Structures of the Sugar Chains of Rabbit Immunoglobulin G: Occurrence of Asparagine-Linked Sugar Chains in Fab Fragment†

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ABSTRACT: The asparagine-linked sugar chains of rabbit immunoglobulin G (IgG) and its Fc and Fab fragments were quantitatively liberated from the polypeptide portions by hydrazinolysis followed by N-acetylation and NaB^3H_4 reduction. After fractionation by paper electrophoresis, lectin chromatography, and gel filtration, their structures were studied by sequential exoglycosidase digestion in combination with methylation analysis. Rabbit IgG was shown to contain 2.3 mol of asparagine-linked sugar chains per molecule distributed in both the Fc and Fab fragments. The sugar chains were of the biantennary complex type containing four cores: Manα1→6(Manα1→3)(±GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(±Fucα1→6)-GlcNAc. A total of 16 distinct neutral oligosaccharide structures was found after sialidase treatment. The galactose residue in the monogalactosylated oligosaccharides was present on either the α1→3 or α1→6 side of the trimannosyl core. The Fab fragments contained neutral, monosialylated, and disialylated oligosaccharides, whereas the Fc fragment contained only neutral and monosialylated structures. The oligosaccharides isolated from the Fab fragments also contained more galactose and bisecting *N*-acetylglucosamine residues than those from the Fc fragments.

Rabbit immunoglobulin G (IgG)[1] is a glycoprotein composed of two types of polypeptide chains with the stoichiometry H_2L_2. A conserved feature of γ-class immunoglobulins (from all species) is the presence of an asparagine-linked oligosaccharide in each heavy chain located at Asn-297 in the C_H2 domain of the Fc region of the molecule (Beale & Feinstein, 1976). The most distinct structural characteristics of the Fc region are the separation of the C_H2 domains and their attached oligosaccharides, which "bridge" the interstitial space between them.

Recent X-ray crystallographic studies on rabbit IgG have shown that the α1→6 arms of the biantennary complex-type oligosaccharides interact with the protein and one of the α1→3 arms of the oligosaccharide interacts with the opposing oligosaccharide to form the bridge (Sutton & Phillips, 1983). The immunoglobulin molecule therefore serves as a model system for protein–carbohydrate and carbohydrate–carbohydrate interactions as well as for the analysis and consequences of oligosaccharide microheterogeneity.

A possible functional role for the Fc fragment oligosaccharides may be to act as specific ligands for recognition by receptors. The presence of oligosaccharide heterogeneity could then lead to diversification of the immunoglobulin molecules with respect to their functional properties. However, it seems likely that the oligosaccharides could act as "spacers" between the two C_H2 domains, thereby imparting stability or protection against proteolysis. It is clear that a systematic analysis of the molar distributions, locations, and dynamics of immunoglobulin oligosaccharides must be a prerequisite to understand their molecular role in the immunoglobulin molecule.

Although Fab glycosylation has been extensively studied in human and mouse IgG myeloma proteins (Abel et al., 1968; Spiegelberg et al., 1970; Sox & Hood, 1970), no quantitative or structural data exist for rabbit Fab glycosylation, although the presence of carbohydrate has been reported (Fanger & Smyth, 1972a). Also, it is not known whether the incidence of Fab glycosylation is species specific.

Recent advances in structural studies of glycoprotein oligosaccharides include the establishment of controlled hydrazinolysis (Takasaki et al., 1982) for the quantitative release of oligosaccharide chains and the use of rapid, sensitive radioactive techniques for fractionating and sequencing these oligosaccharides (Yamashita et al., 1982). We have already reported preliminary results (Rademacher et al., 1983) using these techniques for rabbit IgG, which we now extend in this study to allow the complete definition of all the asparagine-linked oligosaccharides on rabbit IgG. Further, by studying isolated Fab and Fc fragments, we have been able to confirm the presence of glycosylated Fab fragments in normal pooled rabbit serum and to show that the distribution of oligosaccharide structures differs between the Fab and Fc fragments.

Materials and Methods

Chemicals and Enzymes. NaB^3H_4 (347.8 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 (98%) was purchased from Merck, Co., Darmstadt, Germany. α-Mannosidase, β-galactosidase, and β-*N*-acetylhexos-

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose. All sugars mentioned in this paper were of the D configuration except for fucose, which had the L configuration. Subscript OT is used to indicate NaB^3H_4-reduced oligosaccharides.

