in colostrum protects newborn infants whose immune systems are immature (reviewed in 47). sIgA also plays an important role in the innate immune defense against ricin, a particularly toxic galactose-specific lectin from castor beans. Ricin can bind both the SC and isolated IgA1 and IgA2 from sIgA via their galactose-terminating glycans (48). sIgA is a potent inhibitor of adhesion of S-fimbriated *Escherichia coli*, a cause of sepsis and meningitis in newborns (49). The mechanism involves specific interactions between bacterial adhesins and sialylated N- and O-linked glycans (49). IgA also carries glycans that are potential ligands for the mannose-specific lectin of type I fimbriated *E. coli* (50). SC wraps around the Fc of sIgA (**Figure 4**), and this shields the GlcNAc-terminating and oligomannose glycans from lectin binding (44). However, the SC is attached to the Fc by a single disulphide bond and wraps around the Fc via noncovalent interactions. These noncovalent interactions can be disrupted by acidic conditions, such as on the mucosal surface (44), allowing IgA to present GlcNAc and mannose-terminating glycans to bacteria.

J chain glycans required for IgA dimerization and binding to the pIgR. The single IgA J chain is disulphide bridged to the C-terminus of one α -chain from each IgA to form an IgA dimer (44, 45) (**Figure 4**). The J chain glycans (44) occupy one N-linked site and are required for IgA dimerization and binding to the pIgR (43, 51). Over 75% of the J chain glycans are sialylated, and, unusually, the disialylated structures contain one α 2,3-linked and one α 2,6-linked sialic acid residue (44). The presence of significant quantities of α 2,3-linked sialic acid suggests that the B cells that secrete sIgA have an increased expression level of α 2,3-sialyltransferase because α 2,3 sialic acids are rarely found on serum Igs. sIgA- and serum IgA-secreting B cells reside in different environments, highlighting

the influence of environmental factors, such as the presence of signaling molecules and cell-cell interactions, on the cellular glycosylation machinery. Such factors can modulate the glycosylation pathways within a cell, primarily at the level of enzyme expression levels, with the result that secreted glycoproteins carry distinctive sets of glycan structures.

IgA SC binds pathogens through its N-glycans. SC contains seven N-linked sites occupied by a large diversity of structures (44) (**Figure 4**), many of which differ from those on the Ig α -chain, for example by the presence of outer arm fucosylation. The pIgR, from which the SC is derived, is produced in epithelial cells, whereas the Ig is produced in plasma cells. The wide range of N-linked glycans on SC creates a diverse array of glycan epitopes, including all the different Lewis and sialyl-Lewis epitopes (44). These epitopes are targets for lectins and bacterial adhesins, and can thereby inhibit attachment and subsequent infection of epithelial surfaces (50, 52, 53). The SC glycans also anchor sIgA to the mucosal lining of the epithelium (54), where bacteria can be sequestered.

IgD N-linked Glycans Are Required for Assembly and Secretion

IgD (**Figure 4**) is a low-abundance monomer in serum, circulating at $<30 \mu\text{g/ml}$. Membrane-bound IgD is expressed on the surface of B lymphocytes, where it forms part of the B cell receptor (BCR) complex (55). The membrane-bound form has two additional transmembrane segments, located at the C-terminal of each of the constant regions. IgD is predominantly T shaped in structure, with a semiextended hinge (56).

The δ -chain has three N-linked glycosylation sites (57) (**Figure 1**). Asn-354 in the C δ 2 domain is occupied exclusively by oligomannose structures ($\text{Man}_5\text{-}_9\text{GlcNAc}_2$) (31, 58)

(Table 1). Surprisingly, low levels of the monoglucosylated oligomannose structures, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ and $\text{Glc}_1\text{Man}_8\text{GlcNAc}_2$, have been detected at Asn-354 (31, 58). $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ binds to calnexin and calreticulin in the ER (59, 60), acting as a retention signal to ensure the correct folding and assembly of glycoproteins (61, 62). The glucose residue is normally removed by the ER glucosidase II when the protein is fully folded. Monoglucosylated glycans have not been identified on any other Ig, although they are present in low levels on serum complement components (63). Their function in serum IgD is unknown, but they are inaccessible for binding lectins (31). The oligomannose glycans at Asn-354 are inaccessible to any potential lectin interactions because the complex glycans at Asn-445 on the Fc sterically block binding (31) (Figure 4). The oligomannose glycans at Asn-354 are vital for the production of IgD, and elimination of this site through mutagenesis results in incomplete assembly and failure of secretion (15), suggesting that the glycans are required to maintain

the correct structure of the Fc, which is necessary for IgD secretion.

Glycan Epitopes Clustered on the Antigen-Binding Face of IgM Pentamer Are Poised to Agglutinate Microorganisms

In humans, serum IgM is a multimer (Figures 1 and 5), which circulates at ~ 2.5 mg/ml predominantly as pentamers linked by disulphide bonds and a single J chain (45). The J chain is disulphide bridged to two μ -chain C-termini in IgM (45). The μ -chain has five N-linked glycosylation sites (Figure 1). In common with IgA, the C-termini of IgM μ -chains contain a short 18-residue region with no defined secondary or tertiary structure, known as the tailpiece (64) (Figure 5), which is required for multimerization (65). Human serum IgM μ -chain has three N-linked glycosylation sites that contain complex glycans and a small population (4%) of hybrid structures, in which one bi-antennary

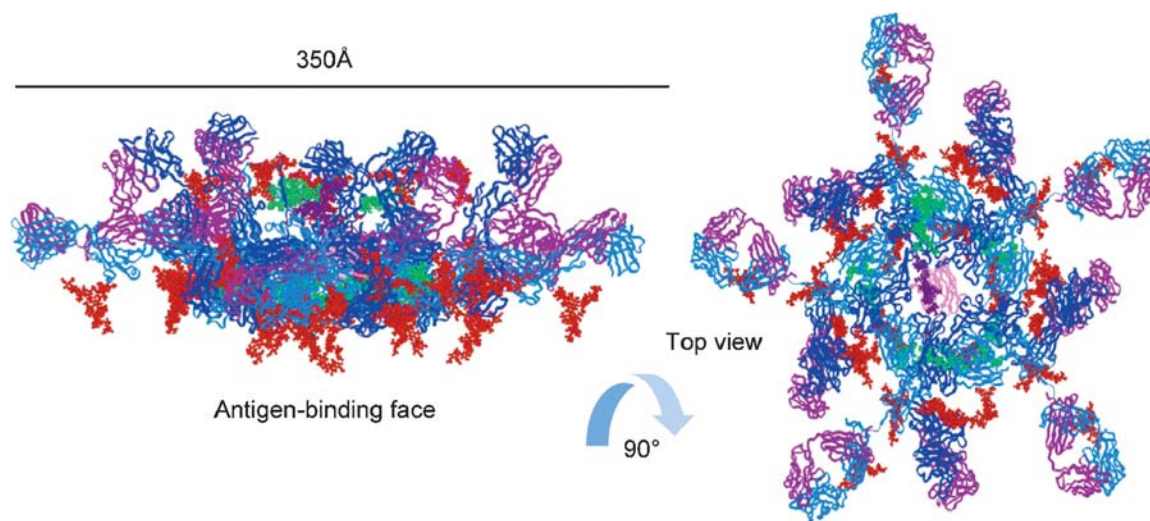


Figure 5

Molecular model of pentameric IgM, based on Reference 66, showing the antigen-binding face (*left*), with the surface of sialylated glycans (*red*) at the bottom and the 90° rotation (*right*) with the nonantigen-binding face facing up. The glycan structures are based on data in Table 1. See Figure 1 for color code. The complex glycan attached to the J chain is dark purple.

arm remains oligomannose and the other is further processed. Monomeric IgM is expressed on the surface of B lymphocytes as part of the BCR complex (55).

