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GLYCOPINION

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The immune response is a major defence system of higher animals. It can specifically recognize and destroy any foreign substance (antigen) present in the body – for example, a pathogenic micro-organism that gains access to the blood stream. The recognition is accomplished by antibodies or immunoglobulins. An antigen stimulates small lymphocytes in the blood stream (B cells) to differentiate and replicate into an expanded population of plasma cells, which produce antibodies that recognize a specific stimulating antigen. Antibodies bound to antigen activate effector systems such as the complement cascade (a series of protein reactions that is triggered to cause lysis of cells to which the antibody is bound) or macrophages (cells that phagocytose antigen/antibody complexes). Antibodies thus label antigens as targets for destruction by the effector systems of the immune response. Hence, the antibody molecule has two functions: the first is to combine specifically with the potentially unlimited range of antigenic structures; the second is to activate the effector systems. Some of the effector functions of IgG are eliminated if the Fc sugars are absent.

An integral feature of all normal IgG class antibodies is the conserved *N*-glycosylation site in the C_H2 domain at Asn-297. The conformation of the oligosaccharides determined by x-ray crystallography is well resolved in rabbit Fc, and some general conclusions can be drawn about the interactions of the sugars with the domains (Fig. 1). Part of the carbohydrate interacts with the domain surface while the remainder extends into the interstitial space. The α(1-6) antenna of each sugar chain interacts with Phe-243 (Man5 and Glc NAc6), Pro-244 (Gal7) and Thr-260 (GlcNAc6 and Gal7) in the domain surface. These constraints result in loss of flexibility associated with 1-6 linkages which are observed in the free sugar. The terminal GlcNAc residue of the α(1-3) arm of one of the oligosaccharide chains interacts with the core (the Manα(1-4)GlcNAc segment) of the opposing oligosaccharide. Because of this steric constraint, this α(1-3) arm is devoid of a galactose residue and terminates with the GlcNAc. The α(1-3) arm of the opposing oligosaccharide extends outwards between the domains with no apparent steric constraints on its length. The Fc is therefore a model for both specific protein – carbohydrate and carbohydrate–carbohydrate interactions.

Given the conservation of the polypeptide structure of the immunoglobulin molecule, and that Fc *N*-glycosylation has been conserved throughout evolution, it might be expected that the Fc oligosaccharides would show limited structual diversity. The large number of glycoforms associated with the Fc presumably results from the concentrations of the processing enzymes involved in biosynthesis, and also any restrictions by which the protein limits their accessibility. This may lead to an asymmetric distribution of oligosaccharides in the intact IgG, since each heavy chain may have associated with it a different set of oligosaccharides. The restricted interstitial space may accommodate only some of the possible combinations of glycosylated heavy chains.

The primary monosaccharide sequences of the oligosaccharides contain important information about the activity of particular glycosyltransferases in plasma cells and also any structural limitations imposed by the immunoglobulin molecule. A comparison of the *N*-glycosylation pattern of serum IgG isolated from normal individuals and patients with rheumatoid arthritis has shown that a decrease in outer arm galactosylation correlates with the disease activity. The galactosylation marker is therefore a convenient biochemical parameter with which to monitor the disease, and has focused attention on the need to understand the significance of

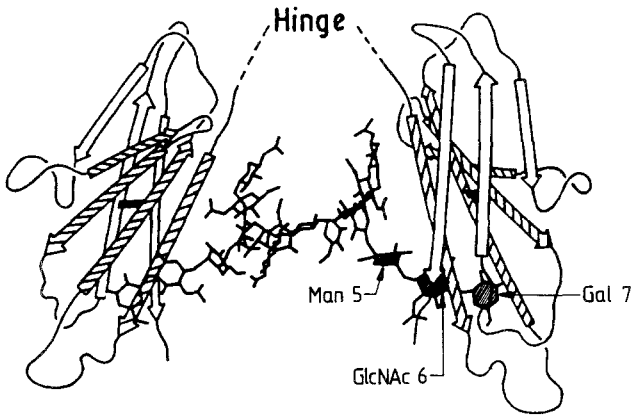

Figure 1. Rabbit immunoglobulin Fc structure highlighting the roles of Man5, GlcNAc6 and Gal7.

glycosylation in the immunoglobulin molecule. Indeed, the second Jenner International Glycoimmunology meeting held last November highlighted the importance of oligosaccharides to immune mechanisms and the potential for the development of novel therapeutic strategies. Although much effort is being directed towards showing whether the change in galactosylation is primary or secondary to the cause of the disease, the results are not yet conclusive.