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aminidase were purified from jack bean meal according to the method of Li & Li (1972). Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were prepared by the method of Glasgow et al. (1977). Sialidase purified from *Arthrobacter ureafaciens* (Uchida et al., 1974) was purchased from Nakarai Chemicals, Ltd., Kyoto. Snail β-mannosidase (Sugawara et al., 1972) was kindly supplied by Seikagaku Kogyo Co., Tokyo. Bovine epididymis α-L-fucosidase was purchased from Sigma Chemical Co., St. Louis, MO. Glycosidase digestion of the radioactive oligosaccharides [(4∼6) × 10^4 cpm] was performed according to Mizuochi et al. (1982b).

Preparation of Rabbit IgG and Its Fragments. IgG was isolated from pooled nonimmune rabbit sera as described by Colomb & Porter (1975). Fragments Fc and Fab were prepared under nonreducing conditions essentially as described by Dower et al. (1975). Two-thirds of the IgG was digested for 20 h at 37 °C with 3% activated papain (Worthington, 25 units) in 0.5 M Tris-HCl buffer, containing 1 mM EDTA, pH 7.3, in the absence of cysteine. Undigested IgG (papain resistant) was separated from the fragments by gel filtration on Sephacryl S-200 (Pharmacia) in 60 mM sodium acetate buffer, pH 5.6 at 4 °C. The Fab and Fc fragments were then separated on CM-cellulose (Whatman), by eluting the Fab in 60 mM sodium acetate, pH 5.6, and the Fc in 500 mM sodium acetate, pH 5.6. Fragments were judged free of contaminants by immunodiffusion against the appropriate antisera and by SDS–PAGE.

Release of the Asparagine-Linked Sugar Chains of Rabbit IgG, Fc Fragment, Fab Fragment, and Papain-Resistant Rabbit IgG Fraction. Each rabbit IgG (40 mg), Fc fragment (1.0 mg), Fab fragment (1.8 mg), and papain-resistant IgG fraction (1.5 mg) was suspended in 0.5 mL of anhydrous hydrazine and subjected to 9-h hydrazinolysis as described previously (Takasaki et al., 1982). One-third of the oligosaccharide fraction obtained from rabbit IgG was reduced with 2.4 μmol of NaB^3H_4 (0.83 mCi) in 100 μL of 0.05 N NaOH at 30 °C for 4 h. Then, 2.5 mg of NaB^2H_4 in 100 μL of 0.05 N NaOH was added, and reduction was continued at 30 °C for another 2 h to complete the reduction. The remaining two-thirds was reduced with 7.5 mg of NaB^2H_4 in 300 μL of 0.05 N NaOH at 30 °C for 4 h to obtain deuterium-labeled oligosaccharides. To facilitate detection of the deuterium-labeled oligosaccharides in further purification procedures, one-tenth of the tritium-labeled oligosaccharides was added to the deuterium-labeled sample. The oligosaccharide fractions from the Fc fragment, the Fab fragment, and the papain-resistant IgG fraction were also reduced with NaB^3H_4 (0.25 mCi each).

In order to determine the number of asparagine-linked sugar chains in one molecule, the time course of liberation of oligosaccharide by hydrazinolysis was studied with 1.5 mg of rabbit IgG and 2-deoxy-D-ribose instead of 6′-sialyllactose as internal standard by a modification of the previous method (Takasaki et al., 1982). 2-Deoxy-D-ribose (25 nmol) was added to the oligosaccharide fractions just before reduction with 2.0 μmol of NaB^3H_4 (0.7 mCi). The radioactive oligosaccharide mixtures were then subjected to paper chromatography in 1-butanol–ethanol–water (4:1:1 v/v). On the basis of the radioactivities incorporated into 2-deoxy-D-ribose and the oligosaccharide mixture and on the basis of the molecular weight of rabbit IgG, 150000, the amount of asparagine-linked sugar chains liberated from 1 mol of rabbit IgG was calculated to be approximately 2.3 mol.