Twenty percent of serum IgM contains a high proportion of GlcNAc-terminating structures (~10% of the complex glycan pool) that, when multiply presented, such as in immune aggregates, provide ligands for MBL (66). Researchers have proposed that IgM monomers are organized in a barrel-like arrangement to form a pentamer (66) so that the complex glycans are largely confined to a single face (**Figure 5**). The location of these glycans renders most of them inaccessible for lectin binding once the Fabs have engaged antigen (66) (**Figure 5**). In the absence of Fab-mediated antigen binding, the complex glycans may allow IgM to agglutinate microorganisms in the serum. For example, the sialic acid-terminating glycans of IgM provide a binding site for viral lectin hemagglutinin, potentially allowing IgM to agglutinate the viral particles.

Asn-402 and Asn-563 are occupied by the oligomannose glycans $\text{Man}_{6-9}\text{GlcNAc}_2$ (64, 67, 68), the significance of which is currently unknown. It is unlikely that they bind lectins in a physiological situation because antigen-bound IgM does not bind MBL (66). Interestingly, in normal human IgM, Asn-563 is only 17% occupied (66). The glycosylation sequon is located close to the C-terminus of the protein, perhaps making it a poor substrate for the oligosaccharyl transferase complex, although in a human-mouse hybridoma human IgM, the site was 75% occupied (69).

The J chain attached to pentameric IgM has a single N-linked glycosylation site containing mainly sialylated bi-antennary glycans with and without core fucose (66). The extent of glycan processing is surprising considering the position of the J chain within the center of the IgM pentamer (**Figure 5**). IgM μ -chain mainly contains monosialylated bi-antennary structures (**Table 1**), suggesting that crowding may prevent the glycosylation machinery from processing all 30 complex N-linked gly-

cans per pentamer to disialylated structures. Possibly the J chain is more flexible as it links the C-termini of the tailpieces of two IgM μ -chains. This location would make the J chain glycans a preferred substrate for the sialyltransferases that process the glycans to disialylated structures.

O-Linked Glycans of IgD and IgA Protect Extended Hinge Regions from Proteases and Bind Pathogens

IgA1 and IgD are the only Igs in humans that have O-linked glycans in the hinge region; however, there is evidence that one is present in a small population of rabbit IgGs (70). The O-glycans are not required for folding or export from plasma cells, although in their absence, IgD is secreted less efficiently (15).

IgD O-glycans increase the angle between the Fabs, expanding the range of hinge conformations. IgD O-glycans are neutral, mono-, and disialylated structures (31, 71). In general, O-glycans extend the peptide backbone to which they are attached and confer a degree of structural rigidity, in this case to the hinge domain. This extension of the hinge favors an increased separation of the Fab domains and, therefore, the antigen-binding sites. The hinge domain in IgD is more extended than in the other Igs (56) (**Figure 4**), and this may allow efficient capture of antigen when IgD forms part of the BCR complex.

IgA1 O-glycans bind pathogens. IgA contains a 23 amino acid hinge region with nine potential O-glycan sites, of which five (Thr-228, Ser-230, Ser-232, Thr-225, and Thr-236) are fully or partially occupied (38, 71, 72) (**Figure 4**). A sixth sugar at Ser-224, Thr-233, Ser-238, or Ser-240 (73) is present on 5%–10% of the glycoforms. The sugars protect the hinge from protease digestion, allowing it to adopt several conformations depending on the structures of the sugars.

Bacteria, including the three leading agents from bacterial meningitis and other clinically important bacterial infections of the respiratory and genito-urinary tracts, secrete proteases that cleave the IgA hinge (74). The activity of some of these proteases is modulated by the glycans, and the hinge containing O-linked glycans terminating in sialic acid has a lower susceptibility to proteolytic cleavage (75).

Serum IgA1 O-glycans impart heterogeneity and rigidity to the hinge region.

The O-glycans are mainly neutral, mono-, and disialylated structures (38), similar to those attached to the hinge of normal human serum IgD (31). The O-glycans, even in a heterogeneous population, are likely to confer a degree of rigidity to the hinge region, particularly because they are able to take part in noncovalent interactions with the protein (56) (**Figure 4**).

Secretory IgA O-glycans present epitopes to decoy pathogens.

The glycans consist of more than 50 different structures containing up to 15 monosaccharide residues (44). They provide an array of surface epitopes, many of which contain terminal galactose (β 1,4- and β 1,3-linked), sialic acid (α 2,3- and α 2,6-linked), and fucose (α 1,4-, α 1,3-, and α 1,2-linked) (44) that act as decoys to prevent pathogenic bacteria binding to mucosal surfaces in the gut (52). In common with IgD, IgA1 has been shown by X-ray scattering to be T shaped (76), a structure that exposes the O-glycans for efficient interaction with bacterial adhesins (**Figure 4**), such as those on S-fimbriated *E. coli* that bind sialylated O-glycans (49).

VARIABLE REGION GLYCOSYLATION CAN MODULATE ANTIGEN BINDING

During Ig synthesis, rearrangement of gene segments and somatic mutations produce variations in amino acid sequence that can

lead to the introduction of N-linked glycosylation sequons in the N-terminal domains, VH and VL. A survey of cDNA sequences indicated that 9% of variable regions had a potential N-linked glycosylation site (77). Each clone of B lymphocytes makes only one type of Ig molecule that contains a variable region unique to that particular B cell clone. O-linked glycans occupy variable region glycosylation sites in λ LC isolated from a plasma cell dyscrasia patient (78), but to date none have been detected on healthy human serum Igs. However, N-linked glycans are present on normal human serum Ig Fab regions (38), where they can significantly influence antigen binding. For example, an anti- α 1,6 dextran antibody had a 15-fold higher affinity for antigen when a glycan was attached at Asn-58 within the variable region (79). Altering the location of glycans within the variable region by site-directed mutagenesis can completely inhibit antigen binding by steric blocking of the interaction by the sugar (80). Most Fab glycosylation sites are located on exposed loop regions and therefore tend to contain fully processed sialylated glycans with a high incidence of bisecting GlcNAc residues (22, 81, 82). Interestingly in follicular lymphoma (FL), variable region glycans containing one or more oligomannose sugars were located within the antigen-binding sites (84). The attachment and processing of sugars is controlled by multiple factors. Within a particular cell, the primary and secondary structure of the protein affects the location of the site and the level of occupancy and, together with the tertiary and quaternary structure, influences the subsequent processing of the attached glycans (85, 86). Closely spaced N-linked glycans inserted into a variable region can be processed differently (83). Moreover, an anti- α 1,6 dextran antibody with a glycan at Asn-60 in the variable region remained oligomannose, although sugars located at Asn-58 or Asn-54 were processed to complex structures (83). These data suggest that the position of the glycan within the local 3D environment exerts a major

FcR: Fc receptor

ADCC:

antibody-dependent
cell-mediated
cytotoxicity

influence because a space of as little as 2 amino acids can make a significant difference to the processing.

