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Review

Carbohydrate moiety of immunoglobulins in health and pathology

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Most of glycoproteins described so far, including immunoglobulins, are glycosylated during post-translational modifications of protein molecules. Current knowledge of the structure of sugar chains in immunoglobulin molecules and their biological role in health and pathology is reviewed.

CARBOHYDRATE CONTENT IN IMMUNOGLOBULINS

Immunoglobulins are a group of glycoproteins present in the serum and tissue fluids of all mammals. They play a role of antibodies, i.e. they are formed as a response of the organism against an antigen. A molecule of immunoglobulin consists of two heavy chains (H-chains) and two light chains (L-chains), which are interconnected in pairs by disulfide and hydrogen bonds. Five distinct classes of immunoglobulins are recognized: IgG, IgM, IgA, IgD and IgE; they differ in amino-acid sequence, size, charge and carbohydrate content [1]. According to Turner [2] the average contents of carbohydrates in human immunoglobulins are as follows:

IgG	2-3%
IgM	12%
IgA	7-11%
IgD	9-14%
IgE	12%

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Abbreviations: CDR, complementarity determining region; TACA, tumor-associated carbohydrate antigen; Ig, immunoglobulin; MBP, mannose binding protein; RA, rheumatoid arthritis; ROS, reactive oxygen species; ADCC, antibody dependent cellular cytotoxicity; CDG, carbohydrate deficient glycoprotein.

These data may vary to some extent due to the native microheterogeneity of glycoprotein glycosylation, to species differences and due to the fact that in some pathological cases the carbohydrate moiety of glycoproteins is affected, e.g. in cancer.

TOPOGRAPHY AND STRUCTURE OF CARBOHYDRATE CHAINS IN IMMUNOGLOBULINS

Immunoglobulin G

Glycosylation is site-specific in Ig molecules. This problem has been most extensively studied in immunoglobulins G, which constitute over 83% of total immunoglobulins in human serum. Figure 1 presents a schematic structure of an IgG molecule. Due to intrachain -S-S- bridges there are several domains present both in the constant (C) and variable (V) regions of the molecule. Immunoglobulin G contains on average 2.4 N-glycans per molecule [3], two of which are conserved at Asn-297 in the C_H2 domain of the Fc region of each heavy chain. The additional oligosaccharides are located randomly in the variable region of the Fab fragment and this glycosylation may be asymmetrical due to the insertion of an oligosaccharide into one of the two Fab arms. This has been shown to cause a functional univalence of non-precipitating antibodies [4, 5]. The best known is N-glycosylation at the conserved site (Asn-297) in the C_H2 domain of the Fc region of each heavy chain in human IgG molecule, with the sugar chain schematically presented in Fig. 2. This is a biantennary, complex-type oligosaccharide with a bisecting GlcNAc residue; it contains also fucose, bound to the innermost GlcNAc. Sialic acid, present in only about 10% of Fc-bound chains, is linked (2-6) to galactose. In most oligosaccharides galactoses are the non-reducing terminals, always accompanied by bisecting GlcNAc and Fuc.

Interestingly, oligosaccharides associated with Fab and Fc regions of IgG are different. Those attached to the Fc fragment of the immunoglobulin molecule are complex-type biantennary chains, poorly sialylated, while those bound in the Fab region are of the more highly sialylated complex-type or are the high-mannose type. In human IgG myeloma proteins oligosaccharides were found in the Fab fragment in 22 out of 76 different samples (29%) [6]. Recently, a computer search revealed that 141 out of 808 amino-acid sequences (17%) of murine IgG V_H regions contained ..Asn-X-Ser/Thr.. , a tripeptide acceptor sequence for N-glycosylation [7]. Oligosaccharides present in variable domains of the Fab fragment of antibodies influence antigen binding; this phenomenon is described below.