Oligosaccharides. Galβ1→4GlcNAcβ1→2Manα1→6-(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→-4GlcNAc$_{OT}$ (Gal_2·$GlcNAc_2$·Man_3·GlcNAc·GlcNAc$_{OT}$), GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)-Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($GlcNAc_2$·Man_3·GlcNAc·GlcNAc$_{OT}$), and Manα1→6(Manα1→3)Manβ1→-4GlcNAcβ1→4GlcNAc$_{OT}$ (Man_3·GlcNAc·GlcNAc$_{OT}$) were obtained from human fibrinogen (Mizuochi et al., 1982a). ±Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→-2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was prepared from ±NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→-6(NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→-4GlcNAcβ1→4GlcNAc$_{OT}$ by sequential digestion with β-galactosidase and sialidase. The sialylated oligosaccharides were obtained from human fibrinogen (Mizuochi et al., 1982a). GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Manα1→3)-Manβ1→4GlcNAcβ1→4(Fucα1-6)GlcNAc$_{OT}$ (GlcNAc·Man_2·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$), GlcNAcβ1→-2Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→-4GlcNAcβ1→4GlcNAc$_{OT}$ (GlcNAc·Man_2·GlcNAc·Man·GlcNAc·GlcNAc$_{OT}$), and Manα1→6(Manα1→3)Manβ1→-4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from human IgG (Mizuochi et al., 1982b). 6′-Sialyllactose was isolated from human milk as described previously (Kobata, 1972).

Analytical Methods. High-voltage paper electrophoresis and Bio-Gel P-4 column chromatography were carried out according to Mizuochi et al. (1982a) and Yamashita et al. (1982). Methylation analyses of oligosaccharides were performed as described by Endo et al. (1979), using a JEOL DX-300 gas chromatograph–mass spectrometer.

Concanavalin A (Con A)–Sepharose column chromatography was performed by a minor modification of the method of Ogata et al. (1975). Separation of radioactive oligosaccharides was obtained on a column (0.5 × 2.5 cm) of Con A–Sepharose. The column was washed with 5 mL of 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, and eluted with 10 mL of the same buffer containing 0.2 M methyl α-mannoside. Five milliliters of buffer containing 1% sodium dodecyl sulfate was then applied to ensure complete removal of all radioactivity. The bound and unbound oligosaccharide fractions were desalted by passage through a column containing Dowex 50W (H^+) and AG 3 (OH^-). Oligosaccharides released from Con A–Sepharose were subjected to Bio-Gel P-4 column chromatography to remove the methyl α-mannoside.

RESULTS

Fractionation of Oligosaccharides by Paper Electrophoresis. The radioactive oligosaccharide mixture obtained from rabbit IgG was subjected to paper electrophoresis at pH 5.4. As shown in Figure 1A, one neutral (N) and two acidic (A1 and A2) components were obtained. Both A1 and A2 were converted to neutral oligosaccharides by sialidase digestion. Mild acid hydrolysis (0.01 N HCl at 100 °C for 3 min) of A2, in which part of the original acidic component still remained, gave another acidic component with the same mobility as A1 together with a neutral component on paper electrophoresis (Figure 1E). In contrast, fraction A1, treated under similar conditions, gave only a neutral product (Figure 1D). The results indicated that A1 and A2 contained one and two sialic acid residues, respectively. When the sialic acid residues released from rabbit IgG by mild acid hydrolysis were analyzed by paper chromatography after NaB^3H_4 reduction as described previously (Yoshima et al., 1980), *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were detected in a ratio of 43:57. The percent molar ratio of neutral, monosialylated, and disialylated oligosaccharide fractions was 74:17:9. The oligosaccharide mixture obtained from rabbit IgG was com-

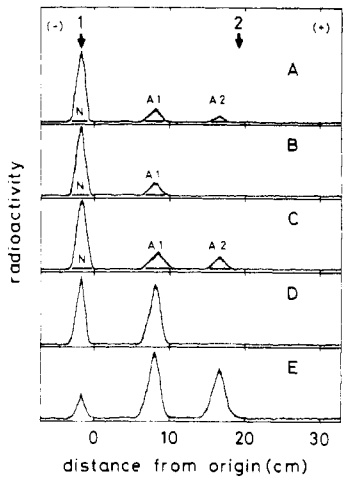

FIGURE 1: Paper electrophoretograms of the radioactive oligosaccharides. The radioactive oligosaccharide mixtures were subjected to paper electrophoresis at pH 5.4. Arrows indicate the positions where authentic oligosaccharides moved: (1) lactitol; (2) 6′-sialyllactitol. (A) Radioactive oligosaccharides liberated from rabbit IgG; (B) those from Fc fragment; (C) those from Fab fragment; (D and E) mild acid hydrolysates (0.01 N HCl at 100 °C for 3 min) of A1 and A2 in (A), respectively.