Fc AND FcR GLYCANS MODULATE Ig EFFECTOR FUNCTIONS

Fc receptors (FcRs) link the cellular and humoral immune responses. The stimulation of cells expressing these receptors by the Fc regions of Igs has dramatic consequences, including antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, oxidative burst, and release of inflammatory mediators.

IgG Fc Glycans Are Required for Optimal Fcγ Receptor Binding

Fcγ receptors are expressed on leukocytes, including macrophages, eosinophils, neutrophils, natural killer cells, and lymphocytes, and there are three distinct classes: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (87). IgG Fc glycans are required for optimal binding of the antibody to all classes of receptor (88) and for the effector functions that eliminate pathogens and control the clinical properties of therapeutic antibodies. The glycans at Asn-297 maintain the quaternary structure and the thermodynamic stability of the Fc (16). Replacing amino acids in IgG Fc within the extensive region that contacts the core hexasaccharide (GlcNAcMan₃GlcNAc₂) demonstrated that noncovalent interactions of multiple amino acid residues with the glycan are necessary for optimal recognition of the protein-binding site by FcγRI (27). More specifically, the presence or absence of core fucose on the Fc glycans modulates the binding of FcγRIIIa (89). A high-avidity interaction is generated when there is no core fucose because more of the Fc protein surface is available for additional interactions with the Asn-162 glycan on the receptor. The presence of this glycan on FcγRIIIa is an additional

requirement for a high-avidity interaction (90).

IgD glycans bind to Fcδ receptors. IgD glycans bind to Fcδ receptors that are expressed on CD4⁺ and CD8⁺ T cells in humans and on CD4⁺ T cells in mice. The receptors display lectin-like properties, and the binding of antibody was abolished by deglycosylation of the IgD and competitively inhibited by monosaccharides (91, 92). Following antigen binding, membrane-bound IgD is cross-linked on the B cell surface, where it presents arrays of O-glycan epitopes that increase interactions between the B cells and the IgδRs of the T cells (93).

IgE glycans allow IgE to select between Fcε receptors. IgE glycans allow IgE to select between Fcε receptors, triggering effector functions (**Figure 6**). The high-affinity receptor (FcεRI) is expressed on the surface of mast cells and blood basophils and plays a key role in degranulating these cells to release proinflammatory mediators. A second, low-affinity IgE receptor (FcεRII) is expressed on monocytes (94), eosinophils (95), B lymphocytes (96), and T lymphocytes (97). On B cells, FcεRII influences the presentation of IgE-bound antigen to T cells (98). FcεRII is heavily glycosylated and, interestingly, contains an IgE-binding region of 128 amino acids that has a high-sequence homology to the carbohydrate-binding domains of C-type (calcium-dependent) animal lectins (99). The glycosylation of IgE is not required for the activity of the receptor (100, 101), and it appears that the lectin-like domain has evolved to interact with a protein moiety rather than an oligosaccharide. IgE glycosylation is in a position to hinder the binding of IgE to FcεRII (101) but not to FcεRI, which recognizes a different site on IgE Fc out of the range of the sugars (31, 102) (**Figure 6**). The location of the IgE glycans, determined by the primary amino acid sequence, is therefore a means of modulating Fcε receptor binding.

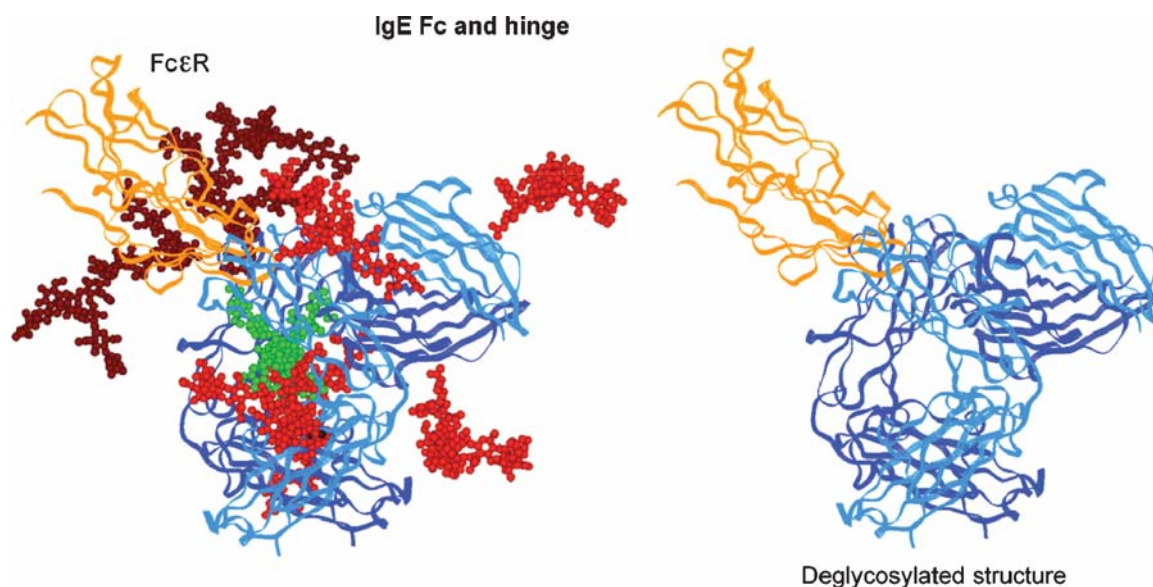


Figure 6

Molecular model of IgE Fc in complex with the FcεR based on the crystal structures of the IgE Fc (138) and the Fc/FcεR (139) (*left*). The FcεR is orange, and its complex glycans are dark red. The glycans attached to IgE Fc are red (complex) and green (oligomannose). The deglycosylated structure is shown for comparison (*right*). The glycan structures are based on data in **Table 1**. For color code of IgE Fc, see **Figure 1**.

IgA glycans modulate Fcα receptor-binding affinities. FcαRs perform different functions. ASGP-R is expressed on hepatocytes and binds to terminal galactose residues on serum IgA (41), after which it conveys the ligand to the intracellular degradation machinery to clear the immune complexes from the serum. (42). IgA binds to CD4⁺ and CD8⁺ T lymphocytes via GlcNAc-terminating N-glycans (103) and the O-linked glycans of IgA1 (91).

