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Structural Study of the Carbohydrate Moieties of Two Human Immunoglobulin Subclasses (IgG2 and IgG4)

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Asparagine-linked sugar chains were quantitatively released as oligosaccharides from human IgG2 and IgG4 myeloma proteins by hydrazinolysis followed by *N*-acetylation and NaB^3H_4 reduction. Each oligosaccharide was isolated by serial lectin column chromatography. Study of their structures by sequential exoglycosidase digestion and methylation analysis, revealed that all of them were of the bi-antennary complex-type containing Manα1-6(±GlcNAcβ1-4)(Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc as core structures, and GlcNAcβ1- , Galβ1-4GlcNAcβ1- and Siaα2-6Galβ1- in their outer chain moieties. However, the molar ratio of each oligosaccharide was different in each IgG sample, indicating that clonal variation is included in the sugar chain moieties of IgG molecules. One of the IgG2 contained four asparagine-linked sugar chains in one molecule, two on the Fc fragment and the remainder on the Fab fragment. The sugar chains in the Fc fragment contained much less galactose as compared with the Fab fragment.

Human immunoglobulin G (IgG) is a glycoprotein which is composed of two types of polypeptide chain, heavy (H) and light (L), with a stoichiometry of H_2L_2, and contains an asparagine-linked sugar chain in the Fc region of each H chain [1-3]. Structures of the desialylated asparagine-linked sugar chains of human serum IgG have been elucidated already as shown in Table 1 [4, 5]. The presence or absence of two galactoses, of bisecting *N*-acetylglucosamine and of fucose residues produced 16 different sugar chains. Human IgG can be divided into four subclasses; IgG1, IgG2, IgG3 and IgG4. In view of the extraordinarily high heterogeneity found in the sugar chain structure of IgG, it is interesting to find out whether these four subclasses contain different sugar chain structures, or not. Mizuochi *et al.* analyzed ten human IgG myeloma proteins and found structural variation in their sugar chains [4]. However, since they analyzed only IgG1 and IgG3, it was not clear whether there

Abbreviations: IgG, immunoglobulin G; AAL, *Aleuria aurantia* lectin; E-PHA, *Phaseolus vulgaris* erythroagglutinin; RCA, *Ricinis communis* agglutinin; Con A, concanavalin A. All sugars mentioned have the D-configuration except for fucose, which has the L-configuration. Subscript OT is used to indicate NaB^3H_4-reduced oligosaccharides.

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Table 1. Structures of desialylated asparagine-linked sugar chains found in human IgG.

Structures	R	
	(Fucα1-6)GlcNAc	GlcNAc
GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4R 3 GlcNAcβ1-2Manα1	1	2
GlcNAcβ1-2Manα1 6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4R 3 GlcNAcβ1-2Manα1	3	4
GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4R 3 Galβ1-4GlcNAcβ1-2Manα1	5	6
GlcNAcβ1-2Manα1 6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4R 3 Galβ1-4GlcNAcβ1-2Manα1	7	8
Galβ1-4GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4R 3 GlcNAcβ1-2Manα1	9	10
Galβ1-4GlcNAcβ1-2Manα1 6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4R 3 GlcNAcβ1-2Manα1	11	12
Galβ1-4GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4R 3 Galβ1-4GlcNAcβ1-2Manα1	13	14
Galβ1-4GlcNAcβ1-2Manα1 6 GlcNAcβ1-4Manβ1-4GlcNAcβ1-4R 3 Galβ1-4GlcNAcβ1-2Manα1	15	16

is any relationship between the oligosaccharide structures and the four subclasses. Therefore, the structural analysis of IgG2 and IgG4 was performed. Furthermore, by studying isolated Fab and Fc fragments of rabbit IgG, differences in the distribution of oligosaccharides in these fragments have been reported [6]. Since one of the IgG myeloma proteins studied in this paper contained a sugar chain in its Fab fragment, a comparative study of the sugar chains of the Fab and Fc portions of this myeloma protein was also performed.