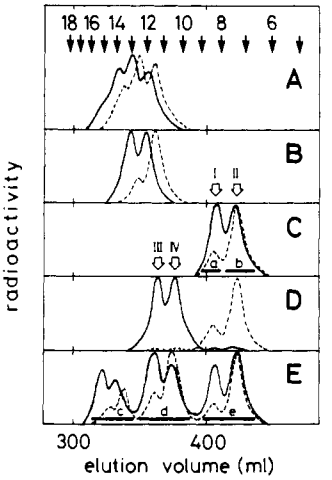

FIGURE 2: Sequential exoglycosidase digestion of oligosaccharides unbound (—) and bound (--) to a Con A–Sepharose column. The radioactive sugars were subjected to Bio-Gel P-4 column chromatography, and the radioactivity in each tube (3 mL/tube) was measured by liquid scintillation spectrometry. The black arrows indicate the elution positions of glucose oligomers (numbers indicate the glucose units) added as internal standards, and the white arrows indicate the elution positions of authentic oligosaccharides: I, Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; II, Man_3·GlcNAc·$GlcNAc_{OT}$; III, GlcNAc·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$; IV, GlcNAc·Man_2·GlcNAc·Man·$GlcNAc_{OT}$. (A) Intact oligosaccharides unbound and bound to Con A–Sepharose; (B) radioactive peaks in (A) after jack bean β-galactosidase digestion; (C) radioactive peaks in (B) after jack bean β-*N*-acetylhexosaminidase digestion; (D) radioactive peaks in (B) after diplococcal β-*N*-acetylhexosaminidase digestion; (E) radioactive peaks in (A) after jack bean β-*N*-acetylhexosaminidase digestion.

pletely freed from sialic acid by exhaustive sialidase digestion, and the neutral oligosaccharide mixture thus obtained was applied to a Con A–Sepharose column. Free and bound fractions were obtained in the percent molar ratio of 38:62 (data not shown).

Fractionation of the Neutral Oligosaccharide Mixtures by Bio-Gel P-4 Column Chromatography and Their Sequential Exoglycosidase Digestion. Free and bound fractions obtained by Con A–Sepharose chromatography were analyzed on columns of Bio-Gel P-4 and gave similar elution patterns (Figure 2A). But the effective sizes of the peaks of the unbound fraction were larger than the respective peaks of the bound fraction by about 0.5 glucose unit.

In order to determine the monosaccharide sequence in each oligosaccharide, both fractions were subjected to sequential exoglycosidase digestion, and the products were analyzed by Bio-Gel P-4 column chromatography. When both radioactive fractions were incubated with jack bean β-galactosidase, the elution patterns shown in Figure 2B were obtained. Further digestion with jack bean β-*N*-acetylhexosaminidase showed conversion to two peaks a and b (Figure 2C), which were eluted at the same positions as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and Man_3·GlcNAc·$GlcNAc_{OT}$, respectively. Sequential digestion with jack bean α-mannosidase, snail β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and bovine epididymis α-L-fucosidase and methylation analysis confirmed that the radioactive peaks a and b have the structures of Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4-(Fucα1→6)$GlcNAc_{OT}$ and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$, respectively.