FcαRI endocytoses IgA that contains sialylated glycans more efficiently than IgA that presents neutral glycans (41). In contrast, the transferrin receptor that selectively binds IgA1 but not IgA2 preferentially binds to IgA1 that presents neutral glycans terminating in GlcNAc (104, 105). Thus, specific glycan processing modulates the affinity of IgA for different receptors, ultimately determining both the serum half-life and the effector function.

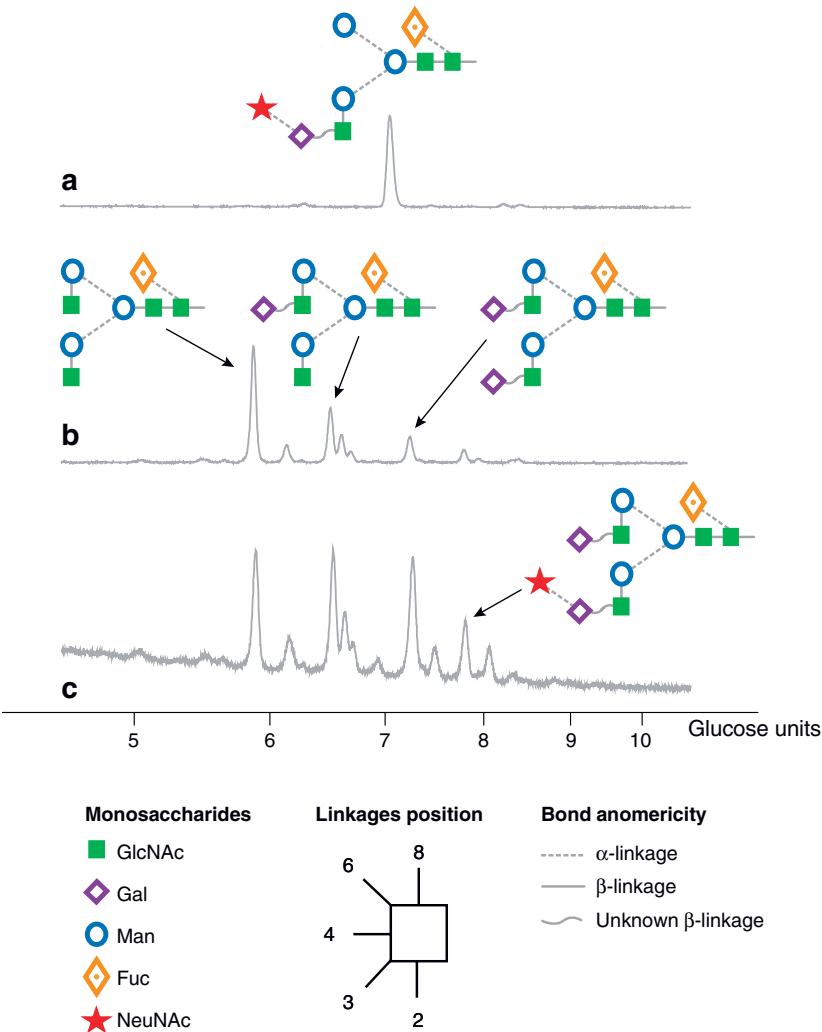
ABERRANT GLYCOSYLATION OF IMMUNOGLOBULINS LINKED TO DISEASE PATHOGENESIS

Levels of Agalactosyl Glycoforms of IgG (IgG-G0) in Rheumatoid Arthritis Correlate with Disease Activity and Contribute to Pathogenesis

In RA, there is a marked increase in the percentage of serum IgG glycans lacking sialic acid and galactose residues (**Figure 7**). The level of these IgG-G0 glycoforms is more than 2 standard deviations above those of age-matched healthy controls (5, 23), they correlate with disease activity (106), and they are directly associated with pathogenesis in a mouse model (107). Levels of IgG-G0 fall during remission of the disease and also during pregnancy, when patients with the disease

Figure 7

Analysis of N-linked glycans from Ig γ -chains from (a) a CDG patient with a mutation in the *Mgat2* gene that encodes GlcNAcT II, (b) a patient with RA, and (c) a healthy control. Glycans throughout the manuscript were analyzed according to References 44, 140.



frequently go into remission (106). Abnormally high IgG-G0 levels in serum are also characteristic of a restricted number of other diseases, including Crohn's disease, juvenile onset chronic arthritis, systemic lupus erythematosus complicated by Sjögren's syndrome, and tuberculosis (5, 108, 109). In a patient diagnosed with seronegative RA and HC deposition disease, the predominant Ig was a truncated glycosylated γ 3 chain that contained fully galactosylated bi-antennary glycans. In contrast, polyclonal IgG in the same patient before and after the episode of HC deposition disease showed an RA-like glycan profile.

These data suggest that specific B cell clones secrete IgGs with characteristic glycosylation (L.A. Omtvedt, L. Royle, G. Husby, K. Sletten, C. Radcliffe, D.J. Harvey, R.A. Dwek, and P.M. Rudd, manuscript submitted). The reduction in galactosylation of IgG glycans is not a consequence of elevated Ig synthesis by B cells as there is no correlation between IgG serum concentration and IgG-G0 in patients with RA (108). Agalactosylated IgG is associated with lowered galactosyltransferase (GTase) activity (110), and although there is a linear correlation between B and T cell GTase activity and IgG-G0 levels

in RA serum (111), the factors that contribute to the reduced GTase activity remain to be established.

In RA patients, inflammation of the joints may, in part, be the result of complement activation mediated by MBL/MBL-associated serine proteases. MBL binds to clustered IgG-G0 glycoforms (6) that deposit on synovial tissue and to IgG-IgG immune complexes that consist mainly of agalactosyl IgG. The interaction of a lectin carbohydrate recognition domain and a single IgG-G0 glycan in the fluid phase is relatively weak and transient. However, when IgG-G0 is clustered, the resulting arrays of exposed GlcNAc residues engage multiple carbohydrate recognition domains simultaneously and become physiologically relevant. The high-avidity interactions generated by this process initiate downstream effector functions, including the complement cascade that leads to inflammation. Garred et al. (112) proposed that the efficiency of MBL binding may determine the course of late-onset and rapidly progressive RA.

Aberrantly Glycosylated IgA Glycoforms: Decreased Clearance Contributes to Pathogenesis in IgA Nephropathy

Aberrant glycosylation has been linked to diseases in which there is reduced clearance of IgA. The ASGP-R removes IgA from serum by binding to terminal galactose residues on the glycans (42). In IgA nephropathy, circulating IgA1 deposits in the glomeruli of the kidney mesangium, leading to renal failure (113). Aberrant O-glycosylation of serum IgA1 in patients (114, 115) is ascribed to a decrease in terminal galactosylation and sialylation (116, 117). Reduced levels of terminal galactose lead to diminished clearance by the liver ASGP-R of both IgA and serum immune complexes containing IgA1, thus promoting their deposition in the kidney (117). In addition, reduced sialylation and galactosylation of IgA O-glycans promotes self-aggregation

(118) and further deposition of IgA on the kidney mesangium. This gives rise to complement activation and inflammation (119). MBL binds effectively to a small population of multiply presented $\text{Man}_5\text{GlcNAc}_2\text{N}$ -glycans that are present on polymeric, but not monomeric, IgA. The inflammatory process is further exacerbated by increased levels of polymeric IgA in patients. The activation of complement by MBL binding to exposed clusters of terminal mannose and GlcNAc residues on IgA deposits on the mesangium provides a significant route to the inflammation associated with IgA nephropathy (B.D. Oortwijn, A. Roos, L. Royle, D.J. van Gijlswijk-Janssen, M.C. Faber-Krol, J.W. Eijgenraam, R.A. Dwek, M.R. Daha, P.M. Rudd, and C. van Kooten, manuscript submitted).