The article by Roy Jefferis sets into context some aspects of the antibody molecule and highlights some of the difficulties, in terms of clones, classes, subclasses, and multiple glycosylation sites in the molecules, that will have to be understood before a comprehensive picture of the antibody molecule and a role for glycosylation can be realized.

The glycosylation of antibody molecules: functional significance

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Introduction

In humans, five classes of antibody molecules have been defined and the nomenclature IgA, IgD, IgE, IgG and IgM universally adopted. Antibody structure is based on a four polypeptide chain basic unit comprised of two light (25 kDa) and two heavy (50–70 kDa) chains. The fundamental 'building block' is 110 amino acid residues that form a compact globular domain through the immunoglobulin fold; a characteristic structure for products of the immunoglobulin supergene family [1]. The N-terminal domains of both heavy (VH) and light (VL) chains are variable in sequence, and determine antigen binding specificity. Heavy chains have three or four constant region domains. The constant regions of the heavy chains determine the profile of effector functions that the antibody class can activate when aggregated as an antigen–antibody complex. The location of glycosylation sites is a conserved characteristic for constant region domains of a given antibody class or subclass, but significant differences between variable domains can be anticipated. This mini-review presents thoughts and conclusions drawn from the author's studies of human IgG. It does not provide an extensive bibliography but direct readers to a series of comprehensive reviews of antibody structure [2–5] and glycosylation [6–9].

Variable regions

Although antibody molecules bearing carbohydrate within variable domains have been reported, the position of attachment and structure of the oligosaccharide moieties has not been the subject of systematic study. Germline VH gene segments have been defined that encode glycosylation sequons, but glycosylated products of these gene segments have not been identified. Analysis of the germline VH gene sequences reported in Tomlinson *et al.* [10] reveals that 5 of 83 germline VH gene segments encode for an N-site; the frequencies being 0/22, 0/5, 2/29, 1/20, 2/6 and 0/1 for VH1, VH2, VH3, VH4, VH5 and VH6 family gene segments, respectively. It is of interest to note that all VH4 genes encode an -N-P-S- sequence; however, it has been reported that the presence of proline at this position is not permissive of glycosylation, and hence the sequence is not a glycosylation sequon. Currently, no protein has been reported in which -N-P-S- functions as an acceptor site for glycosylation (A. Dell, personal communication).

Inspection of 37 VH region protein sequences revealed 15 glycosylation sequons, 1 in FR1, 4 in CDR2 and 10 at a common site in FR3 of VHIII subclass proteins. The heavy chain variable regions having potential N-sites are not products of germline genes that encode the glycosylation sequon. It appears, therefore, that glycosylation sequons in expressed proteins arise as a result of somatic mutation and positive selection by antigen, while protein sequences expressing germline encoded glycosylation sequons may be selected against. This analysis did not include D and J regions, so the proportion of VH sequences having glycosylation sequons will be higher than indicated. Similar analysis of VL gene and protein sequences is certain to reveal additional potential N-sites and, hence, the proportion of Ig molecules glycosylated in the variable regions may be substantial, suggesting a significant functional role. An early estimate of the proportion of IgG molecules bearing carbohydrate in the Fab regions gave a value of 30% [8].

Constant domains

In man, mouse and rat, 4 subclasses of IgG are defined (IgG1, IgG2, IgG3, IgG4; IgG1, IgG2a, IgG2b, IgG3 and IgG1, IgG2a, IgG2b, IgG2c, respectively) that differ in the primary sequence of the constant regions of the heavy chains, and are products of distinct genes. Each subclass has a unique profile of Fc mediated biological activities [11, 12]. By contrast, the rabbit has a single gene encoding for IgG and, consequently, does not have subclasses. x-Ray crystallography of a human IgG1 subclass Fc fragment [13] and a rabbit Fc fragment [14] reveals that the oligosaccharide moiety is integral to the protein structure and forms multiple hydrogen bonds and other non-covalent contact with amino acid side chains exposed on the Fx face of the C_H2 domains. Throughout the remainder of the IgG molecule the Fx faces of homologous domains pair through hydrophobic interactions. The oligosaccharide interaction with the Fx face of the C_H2 domain protects hydrophobic side chains from exposure to the aqueous environment. The oligosaccharide is of the complex biantennary type, and may be viewed as having a core structure with variable additional sugar residues attached. There are significant differences in the 'non-core' residues between species. Mouse IgG does not bear the bisecting *N*-acetylglucosamine residue; however, a proportion of molecules bear the Gal 1-3 Gal structure that is never present in human IgG. In fact, higher primates and man lack the transferase for generating this structure, and high levels of anti-Gal 1-3 Gal antibodies have been reported for all normal individuals [15]. However, chimeric mouse/human IgG produced in mouse J558L cells can express >20% of molecules bearing the Gal 1-3 Gal structure [16]. These findings have an obvious significance for the production of recombinant proteins intended for application in humans, since glycosylation differences may affect function, pharmacokinetics and antigenicity.