Because the elution positions of samples, shown by the solid and dotted lines in Figure 2B, were different, both samples were incubated with diplococcal β-*N*-acetylhexosaminidase, which has a different substrate specificity from the jack bean enzyme. The Con A bound fraction (dotted line) moved to the same elution position as a and b in Figure 2C, whereas the Con A unbound fraction (solid line) gave two peaks at the same elution position as those of authentic GlcNAc·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$ and GlcNAc·Man_2·GlcNAc·Man·GlcNAc·$GlcNAc_{OT}$ (Figure 2D). Diplococcal β-*N*-acetylhexosaminidase releases only one *N*-acetylglucosamine residue from GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAcβ1—and converts it to GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)-(Manα1-3)Manβ1→4GlcNAcβ1—(Yamashita et al., 1981). Therefore, the results of the two β-*N*-acetylhexosaminidase digestions indicated that the solid-line peaks in Figure 2B derived from the Con A unbound fraction are composed to two oligosaccharides: GlcNAcβ1→2Manα1→6-(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→3)Manβ1→-4GlcNAcβ1→4(±Fucα1→6)$GlcNAc_{OT}$, whereas the dotted-line peaks in Figure 2B from the Con A bound fraction are composed of two oligosaccharides: GlcNAcβ1→2Manα1→-6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(±-Fucα1→6)$GlcNAc_{OT}$.

In order to determine the number of β-galactose residues in the outer chain moieties, both samples in Figure 2A were incubated with 5 units of jack bean β-*N*-acetylhexosaminidase. γ-D-Galactonolactone (2.5 mg) was added to each reaction mixture to inhibit the action of contaminating β-galactosidase, if any. The products gave the elution patterns shown in Figure 2E. By sequential digestion with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase followed by analysis of the radioactive products by Bio-Gel P-4 column, the radioactive oligosaccharides eluted in area c (shown by a bar in Figure 2E) were found to contain two galactose and two *N*-acetylglucosamine residues in their outer chain moieties (data not shown). The oligosaccharides eluted in area d in Figure 2E were found to have one galactose and one *N*-acetylglucosamine residue in their outer chain moieties by the same analytical procedure (data not shown). After this sequential digestion, all radioactive components that were eluted in areas c and d were converted to smaller oligosaccharides with the same mobility as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and Man_3·GlcNAc·$GlcNAc_{OT}$. Therefore, the percent molar ratios of oligosaccharides with two, one, and

Table I: Molar Ratios of Alditol Acetates Obtained from Hydrolysates of Permethylated Oligosaccharides

partially methylated sugar	molar ratio[a] intact[b]	desialylated[c] Con A unbound fraction	desialylated[c] Con A bound fraction
fucitol			
2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl	0.2	0.4	0.1
galactitol			
2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl	0.5	0.7	0.8
2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetyl	0.3	0	0
mannitol			
3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl	2.0	2.0	2.0
2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetyl	0.6	tr[d]	0.9
2-mono-*O*-methyl-1,3,4,5,6-penta-*O*-acetyl	0.4	1.1	tr
2-(*N*-methylacetamido)-2-deoxyglucitol			
1,3,5,6-tetra-*O*-methyl-4-mono-*O*-acetyl	0.6	0.5	0.7
1,3,5-tri-*O*-methyl-4,6-di-*O*-acetyl	0.3	0.5	0.2
3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl	1.5	2.1	1.1
3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl	1.7	1.8	1.7

[a] Numbers were calculated by making the values of 3,4,6-tri-*O*-methylmannitol as 2.0. [b] Oligosaccharides mixture liberated from rabbit IgG by hydrazinolysis. [c] The desialylated oligosaccharides were the samples corresponding to the mixtures of peaks in Figure 2A. [d] tr, less than 0.1.

zero galactose residues were calculated from the radioactivities in areas c, d, and e, respectively: the values were 23%, 42%, and 35% for the Con A bound fraction and 13%, 37%, and 50% for the Con A unbound fraction, respectively. The molar ratio of oligosaccharides with and without fucose residues in each area was calculated from the radioactivities in the fast- and slow-moving peaks of the area and are summarized in Figure 3. These results indicated that (i) the bound and unbound fractions were mixtures of six biantennary complex-type oligosaccharides with different numbers of β-galactosyl residues linked to the common structures of GlcNAcβ1→Manα1→(GlcNAcβ1→Manα1→)Manβ1→GlcNAcβ1→(±Fucα1→)$GlcNAc_{OT}$ and (ii) the oligosaccharides in the Con A unbound fraction contained a bisecting *N*-acetylglucosamine residue.