In primary Sjögren's syndrome, the levels of serum IgA1 and IgA2 are elevated as a result of increased sialylation of the glycans. The sialic acids cap the terminal galactose residues, thus reducing the uptake of IgA into the liver hepatocytes and decreasing the rate of clearance (41, 120).

Aberrant IgG Glycoforms Are Diagnostic for Congenital Disorders of Glycosylation

CDGs are a group of genetic disorders characterized by low efficiency of one of the steps in the glycan processing pathway. These disorders affect the glycosylation of all glycoproteins produced by the patients and have severe pathological consequences. CDGs can arise from genetic mutations leading to molecular defects in glycan processing enzymes, sugar nucleotide donors, or protein trafficking machinery within a cell (20). Glycan analysis of serum proteins, such as the Igs that collectively account for up to 50% of the serum glycoproteins, provides diagnostic information that can pinpoint the faulty step in the pathway.

Accordingly, the glycosylation of IgG was analyzed in a patient with a mutation

in the *Mgat2* gene that codes for the UDP-N-acetylglucosaminyl: α -6-D-mannoside- β -1-2-N-acetylglucosaminyl-transferase-II (GlcNAcT-II) enzyme that catalyzes the conversion of a mono-antennary to a bi-antennary glycan (Figure 7). The mutation leads to >98% loss of enzyme activity, which has dramatic implications for the glycosylation of all serum glycoproteins. In particular, the IgG isolated from the patient contained a very restricted pool of glycans in which only mono-antennary structures that were predominantly fucosylated and sialylated on the α 1,3 arm were detected (20) (Figure 7). These glycans are completely absent in healthy serum glycoproteins. The mutation leads to the absence of the GlcNAc and galactose residues on the α 1,6 arm and the loss of noncovalent interactions between these residues and the protein surface of the C γ 2 domains. These interactions normally restrict the orientation of the glycans, which accounts for the incomplete processing of normal IgG sugars (Figure 7). In CDG type II IgG, the accessibility of the galactosyl- and sialyltransferases to the α 1,3 arm glycan is increased, resulting in the processing of fully galactosylated and sialylated mono-antennary glycans. The alterations in sugar structure could well contribute to suboptimal effector functions, contributing to disease pathogenesis (20).

Variable Region Glycosylation Is a Feature of Follicular Lymphoma

FL is a B cell malignancy that represents about 40% of all non-Hodgkin lymphomas. cDNA sequencing (77) indicated a significant increase in potential glycosylation sites in the variable region of the HCs expressed by FL cells (79%) compared with normal memory B cells (9%). Diffuse large B cell lymphomas and endemic and sporadic Burkitt's lymphoma are also characterized by an increased frequency of N-linked glycosylation sites in the variable regions of the HCs (121). The glycans

attached to these sites in FL IgG and IgM are mainly oligomannose (84). Intriguingly, most are located within the antigen-binding sites, where they can interact with soluble or cell surface mannose-specific lectins. In the germinal center, this may lead to cross-linking of the BCR, resulting in the inappropriate activation, proliferation, and survival of the lymphoma cells.

NONHUMAN SUGARS ON PATHOGENS ARE THERAPEUTIC TARGETS

Igs can specifically bind antigenic sugars, even though individual monosaccharide-protein interactions are notoriously weak (K_d in the mM range). Structural characterization of Fabs complexed with sugar antigens indicates that protein-glycan interactions are exploited to increase the avidity of the interaction, as with *Salmonella* B LPS (122, 123). In schistosomiasis, the monoclonal antibody 114-4D12 (124) has affinity for the glycan epitopes of soluble egg antigens of *Schistosoma mansoni*, specifically the unusual O-glycan Fuc1-2Fuc1-3GalNAc1-4(Fuc1-2Fuc1-3) (M.L.M. Robijn, C.A.M. Koeleman, M. Wührer, L. Royle, R. Geyer, R.A. Dwek, P.M. Rudd, A.M. Deelder, and C.H. Hokke, manuscript submitted), and, in common with the bacterial capsular polysaccharide of *Haemophilus influenzae* type b, the nonhuman sugars may provide the basis for a vaccine (125).

New Antibody Structure: Domain-Swapped IgG 2G12 Recognizes an Oligomannose Cluster on HIV gp120

The monoclonal antibody 2G12 (126) uses a different method of maximizing the number of glycan-protein interactions. 2G12 is a broadly neutralizing antibody isolated from an HIV-1 patient that can neutralize many isolates of HIV through binding to an unusually

dense cluster of oligomannose glycans on the viral envelope glycoprotein gp120 (11) (**Figure 8**). Viruses exploit the glycosylation machinery of the host; therefore, it is highly unlikely that the host immune system will raise antibodies to host sugars on viral envelope proteins. However, gp120 has a tight cluster of glycosylation sites on the immunologically silent face; this oligomannose cluster is distinct and not typical of human glycoproteins. Moreover, to date, the structure of 2G12 IgG is unique because the Fab region consists of an interlocked VH domain-swapped dimer as a result of specific mutations (**Figure 8**). This remarkable structure places the combining sites in close proximity, and these, together with an extended groove, provide an extended paratope of approximately 19 Å; this paratope can engage in multivalent interactions with the clustered oligomannose glycans of gp120 (11) (**Figure 8**). The oligomannose cluster is a potential therapeutic target, and, among other approaches researchers have used to exploit this finding, α -mannosidase inhibitors have been used to manipulate the N-glycosylation of gp120 into a homogeneous oligomannose glycoform that has enhanced binding for 2G12 (G.E. Ritchie, J. Rawling, M. Crispin, C.N. Scanlan, M.R. Wormald, D.R. Burton, P.M. Rudd, and R.A. Dwek, manuscript submitted). New ways of synthesizing oligomannose glycans and clustering them on scaffolds using linkers are also being designed.