Immunoglobulins G secreted by murine hybridomas also have conserved N-glycosylation sites at Asn-297 of heavy chains. These oligosaccharides are similar to those found in human serum IgG (shown in Fig. 2), with the exception that they lack the bisecting GlcNAc. The predominant structure is also devoid of galactose in the branch 1-3 linked to β-mannose [8]. In a mouse monoclonal OKT3 antibody, which belongs to the IgG2a isotype, this predominant structure was accompanied by eight additional oligosaccharide structures, among them two triantennary oligosaccharides with four sialic acid residues [9]. It has also been revealed that different conditions of hybridoma culture may significantly influence IgG glycosylation, for example the use of serum-free media yields the structures containing more sialic acid [10]. Immunoglobulins G from rabbit [11] and bovine [12] serum have N-glycans similar to human IgG. The presence of GalNAcβ1-4GlcNAc sequence is an interesting feature of the bovine species. O-glycosylation in IgG is rare; recently this type of sugar-protein linkage was found in the hinge region of mouse immunoglobulin G2b [13].

Immunoglobulin M

In the IgM monomer there are five conserved sites of N-glycosylation in each heavy chain constant regions: four of them are the same for human and mouse, i.e. Asn-171 (complex-type), Asn-332 (complex-type), Asn-402 (high-mannose in human, complex-type in mouse) and Asn-563 (high-mannose or complex in human, high-mannose in mouse). The fifth N-glycosylation site is different: Asn-395 in human and Asn-364 in mouse, in both species occupied by a complex-type chain [14, 15]. Indications of the presence of O-linked glycans in immunoglobulins M are rather rare; among these exceptions is a mouse monoclonal IgM antibody, directed against blood-group B antigen [16]. The structure of N-glycans in this antibody include bi- and triantennery chains, partially fucosylated, having two different linkages of sialic acid, (2-3) and (2-6), in one oligosaccharide.

Immunoglobulin A

In IgA1 from human serum two conserved N-glycosylation sites are present in each heavy chain at Asn-263 and Asn-459. Over 90% of these chains are sialylated. Besides, IgA1 contains a proline-rich hinge region. Within this sequence there are nine potential O-glycosylation sites; the exact number of O-glycans is not known, but it is well established that they are predominant carbohydrate structures in this type of immunoglobulin molecules [17]. In a mouse monoclonal IgA antibody, specific for human blood group A determinant [18], nine different N-glycans were shown; two of them contained Galα1-3Gal- non-reducing sequence, which is a rather rare feature in this type of proteins. No O-glycans in this antibody were detected.

Immunoglobulins D and E

The carbohydrate moiety of these two immunoglobulin classes has been less extensively investigated. Immunoglobulins D possess, similarly to human serum IgA1, several O-glycans in the hinge region and three N-glycosylation sites in the Fc portion of the molecule [2]. Human IgE has no hinge region; it has three N-glycosylation sites in the Fc fragment and several glycosylation sites in the Fab fragment [2]. In a report on rat monoclonal IgE the presence of high-mannose and complex-type chains was evidenced, the latter structures as bi- and triantennary oligosaccharides with sialic acid bound to galactose both by (2-3) and (2-6) linkages in one oligosaccharide [19].

THE SIGNIFICANCE OF CARBOHYDRATES PRESENT IN IMMUNOGLOBULIN MOLECULES

Sugar chains, present in immunoglobulins, influence *physicochemical* properties of these molecules [20] in the same manner as in any other type of glycoprotein: 1 – increase solubility in polar solvents (e.g. water), 2 – increase thermal resistance, 3 – protect against protease digestion, 4 – are responsible, to some extent, for the conformation of immunoglobulin molecules, which affects, for example, secretion of immunoglobulins from the cells. The role of glycosylation in Ig secretion has been extensively studied with tunicamycin (an inhibitor of N-glycosylation). The results show that secretion of aglycosylated species by mouse plasmacytomas was totally abolished in the case of IgE, whereas secretion of IgG and IgD was largely unaffected. IgM and IgA secretion was reduced to a moderate extent [21]. This different behaviour suggests that the key function of carbohydrates in immunoglobulin secretion is to maintain a proper conformation of the molecule, 5 – there is some debate about the role of the carbohydrate moiety of immunoglobulins in the clearance of antibodies from circulation, but currently it is assumed that sugars in immunoglobulins may have only a limited effect on this property [22].