Methylation Analysis. The location of each glycosidic linkage was confirmed by methylation analysis of deuterium-labeled oligosaccharides derived from rabbit IgG. Half of each of the deuterium-labeled oligosaccharides was digested with sialidase and subjected to Con A–Seharose column chromatography, and the bound and unbound fractions were also subjected to methylation analysis.

Detection of 1,3,5,6-tetra- and 1,3,5-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol indicated that the *N*-acetylglucosamine residues at the reducing termini of a part of oligosaccharides of the three samples are substituted at the C-4 position and the remaining part of oligosaccharides at C-4 and C-6 positions (Table I). The C-6 position in the latter group should be occupied by an α-fucosyl residue. As 2,3,4-tri-*O*-methylgalactitol was detected in intact sample, but not in the two desialylated samples, sialic acid should be linked at the C-6 position of the galactose residues of all oligosaccharides.

In the case of desialylated oligosaccharides, 2,3,4,6-tetra-*O*-methylgalactitol and 3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol were detected as the nonreducing terminal sugars. The galactose residue is linked at the C-4 position of *N*-acetylglucosamine because 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol was detected as the only di-*O*-methyl derivative of *N*-acetylglucosamine. That 3,4,6-tri-*O*-methylmannitol is derived only from α-mannosyl residues and 2,4-di- and 2-mono-*O*-methylmannitols from β-mannosyl residues of the trimannosyl core was estimated as follows. The β-mannosyl residue should at least be substituted at the C-3 and C-6 positions by two α-mannosyl residues. By taking the value of 3,4,6-tri-*O*-methylmannitol as 2.0, the sum of 2,4-di- and 2-mono-*O*-methylmannitols in the three samples became approximately 1.0. The Con A unbound fraction contained only 2-mono-*O*-methylmannitol, while the Con A bound fraction contained only 2,4-di-*O*-methylmannitol. These results are in accord with the conclusion obtained by sequential glycosidase digestion, viz., that all oligosaccharides in the Con A unbound fraction contain a bisecting *N*-acetylglucosamine residue and those in the Con A bound fraction do not contain this residue. From the results of methylation analysis and sequential exoglycosidase digestion, the structures of the neutral oligosaccharides derived from rabbit IgG were confirmed as shown in Figure 3.

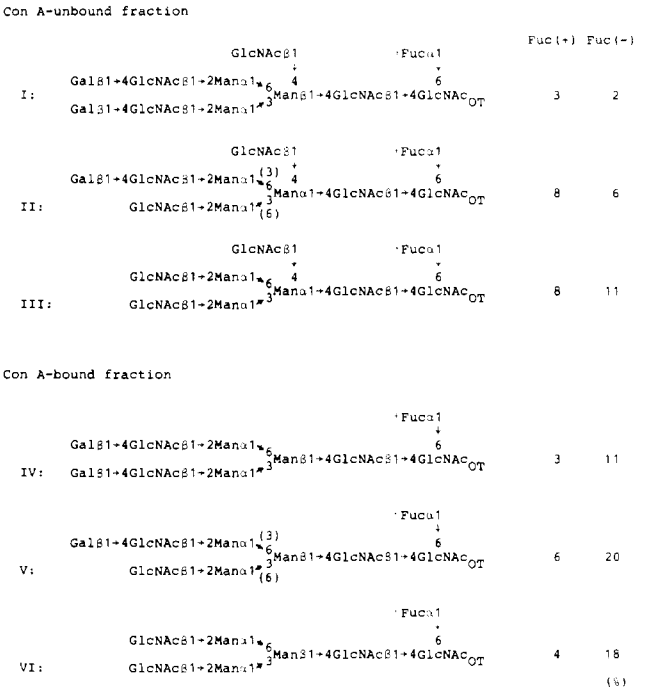

FIGURE 3: Proposed structures of desialylated oligosaccharides of rabbit IgG and their percent molar ratio.