Materials and Methods

Chemicals and Enzymes

NaB^3H_4 (348 mCi/mmol) was purchased from New England Nuclear, Boston, MA, USA, and NaB^2H_4 (98%) from Merck Co., Darmstadt, W. Germany. *Arthrobacter ureafaciens* sialidase was purchased from Nacalai Tesque Inc., Kyoto. *Aleuria aurantia* lectin (AAL)-Sepharose was prepared as reported previously [7]. *Phaseolus vulgaris* erythroagglutinin (E-PHA)-Sepharose, concanavalin A (Con A)-Sepharose and a *Ricinus communis* agglutinin (RCA) 120-WG003 HPLC column were purchased from Hohnen Oil Co., Tokyo. Human myeloma proteins were kindly provided as follows; Til (IgG2, *k*) was from Dr. A.C. Wang (Medical University of South Carolina): Dom (IgG2, *k*) and Heb (IgG4, l) were from Dr. H.L. Spiegelberg (Research Institute of Scripps Clinic). Fragments Fab and Fc of Dom were prepared as described by Frangione *et al.* [8]. Glycosidase digestion was performed as reported in a previous paper [4].

Oligosaccharides

Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc$_{OT}$ (Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc$_{OT}$ (Gal·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4 (Fucα1-6)-GlcNAc$_{OT}$ (GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from human serum IgG [4]. 6′-Sialyllactose was isolated from human milk as described previously [9].

Release of the Asparagine-linked Sugar Chains of Myeloma Proteins as Oligosaccharides

Myeloma proteins, Dom (4.0 mg), Til (4.8 mg) and Heb (6.4 mg) were subjected to 9 h hydrazinolysis as described previously [4]. One-tenth of the oligosaccharide fraction obtained from each sample was reduced with 2.4 mmol of NaB^3H_4 (0.83 mCi) in 100 ml of 0.05 N NaOH at 30°C for 4 h. The remainder was reduced with 2 mg of NaB^2H_4 for the methylation analysis. Lactose (10 nmol) was added as an internal standard to the oligosaccharide fractions just before reduction with NaB^3H_4. The radioactive oligosaccharide mixtures were then subjected to paper chromatography using butan-1-ol/ethanol/water, 4/1/1 by vol, as a solvent. Based on the radioactivities incorporated into lactitol and oligosaccharide fractions, the amounts of total sugar chains released from 1 ml of myeloma proteins (M.W., 150,000) were determined as listed in Table 2. The oligosaccharide fractions from the Fab fragment (1.1 mg, M.W. 50,000) and the Fc fragment (0.8 mg M.W. 50,000) of Dom were also reduced with NaB^3H_4.

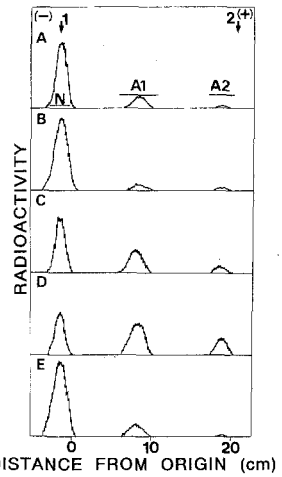

Figure 1. Paper electrophoretograms of the radioactive oligosaccharides. The radioactive oligosaccharide mixtures were subjected to paper electrophoresis at pH 5.4. Arrows indicate the positions to which authentic oligosaccharides migrated: 1, lactitol; 2, 6′-sialyllactitol. A, radioactive oligosaccharides liberated from Heb; B, those from Til; C, those from Dom; D, those from the Fab fragment of Dom; those from the Fc fragment of Dom.

Analytical Methods

High-voltage paper electrophoresis was carried out according to a previous paper [4]. Methylation analysis of oligosaccharides was performed as described previously [10], using a JEOL DX-300 gas chromatograph-mass spectrometer. Lectin column chromatography was performed according to the previous papers [7, 11, 12].