CONTROLLING GLYCOSYLATION FOR ANTIBODY THERAPY

Many expression systems are currently used to produce therapeutic antibodies, including animal cell culture and plants. Controlling glycan processing is a major hurdle to be overcome because nonhuman systems may secrete Igs with antigenic glycan structures or sugars, which give rise to suboptimal effector functions. The novel technologies discussed in this

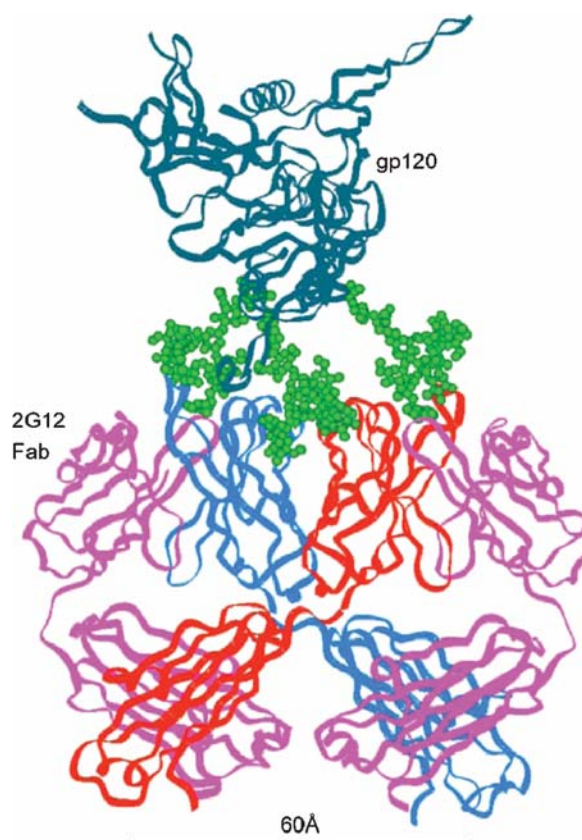


Figure 8

Model of the domain-swapped Fabs of the IgG 2G12 antibody complexed with the oligomannose cluster (*green*) on the silent face of HIV gp120 (*turquoise*). Model adapted from Reference 11. The IgG LCs are purple, and the γ -chains are blue and red. The VH regions are exchanged in this unique domain-swapped structure and form an extended paratope that can accommodate the D1 arms of three oligomannose sugars.

section have been designed to produce fully humanized therapeutic antibodies.

Humanized mammalian cell culture systems. Culture conditions significantly influence the glycosylation and hence the activity of monoclonal antibodies (127). The humanized anti-CD52 (CAMPATH-1H) used in the treatment of lymphoma, leukemia, and RA has a different glycosylation pattern and altered biological activity (measured by ADCC), depending on whether it is expressed in CHO, Y0 myeloma, or NS0 myeloma cell

lines (128). Mouse- and hamster-derived cell lines used for the production of monoclonal antibodies process oligosaccharides similar to those on human serum IgG, but they often secrete predominantly fucosylated IgG-G0 glycoforms that, when clustered, have the potential to activate complement through MBL binding (6). Mouse- and hamster-derived cell lines do not express $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GNTase III), a GTase that catalyzes the addition of a bisecting GlcNAc to bi-, tri-, and tetra-antennary structures. This monosaccharide residue increases ADCC activity (127, 128); therefore, CHO cells producing an antineuroblastoma chimeric IgG (chCE7) were engineered to express GNTase III. The resulting antibody killed neuroblastoma cells at 10- to 20-fold lower concentrations. The presence of the bisecting GlcNAc on the Fc glycans alters the surface exposure of the structural epitope for the Fc γ R on the killer cells, increasing the avidity of the interaction.

Customized glycan processing achieved in *Pichia pastoris*. The yeast glycosylation pathway was modified by the introduction of several human glycosylation enzymes, including mannosidase I and II, N-acetylglucosaminyl transferases I and II, and the uridine 5'-diphosphate (UDP)-N-acetylglucosaminyl transporter. These were successfully localized in the yeast Golgi to generate a synthetic glycosylation pathway that generated glycoproteins with homogeneous N-glycan pools containing the human oligosaccharide GlcNAc₂Man₃GlcNAc₂ instead of the oligomannose series (GlcNAc₂Man₁₀₋₁₅), which is naturally present on the K3 strain of *Pichia Pastoris* glycoproteins (129). This approach represents a significant step forward in the production of therapeutic antibodies with "humanized" glycan structures.

Anti-CD3 monoclonal antibodies are immunosuppressants that can reverse transplantation rejection episodes. The use of these

therapeutics are limited because they are immunogenic and have a propensity to promote T cell activation and cytokine release by cross-linking the T cell receptor (TCR) complex following FcR binding. A humanized rat anti-CD3 IgG was made in which the Asn-297 N-linked glycosylation site was deleted, generating an aglycosylated, nonimmunogenic antibody that did not elicit any significant cytokine release (130, 131). The IgG was unable to bind and activate Fc γ R because these interactions require the structural epitopes on the Fc to be maintained by the Asn-297 glycans (26).

Plantibodies are a source of "green" humanized glycotherapeutics. Plantibodies are antibodies expressed in bioengineered plants that offer an important source of therapeutics to combat animal and human diseases. Even complexes with many subunits, such as sIgA, can be correctly assembled (132); however, differences between mammalian and plant glycosylation systems are a major concern. Plant glycans often contain α 1,3-linked fucose and β 1,2-linked xylose, both unknown in mammalian systems, that can be immunogenic in humans (133). To avoid this problem, a neutralizing monoclonal anti-rabies virus antibody was grown in transgenic tobacco plants engineered with a KDEL (Lys-Asp-Glu-Leu) ER retention signal. This motif prevented most of the antibody from trafficking into the Golgi, avoiding the attachment of the potentially antigenic α 1,3-linked fucose to the glycans (126, 134). As expected, the N-linked oligosaccharides at Asn-297 were predominantly of the oligomannose type, and this antibody was as effective at neutralizing rabies virus as when produced in mammalian cells (134).

Advances in humanizing glycosylation pathways in other organisms means that bioengineering cells and plants to produce therapeutic antibodies with human glycosylation is now feasible (135). A full appreciation of the biochemical properties of the oligosaccharides attached to the Igs is

needed to enable the design of improved nonantigenic therapeutic antibodies, with higher expression levels, activities, and serum half-lives.

CONCLUSION

In this review, we have brought together both well-established and more recently explored aspects of Ig glycosylation to demonstrate the importance of the sugars for the biological functions of antibodies. First, the glycans provide recognition epitopes and maintain the structure and stability of the Igs, enabling them to select and modulate important Fc effector functions such as IgE high- and low-affinity receptor binding and ADCC. Second, specific terminal glycans interact with lectins of the innate immune system, providing a mechanism for cross-talk between the innate and adaptive arms of immunity. Third, glycosylation processing pathways dictate the processing of numerous glycan epi-

topes that endow the Igs with an expanded repertoire of physiologically important functions. In particular, the heterogeneity of the N- and O-glycans protect the host by displaying decoys for bacterial, parasitic, and viral pathogens, adding to the diversity of the antibody molecule. Fourth, the distribution of the glycoform populations is well controlled and, in a given state, is not random but reproducible. Alterations in glycosylation can therefore be used to diagnose and monitor diseases such as RA and IgA nephropathy. Fifth, anticarbohydrate antibodies are at the leading edge of a new generation of therapeutics, especially for use in targeting pathogens such as bacteria, parasites, or viruses that display clusters of nonhuman glycans on their surfaces. Sixth, advances in modifying glycosylation pathways in plants, yeast, insect, and animal cells provide a means of expressing large quantities of nonantigenic therapeutic antibodies with optimized effector function activity.