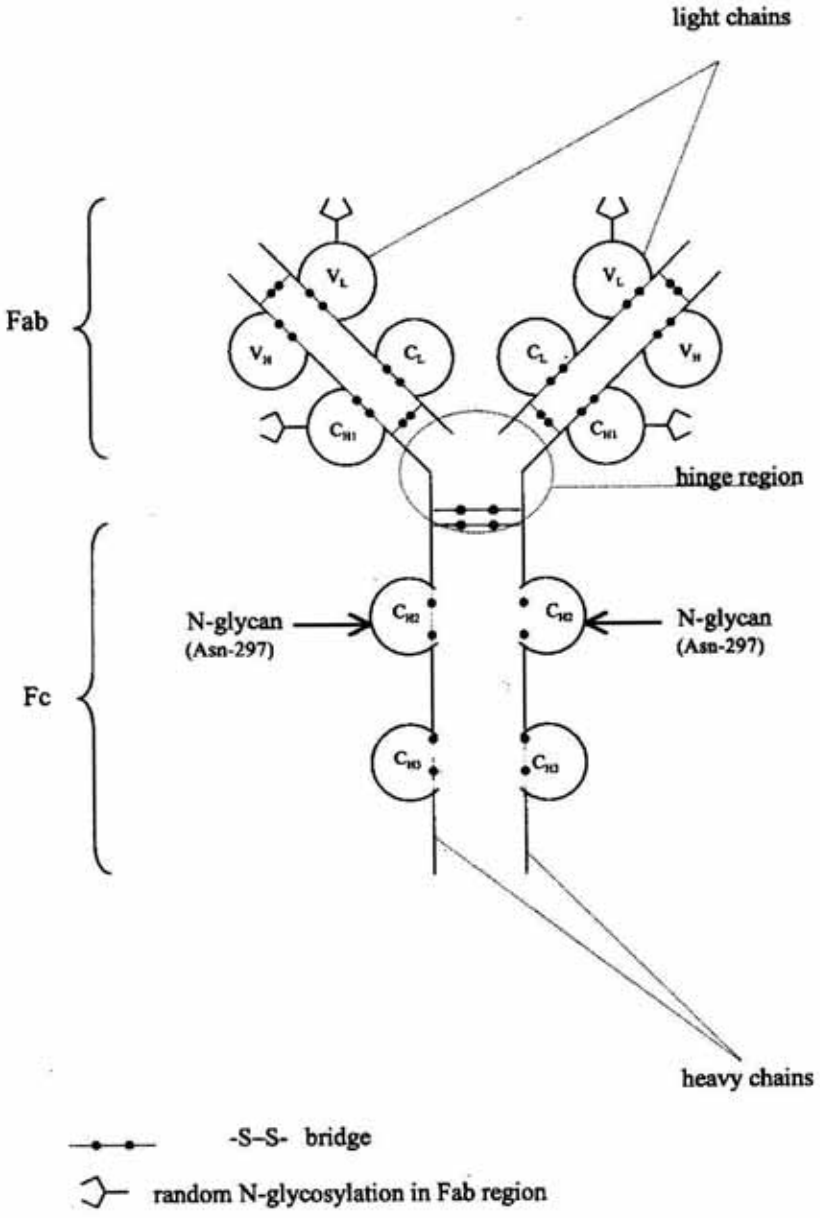

Figure 1. Schematic structure of IgG molecule.

Carbohydrates influence also *effector* properties of immunoglobulins [23]. As it was mentioned above, all heavy chains of IgGs contain N-glycans attached at the conserved position in the C_H2 domain (Asn-297). These oligosaccharides contribute to several biological activities of immunoglobulins G: 1 – influence the complement activation *via* the classical pathway, which starts with the binding of C1q component of the complement to the C_H2 domain of IgG molecule. Aglycosylated IgGs do not exhibit this property, 2 – contribute to the induction of antibody-dependent cellular cytotoxicity (ADCC), 3 – influence the binding of IgGs to Fc receptors, 4 – contribute to the rapid elimination of antigen-antibody complexes from circulation. However, conserved glycosylation of IgGs at Asn-297 is not required for their binding to *Staphylococcus aureus* protein A [24].