Results

Fractionation of Oligosaccharides by Paper Electrophoresis

The radioactive oligosaccharide mixtures obtained from Heb, Til and Dom were subjected to paper electrophoresis at pH 5.4. As shown in Fig. 1A-C, each contained one neutral (N) and two acidic (A1 and A2) components). The molar percentage distributions of radioactive components from each sample were calculated on the basis of their radioactivities and are summarized in Table 2.

All acidic components obtained from the three myeloma IgGs were completely converted to neutral components by sialidase digestion, indicating that the acidic nature of the oligosaccharides in fractions A1 and A2 can be ascribed to sialic acid residues. After mild acid hydrolysis (in 0.01 N HCl at 100°C for 3 min) for partial removal of sialic acid residues, only a neutral oligosaccharide fraction was obtained from A1 in addition to the original acidic fraction. In contrast, A2 gave another acidic fraction together with a neutral fraction (data not shown). These results indicated that fractions A1 and A2 contain mono- and disialyl oligosaccharides, respectively.

Table 2. Characteristics of asparagine-linked sugar chains of human IgG myeloma proteins.

	Molar ratio (%) of sugar chains[a]			Number of sugar chains
	N	A1	A2	
Heb	77.1	18.9	4.0	1.8
Til	89.9	9.4	0.7	2.0
Dom	65.8	26.5	7.7	4.0
Fab (Dom)	52.3	35.5	12.2	1.1
Fc (Dom)	86.7	13.1	0.2	2.0

[a] Values in the table were calculated from the radioactivities incorporated into each fraction.

Structural Analysis of the Desialylated Oligosaccharides

Neutral oligosaccharide mixtures obtained by exhaustive sialidase treatment were analyzed by Bio-Gel P-4 column chromatography (Fig. 2A-C). Methylation analysis of the neutral oligosaccharide mixtures obtained by sialidase treatment of the deuterium-labeled oligosaccharide fractions from three myeloma IgG samples gave 2,3,4-tri-*O*-methyl fucitol, 2,3,4,6-tetra-*O*-methyl galactitol, 3,4,6-tri-, 2,4-di- and 2-mono-*O*-methyl mannitols, 3,6-di-, 3.4.6-tri-, 1,3,5-tri- and 1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitols in all cases (data not shown). These results were qualitatively the same as the methylation data obtained from the neutral fractions of human serum IgG [4].

A preliminary structural study together with the methylation data indicated that the oligosaccharides from the three myeloma IgG samples were a mixture of bi-antennary complex-type oligosaccharides as were found in human serum IgG [4, 5]. The 16 different oligosaccharides found in the human serum IgG can be separated by serial lectin column chromatography [11]. Therefore, the tritium-labeled neutral oligosaccharide mixtures were subjected to this procedure. The fractionation scheme and the % molar ratio of oligosaccharide in each fraction are summarized in Fig. 3 and Table 3. Since the specificity of each lectin column has been reported previously [11, 13, 15], it will not be repeated here.

The structure of oligosaccharide in each fraction was further confirmed as shown in Table 1 by sequential exoglycosidase digestion. Since the results obtained were the same as those reported in a previous paper [4], details will not be described here.

Structures of Oligosaccharides Included in the Fab and Fc Fragments of Dom

The radioactive oligosaccharide fractions obtained from Fab and Fc fragments of Dom were analyzed by paper electrophoresis (Fig. 1D and E, respectively). The fractionation patterns were very similar to that of whole molecule of Dom giving a neutral and two acidic fractions (Fig. 1C). The molar ratio of oligosaccharides in the three fractions was calculated from their radioactivities and the data are listed in Table 2.