Glycoforms of human IgG produced *in vivo* and *in vitro*

Analysis of normal polyclonal human IgG (Sandoglobulin) reveals that approximately 25% of oligosaccharide moieties are monosialylated, with a negligible proportion being disialylated (N. Takahashi, personal communication). It should be noted that this immunolglobulin preparation is produced by a multi-step isolation procedure which may result in loss of sialic acid residues; significant levels of disialylated IgG have been observed within other IgG preparations (R. A. Dwek, personal communication). Following removal of sialic acid, analysis of the neutral oligosaccharides allows the structures shown in Fig. 2 to be identified and quantified [17]. Structures E, F, G and H predominate, and account for 65% of oligosaccharide moieties. It is of interest to note that structure F predominates over G, suggesting preferred galactosylation on the Man(1-6) arm over the Man(1-3) arm. Analysis of myeloma proteins shows that each has an essentially unique glycosylation profile which it is assumed reflects the unique properties of the clones producing them [17]. This suggests that the profile observed for polyclonal IgG is the sum of all the unique clones contributing to IgG production. One of the parameters influencing glycosylation will be the polypeptide chain itself which may act as a template directing or being permissive of glycosylation pathways. Data suggesting template direction was obtained from analysis of a panel of human IgG paraproteins comprising each of the four subclasses. In all IgG2 proteins structure G predominated over F, demonstrating an altered preference within the galactosylation pathway. Interestingly,

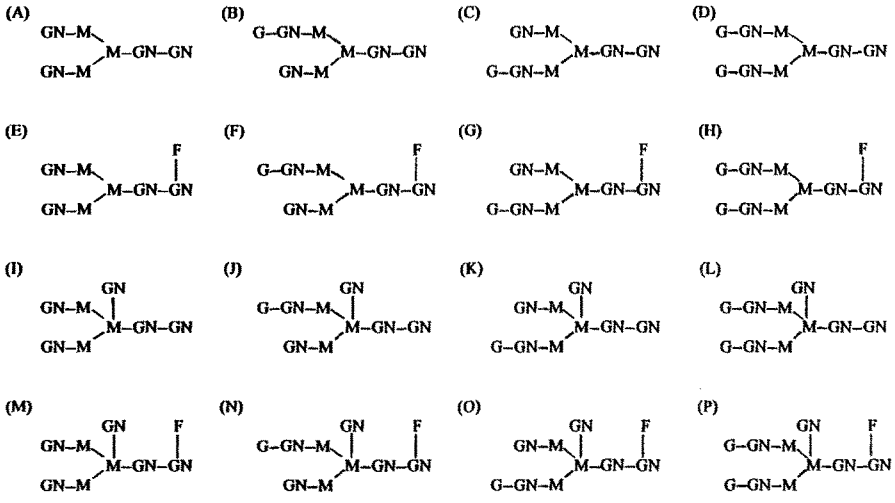

Figure 2. Profile of neutral oligosaccharide structures present in human IgG molecules (G, galactose; M, mannose; F, fucose; GN, *N*-acetylglucosamine).

this preference was maintained for IgG2 produced as a chimeric antibody in mouse J558L cells [16], a cell line derived from a plasmacytoma that was originally producing mouse IgA anti-dextran antibodies.