SUMMARY POINTS

1. Detailed, quantitative glycan analysis has demonstrated that each Ig has a distinctive set of glycan structures that is nonrandom and reproducible in a given physiological state (**Table 1**).
2. The combination of oligosaccharide sequencing data, monosaccharide linkage information from the Oxford Glycobiology Institute structural database, and 3D protein structural information available in "The Protein Data Bank" (136) has allowed the construction of molecular models of the complete family of glycosylated Igs to visualize these molecules as glycoproteins (**Figures 2–5** and **Supplemental Figures 1–3**; to view the Supplemental Figures, follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org>).
3. The oligosaccharides contribute to the maintenance of both the quaternary structure and the stability of the Igs, allowing optimal binding of protein epitopes to receptors that initiate effector functions following antigen binding.
4. The oligosaccharides provide important recognition epitopes that participate directly in binding events with serum and cell surface lectins to elicit further responses to infection by pathogens.
5. Aberrant glycosylation of Igs is associated with the pathogenesis of diseases such as RA, CDGs, IgA nephropathy, and FL. The levels of the serum IgG-G0 glycoform provide a diagnostic marker for RA that reflects disease activity and progression (**Figure 7**).

6. Anticarbhydrate antibodies are providing powerful therapies directed against bacterial, parasite, viral, and fungal infections as well as against tumors. In an HIV patient, the generation of a unique domain-swapped antibody, IgG 2G12, that recognizes an unusual cluster of oligomannose glycans on the surface envelope glycoprotein gp120 signals a novel approach to antiviral therapy and antibody design (**Figure 8**).
7. The “humanization” of therapeutic antibody glycosylation using bioengineered cell culture systems and plants provides a technological breakthrough for the generation of nonantigenic therapeutic antibodies with optimized effector functions.

FUTURE ISSUES

1. Diagnostics and biomarkers: Further development is needed of high-throughput, quantitative, detailed glycan analysis of aberrant Ig glycosylation to identify and monitor disease biomarkers and to give insights into pathogenesis.
2. Vaccine design: Structural analysis of the binding sites of high-affinity anticarbhydrate antibodies complexed with their ligands would facilitate rational design of vaccines that will raise antibodies targeted to glycan epitopes in microorganisms and in cancer.
3. Therapeutic antibodies: Continued development of antibodies with fully “humanized” glycosylation in plants and yeast systems could further the production of cheap, nonimmunogenic therapeutic antibodies.
4. Oligosaccharide libraries on chips: These libraries can be used to screen for antibodies (e.g., in serum) that bind to aberrant glycans in diseases such as cancer.

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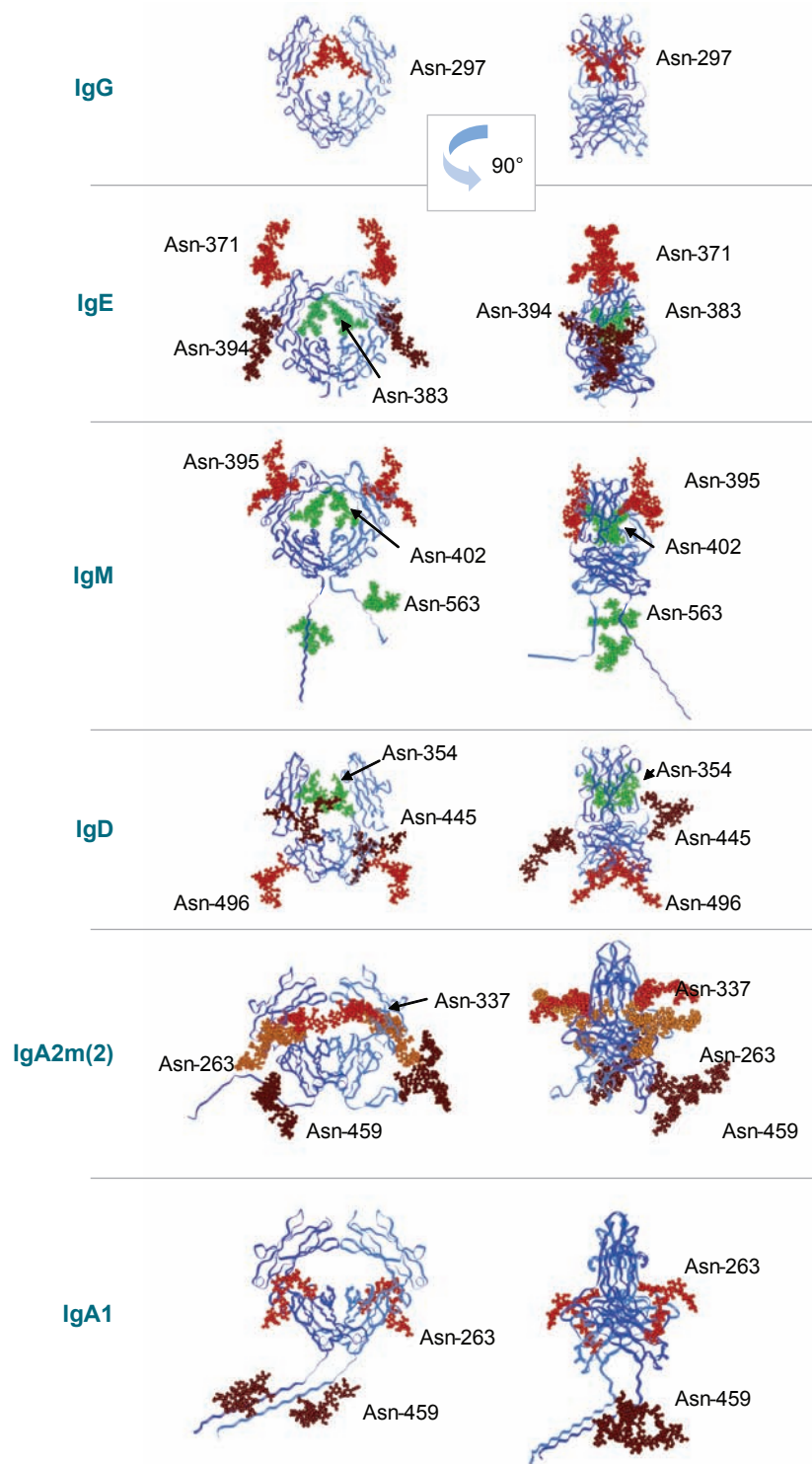
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Dwek RA, Edge CJ, Harvey DJ, Wormald MR, Parekh RB. 1993. Analysis of glycoprotein-associated oligosaccharides. *Annu. Rev. Biochem.* 62:65–100