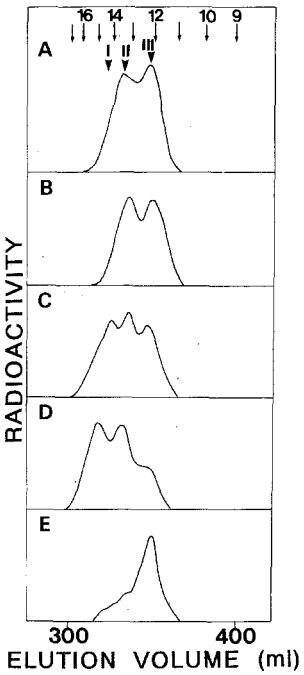

Figure 2. Bio-Gel P-4 column chromatography of radioactive neutral oligosaccharides. The black arrows indicate the elution positions of glucose oligomers, and the numbers indicate glucose units. Arrow heads indicate the positions where authentic oligosaccharides eluted: I, Gal_2 $GlcNAc_2$ Man_3 GlcNAc Fuc $GlcNAc_{OT}$; II, Gal $GlcNAc_2$ Man_3 GlcNAc Fuc $GlcNAc_{OT}$; III, $GlcNAc_2$ Man_3 GlcNAc Fuc $GlcNAc_{OT}$. A, neutral oligosaccharides obtained by sialidase treatment of radioactive oligosaccharide fraction from Heb; B, those from Til; C, those from Dom; D, those from the Fab fragment of Dom; E, those from the Fc fragment of Dom.

The radioactive oligosaccharide fractions obtained from each fragment were exhaustively digested with sialidase, and the neutral oligosaccharide fractions thus obtained were analyzed in the same manner as described in the previous section. The elution patterns of them on Bio-Gel P-4 column chromatography were shown in Fig. 2D and E. The structures of oligosaccharides in the neutral fractions were summarized in Table 1.

Discussion

Evidence reported in this paper indicated that the desialylated asparagine-linked sugar chains of the three myeloma IgGs consist of 16 different bi-antennary complex-type oligosaccharides with one of the four different core structures: Manα1-6(±GlcNAcβ1-4)(Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc. However, the molar ratios of the 16 oligosaccharides were different from myeloma proteins as reported previously by Mizuochi *et al.* [4] from the analysis of eight IgG1 and two IgG3 human myeloma proteins. The structural variation of the sugar moieties of IgG myeloma proteins cannot be correlated with any subclasses. Therefore, accumulation of more data is necessary to find out whether

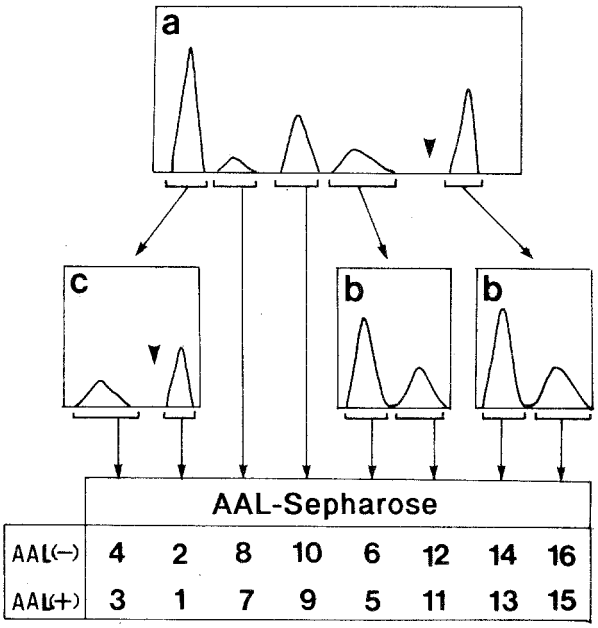

Figure 3. Scheme for fractionating oligosaccharides by lectin-affinity chromatography. The radioactive desialylated oligosaccharides liberated from human IgG myeloma proteins were subjected to sequential chromatography using columns of RCA-HPLC (a), E-PHA-Sepharose (b), Con A-Sepharose (c) and AAL-Sepharose as described in "Materials and Methods". The arrow heads within the graphs indicate the points where buffers were switched to those containing haptenic sugars to elute the bound oligosaccharides. AAL(-) and AAL(+) indicate the "pass through" and "bound" fractions obtained from an AAL-Sepharose column respectively. The numbers in the Figure correspond to those in Table 1. The ordinate and the abscissa represent radioactivity and retention, respectively.

monoclonal IgGs can be classified by their sugar chain structures or not. However, the evidence is of interest, because the sugar chains in the Fc portion of IgG molecules seem to affect the biological functions of antibodies, such as complement activation, binding to the Fc receptors, induction of antibody dependent cellular cytotoxicity and rapid elimination of antigen-antibody complexes from the circulation [16-18].