Human IgG, Fc effector functions

Aglycosylated IgG has been produced by several groups and there is a consensus that Fc receptor, C1q binding, C1 activation and susceptibility to proteolytic degradation are all compromised; however, binding to staphylococcal protein A and reactivity with rheumatoid factors are retained [18–24]. The precise effects of aglycosylation on antibody function may differ with the antibody isotype and the assay systems employed. Thus, Nose and Wigzell [19] reported that aglycosylated mouse IgG2a retained an ability to activate C1, but higher levels of sensitization were required than for the glycosylated form. Dorai *et al.* [23] reported a similar finding for an aglycosylated mouse/human IgG1 antitumour antibody. For mouse IgG2b, Duncan and Winter [20] reported a threefold lowering of the association constant but a complete loss of C1 activation, while Tao and Morrison [24] reported a similar loss of C1 activation for chimeric mouse/human IgGl. Our studies emphasize the need to combine binding studies with biological assays. Thus, while haptenized erythrocytes sensitized with aglycosylated chimeric human IgG3 antibodies failed to rosette with Fcγ RI expressing U937 cells [21], they both rosetted and were able to trigger superoxide release by U937 cells stimulated with interferon. However, in order to obtain a 50% maximal response, sensitization levels 70% higher than for glycosylated IgG were required. The effects were more apparent at lower levels of sensitization, and the threshold response was obtained with 60 and 16000 molecules/erythrocyte for glycosylated and aglycosylated IgG3, respectively [22].

Aglycosylation resulted in abrogation of Fcγ RII recognition, as revealed by rosette formation between sensitized erythrocytes and the Fcγ RII expressing cell lines Daudi and K562 [21]. Rosette formation through Fcγ RIII expressed on K cells was reduced by 60%; however, antibody dependent cellular cytotoxicity (ADCC) was completely abrogated [25]. Thus, the biological consequences of aglycosylation are profound, and require a mechanistic explanation. The two most obvious possibilities are (i) in the absence of the carbohydrate moiety the tertiary and quaternary structure of the protein moiety is compromised, and (ii) the carbohydrate moiety is an integral component of the ligand binding site. Since the Fc receptor [26, 27] and C1q [20] binding sites have been 'mapped' to different topographical areas of the C_H2 domain, explanation (i) may appear the more likely. No differences in epitope expression between glycosylated and aglycosylated IgG,Fc could be distinguished with a panel of twenty mouse monoclonal antibodies to human Fc [21]. This suggests that there are no gross conformational differences between the two forms. This was confirmed by high field NMR studies using histidine residues as reporter groups for conformational differences between the two forms. The environment of His-268 was the only evident difference [28]. This residue is on a beta bend at the N-proximal end of the C_H2 domain in the vicinity of the lower hinge region which has been proposed to be directly involved in Fcγ R recognition [27].

Agalactosylated IgG

It has been established that the proportion of human IgG molecules lacking galactose (Go, IgG) is increased in patients with rheumatoid arthritis and certain other chronic inflammatory diseases [8, 29]. It has been demonstrated further that increased Go levels reflect a tissue specific (B cell) lowered galactosyltransferase activity [30]. It has been suggested, therefore, that Go, IgG may have a role in the pathogenesis of diseases in which its level is increased [29]. Superficially this may seem unlikely since the difference in Go, IgG levels between normals and patients is small. However, IgG antibody responses can be subclass and clonally restricted [12], consequently glycosylation profiles will also be restricted; as for IgG myeloma proteins [17]. If, therefore, receptor–ligand interactions are modulated by glycosylation differences, the protective effector mechanisms activated by an oligoclonal specific antibody population could be dependent on the predominant glycoforms present.

Lowered C1q and Fcγ RI binding activity has been reported for agalactosylated IgG [31], while SpA and rheumatoid factor binding was unaffected [31, 32]. We have investigated Fcγ RI binding and activation with an IgG4,Fc fragment that in its isolated form is comprised of >90% Go,IgG and which was fully galactosylated using bovine milk galactosyltransferase. We could not demonstrate any difference in the ability of these proteins to inhibit binding or activation through FcγI. Similarly, we could not demonstrate differences in recognition by monoclonal IgM and IgA rheumatoid factors.

Many studies have employed mouse/human chimeric antibodies produced in different rodent cell lines that do not attach bisecting *N*-acetylglucosamine sugar residues, suggesting that it does not contribute significantly to the activities investigated. Similarly, human IgG,Fc molecules lacking fucose have exhibited undiminished Fcγ RI inhibitory activities. Thus, the precise biological significance of glycosylation heterogeneity at the Asn-297 glycosylation sequon of IgG remains to be established. However, it is apparent that a full understanding will require a deep yet subtle appreciation of glycoprotein structure–function relations and *in vivo* biology, in health and disease.

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Letters or comments relating to this article would be received with interest by Pauline Rudd, Assistant to the Special Advisory Editor, R. A. Dwek.