In contrast to Fc-associated carbohydrates, Fab glycosylation influences the basic biologi-

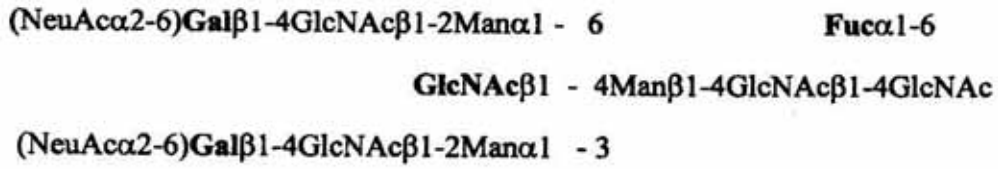

Figure 2. Structure of N-linked glycan, attached to Asn-297 in the C_H2 domain of the Fc region in human IgG molecule.

Only about 10% of these chains are sialylated. In asialo structures four monosugar residues, marked in bold, are responsible for microheterogeneity of this oligosaccharide, giving rise to 16 possible, different structures.

cal property of immunoglobulins, i.e. antigen binding. There is no general rule regarding this effect, since it may be positive (enhancing) or negative (diminishing the binding of antigen); it depends on the structure and specificity of the antibody and structure of the antigen. The following examples should illustrate the problem. A mouse monoclonal antibody (14.6b.1) was generated against α(1-6) dextran [25], it contained N-glycan in its native form at Asn-58 of the V_H region. This position is located in the binding site of the antibody, which is formed by six complementarity determining regions (CDRs): three from the variable region of light chains (V_L) and three from the variable region of heavy chains (V_H). Two other antibodies were also constructed, using site-directed mutagenesis, with amino-acid sequence changed in one and two amino-acid positions, respectively, in their CDRs of heavy chains, which resulted in the absence of this N-glycan. Comparison of these two additional antibodies with the native one demonstrated about 10-fold or even greater reduction in their binding constants for dextran. Further experiments performed by the same group [26] also showed that the presence of the oligosaccharide in the second CDR of V_H of anti-carbohydrate antibody, at Asn-58, increases its affinity for antigen. Three antibodies, derived from the same native one (14.6b.1), were also constructed using site-directed mutagenesis: first – without N-glycan at Asn-58, second – with N-glycan at Asn-60 only (a high mannose-type oligosaccharide) and the third – with N-glycan at Asn-54 only (a complex-type oligosaccharide). Determination of apparent binding constants for dextran showed that the native antibody had the highest affinity for the antigen, its non-glycosylated form was about 10 times less active, the antibody with N-glycan at Asn-60 was about 3 times less active, whereas glycosylation of the last antibody at Asn-54 reduced dextran binding almost totally. The clear conclusion from these experiments was that even small changes in the position and type of N-linked glycan in the CDR2 of anti-dextran antibody may result in substantial reduction of its affinity for antigen.

Somewhat opposite results were obtained for another mouse monoclonal antibody, directed against the CD33 antigen, expressed on early myeloid progenitor cells, some monocytes and the cells of most myeloid leukemias [27]. This antibody was natively N-glycosylated at Asn-73 in the heavy-chain variable region. In the course of experiments a humanized antibody was constructed (it combined the CDRs of the original murine antibody with V and C regions of a human antibody), which was devoid of Asn-73-linked glycan. Surprisingly, the humanized antibody showed an increase in binding affinity to the CD33 antigen. Therefore, it could be concluded that by removing glycosylation of CDR of a given antibody its affinity to antigen increases. The two sets of experiments mentioned above suggest clearly that the reaction of a given antibody with an antigen may de-

pend on the presence of glycans in complementarity determining regions, i.e. the glycans may either increase or decrease this reaction. It is important, therefore, to determine this correlation especially in respect to the antibodies used for diagnostic or therapeutic purposes, because by optimizing the affinity of the antibody to antigen we can minimize the desired amount of the antibody, which means less expensive and more efficient treatment.

THE CARBOHYDRATE MOIETY OF IMMUNOGLOBULINS IN PATHOLOGICAL CASES

The growing amount of knowledge on modifications of the carbohydrate moiety of glycoproteins in pathological cases is referred to as *glycopathology* [28]. The subject becomes most important when sugar structures are shown to be directly involved in the tumor phenotype of cells (tumor-associated carbohydrate antigens, TACAs) [29]; Tn antigen [30] and sialyl-Le^a together with sialyl-Le^x structures [31] are well known examples of these antigens. However, TACAs have not been identified in immunoglobulins so far. Nevertheless, Igs as soluble glycoproteins were and still are the objects of investigation of changes in glycosylation in the diseases discussed below.