Studies reported in this paper together with our previous data suggested that the structural variations observed in the sugar chains of monoclonal IgGs are produced by the difference in the steric effects of their polypeptide moieties as well as the differences in the complement of glycosyltransferases present in each monoclonal B cell line. Two important pieces of evidence were obtained by the comparative study of the sugar chain structures of three glycosylated Bence Jones proteins [19-21].

One is that the sugar chains of these glycoproteins are quite homogeneous in contrast to those of IgG myeloma proteins. This difference might be produced by the structural difference of the light and heavy chains of the IgG molecule. Because the light chain is

Table 3. Proposed structures for the asparagine-linked sugar chains of human myeloma IgG2 and IgG4, and Fab and Fc fragments of Dom.

Oligosaccharides[a]	Heb	Til	Dom	Fab	Fc
1	53.9	51.4	26.7	12.2	45.4
2	13.0	2.9	3.2	1.2	4.9
3	8.0	4.2	12.2	12.2	13.7
4	1.6	1.0	1.0	1.1	1.0
5	9.1	17.7	4.2	4.9	—[c]
6	1.7	1.3	0.1	0.2	—
7	0.3	0.3	18.6	13.3	31.4
8	tr[b]	tr	1.8	0.6	3.0
9	7.7	12.4	1.5	1.5	—
10	0.8	1.4	tr	tr	—
11	0.2	0.1	8.7	13.3	—
12	tr	tr	0.5	0.7	—
13	3.0	6.2	5.5	8.5	0.1
14	0.5	0.9	0.3	0.4	tr
15	0.1	0.1	14.8	28.4	0.4
16	tr	tr	0.8	1.5	tr

[a] Structures of the oligosaccharides 1 to 16 are shown in Table 1.
[b] less than 0.05.
[c] not detected.

smaller and simpler in its molecular construction than the heavy chain, the glycosylation site may be more accessible for glycosyltransferases than that of the CH2 domain of the heavy chain. This assumption was supported by the data reported in this paper. A comparative study of the sugar chains of Fab and Fc portion of an IgG2 myeloma protein revealed that the sugar chains of the latter were far less galactosylated. Because of this situation, more sialylated oligosaccharide were detected in the Fab fragment than the Fc fragment. This finding agrees with our previous finding that the sugar chains of the Fab fragment obtained from rabbit serum IgG was more enriched in sialylated oligosaccharide than its Fc fragment [6] and with other reports that higher amounts of sialic acid were present in the Fab fragment than in the Fc fragment of some human IgG myeloma proteins [22, 23].

Because the glycosylation sites of both Fab and Fc portions of IgG are equally exposed to the same set of glycosyltransferases, the structural difference should be produced by the different steric effects of polypeptide moieties surrounding the glycosylation sites. The possible interaction of the α(1-6)-arms of the bi-antennary oligosaccharides of rabbit IgG with its polypeptide moiety and one of the α(1-3)-arms of the oligosaccharide interact with the opposing oligosaccharide to form a bridge was proposed from the X-ray crystallographic studies of this glycoprotein [24].

Another important piece of evidence revealed by the study of glycosylated Bence Jones proteins is that they all contain different sugar chains [19-21]. Since the peptide moieties of two of them were very similar, it is less likely that the structural difference is produced by

the differences in the steric effects of their polypeptide moieties. Instead, the difference might be produced by the difference in the complement of glycosyltransferases equipped in each B cell that produced the glycosylated Bence Jones protein. Since a significant extent of microheterogeneity was found in the sugar chains of Fab portion of myeloma IgG Dom, the glycosylation site of the light chains in an IgG molecule might be less accessible for glycosyltransferases than a free light chain.

Therefore, the variation found in the oligosaccharide patterns of IgG myeloma proteins is probably produced by such different sets of enzymes and is further enhanced by the steric effects of peptide moieties, especially in the Fc portion.

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