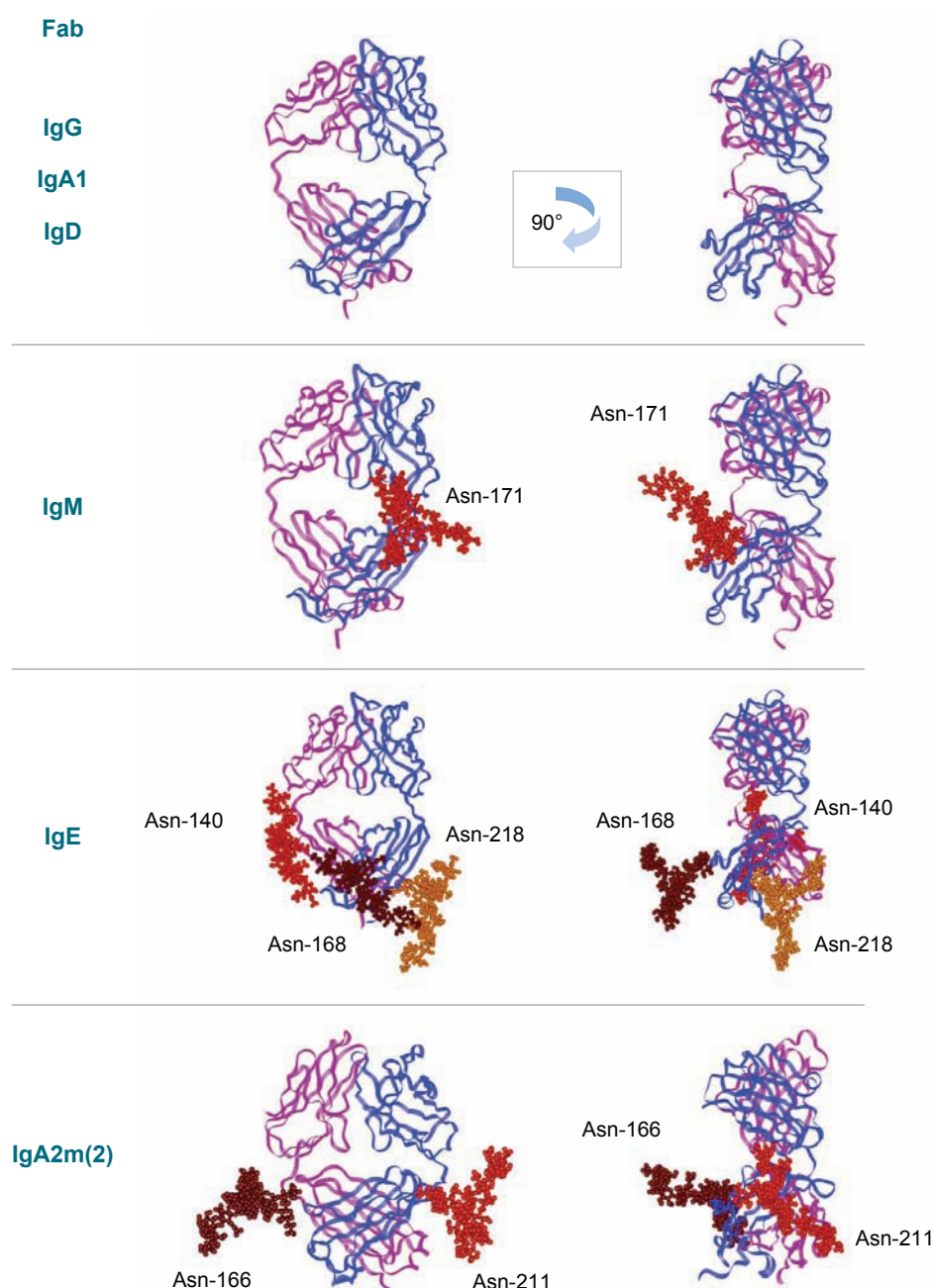
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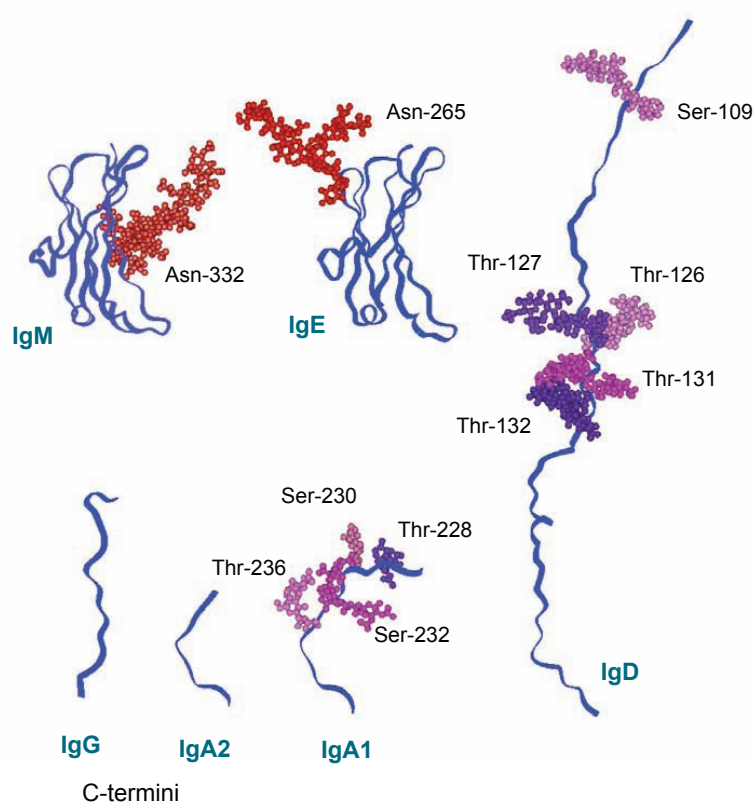
Currently the Oxford Glycobiology Institute is developing a publicly accessible database (GlycoBase) for computer-assisted quantitative glycan analysis based on NP HPLC (L. Royle, C.M. Radcliffe, M.P. Campbell, R.A. Dwek, and P.M. Rudd).



Supplemental Figure 1 Models of the glycosylated Ig Fc regions. The complex glycans are red, orange, and dark red, and the oligomannose glycans are green. The Asn residues to which they are linked are numbered. V and C 1 domains are blue, and variable light (VL) and constant light (CL) domains are purple. The IgG, IgA, and IgD Fcs were constructed from the IgG Fc crystal structure (4). IgE and IgM Fcs were constructed from the crystal structure of IgE Fc and hinge domain (138). The disulphide bridging was modeled according to the amino acid sequence of each Ig.



Supplemental Figure 2 Models of the glycosylated Ig Fab regions. The antigen-binding regions are orientated to the top of the figure. The Asn residues to which the glycans are linked are numbered. The Fabs were constructed from the IgG Fab crystal structure (137) by amino acid substitution. The disulphide bridging was modeled according to the amino acid sequence of the Ig (see Figure 1 for color code).



Supplemental Figure 3 Models of the glycosylated Ig hinge regions. The complex N-linked glycans are red and the O-linked glycans are dark purple, purple, and pink. The N-termini are orientated to the top of the figure. The Asn residues to which the glycans are linked are numbered. The hinge of IgM is shown fully extended (~16 nm) for clarity; however, it is generally more compact (7-9 nm) (56). The models of the hinge regions of IgE and IgM were based on the crystal structure of the IgE hinge domain (138), and that of IgA1 was based on Reference 38.