Rheumatoid arthritis (RA)

N-glycans, attached to the Fc region of IgG, have been thoroughly investigated in this disease. N-glycans from healthy individual and RA patient, separated by gel filtration, differ in proportions of oligosaccharides designated G(2), G(1) and G(0). These oligosaccharides, corresponding to the structure shown in Fig. 2, have two, one or no galactose residues, respectively. In oligosaccharides derived from RA IgG the most abundant is G(0), i.e. oligosaccharides terminated with GlcNAc residues. Detailed investigations proved that the reduced level of galactosylation of the outer-arm of RA IgG is due to the diminished activity of galactosyl transferase in B cells. Because of clinical significance, N-glycans from IgG of RA patients were investigated using different methods: GlcNAc-specific lectin from *Psathyrella velutina* [32, 33] or various gel filtration procedures [34]. It was shown that in rheumatoid arthritis the IgG glycoform lacking two galactoses (referred to as G(0) glycoform) is markedly increased. Interestingly, it was recently shown that non-substituted, terminal GlcNAc residues in G(0) glycoform bind to a serum lectin – the mannose binding protein (MBP), which activates complement [35]. This protein is active in immune defence. In general, it recognizes oligosaccharides on the surface of invading pathogens by reacting with terminal fucose, glucose, mannose or *N*-acetylglucosamine. In the classical complement cascade the first step of events is the binding of C1q to the C_H2 domain of IgG. Otherwise, complement activation takes place by a second route, which does not involve C1q but MBP. This result indicates that even pathological IgG molecules may play a positive, physiological role in the human organism. Although MBP resembles C1q in structure, the mechanism of complement activation by MBP is less well documented and further investigations on this interesting subject are expected.

Other chronic inflammatory diseases

Inflammation sites are characterized by the presence of a large number of phagocytes, which release a variety of reactive oxygen species (ROS, previously known as *oxygen free radicals*). Griffiths and Lunec [36], studying the effect of oxygen free radicals, generated by activated phagocytic cells, on carbohydrate moiety of IgG found that galactose residues in N-glycans were destroyed. These results suggest that agalactosylation of N-glycans of C_H2 domain in the Fc region of IgG, occurring in

chronic inflammation may be a consequence of certain degradation processes, caused by reactive oxygen species. This is a different reason of poor galactosylation of the C_H2 domain N-glycans than in RA patients, where the activity of galactosyltransferase in B cells is decreased.

Leukemia patients

A detailed analysis has been performed on N-glycans isolated from serum IgG, derived from patients with two different leukemias: chronic lymphocytic leukemia and acute myelocytic leukemia [37]. The oligosaccharides were isolated from IgG preparations by hydrazinolysis, pyridylaminated and separated by HPLC. In comparison to normal serum IgG oligosaccharides, those from lymphocytic leukemia patients showed significant abundance of agalacto-, biantennary structure without bisecting GlcNAc and with Fuc residue bound to the innermost GlcNAc. In acute myelocytic leukemia a similar oligosaccharide lacks fucose. The authors of this report [37] suggest that the analysis of fucose and galactose content in IgG N-glycans may be useful in classification of leukemias.

CDG syndrome

This is a family of multisystemic genetic diseases in which secretory glycoproteins, lysosomal enzymes and most probably also membrane proteins are underglycosylated [38]. Many different serum glycoproteins from CDGS patients were analyzed, among them IgG N-glycans from two patients [39]. It was found that the content of galactose was decreased, while that of mannose and GlcNAc was not affected. Comparing the carbohydrate composition of IgG N-glycans originating from CDGS patients and a healthy subject of similar age it was found that hypogalactosylation is a characteristic feature of IgG N-glycans in CDGS patients. In transferrin of CDGS patients reduced number of N-glycans was reported [40].

Glycoprotein nature of immunoglobulins is being investigated by adjusted old and improved new specific analytical methods; most probably carbohydrate biochemists may detect some new sugar structures, also those existing in very small amounts but of great biological significance [41]. In many pathological cases, especially cancer diseases, there is a growing need to detect, as early as possible, the tumor phenotype of the cells and/or modified carbohydrate component of secretion glycoproteins. This might be a basis for good diagnostic procedures and, as a consequence, a promising clinical treatment.

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