That a galactose residue in the monogalactosylated oligosaccharides is linked to the β-*N*-acetylglucosamine residues of both α1→3 and α1→6 sides (II and V in Figure 3) was confirmed as follows. When each radioactive peak eluted at area d in Figure 2E was incubated with jack bean α-mannosidase, about one-third of the oligosaccharides released one mannose residue in both cases (data not shown). The undigested fraction was completely resistant to the second cycle

Table II: Summary of the Oligosaccharides Obtained from Rabbit IgG, the Papain-Resistant IgG Fraction, Fc Fragment, and Fab Fragment

	ratio of acidic sugar chains[a]			galactose[b]				
	A-1	A-2	total	2	1	0	bisecting GlcNAc[b]	fucose[b]
intact	18.0	3.2	21.2	19.0	40.0	41.0	36.3	33.0
papain-resistant fraction	16.6	3.1	19.7	18.5	39.8	41.7	39.0	34.2
Fc	13.2	0	13.2	16.1	41.8	42.1	32.5	32.6
Fab	19.6	15.0	34.6	44.0	34.1	21.9	46.5	34.9

[a] Values are percent molar ratio. [b] Percent molar ratio of the oligosaccharides that contained two, one, and zero galactose, bisecting GlcNAc, and fucose residues.

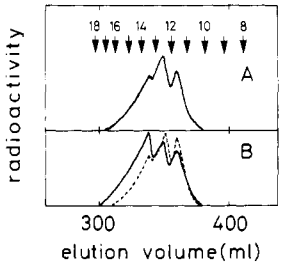

FIGURE 4: Bio-Gel P-4 column chromatograms of the desialylated oligosaccharides liberated from rabbit IgG (A), Fc fragment [dotted line in (B)] and Fab fragment [solid line in (B)].

of the α-mannosidase digestion (data not shown). Since the oligosaccharides eluted at area d in Figure 2E should have the structures of Galβ1→4GlcNAcβ1→2Manα1→6(Manα1→-3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$ and/or Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→-4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$, and jack bean α-mannosidase can liberate one mannose residue from the former butnot from the latter (Yamashita et al., 1980), the results indicated that a galactose residue is distributed on both α1→3 and α1→6 sides of the trimannosyl core in the monogalactosylated oligosaccharides.

Distribution of the Asparagine-Linked Sugar Chains in the Rabbit IgG Molecule. In order to determine the distribution of the asparagine-linked sugar chains in the IgG molecule, Fc and Fab fragments isolated from rabbit IgG after papain digestion were subjected to hydrazinolysis, and the radioactive oligosaccharide fractions, thus obtained, were analyzed by paper electrophoresis. The Fc fragment gave one neutral component and an acidic component with the same mobility as monosialylated oligosaccharides (A1) of rabbit IgG but did not give any disialylated oligosaccharide (Figure 1B). In contrast, the radioactive oligosaccharides fraction obtained from the Fab fragment was separated into a neutral and two acidic components with the same mobility as the components A1 and A2 derived from rabbit IgG (Figure 1C). The mole percent of N and A1 from the Fc fragment was 87:13 and the ratio of N, A1 and A2 from the Fab fragment was 65:20:15. On the basis of the radioactivities incorporated into the acidic and the neutral oligosaccharide fractions and the specific activity of the NaB^3H_4 used, the amounts of the total sugar chains liberated from 1 mol of Fc and Fab fragments were calculated to be 1.8 and 0.2, respectively.

The radioactive oligosaccharides mixtures obtained from each fragment were digested by sialidase, and the neutral oligosaccharides mixtures thus obtained were subjected to Bio-Gel P-4 column chromatography (Figure 4). The elution pattern of the sample from the Fc fragment (dotted line in Figure 4B) was similar to that of intact rabbit IgG (Figure 4A), but not to that of Fab fragment (solid line in Figure 4B). Sequential glycosidase digestion of both samples indicated that the Fab fragment contained more digalactosylated sugar chains and bisecting *N*-acetylglucosamine residues than the Fc fragment as summarized in Table II.

The papain-resistant IgG fraction was also subjected to hydrazinolysis in order to investigate the relationship between papain resistance and sugar chain pattern. The radioactive oligosaccharide fraction, thus obtained, was analyzed by paper electrophoresis, Bio-Gel P-4 column chromatography, and sequential exoglycosidase digestion. The content of sialic acid and the carbohydrate structures of the papain-resistant IgG faction were almost the same as those of intact IgG as summarized in Table II.

DISCUSSION

Column chromatography with Con A–Sepharose effected the separation of oligosaccharides with and without bisecting *N*-acetylglucosamine residues, and this facilitated sequencing. The oligosaccharides not retained by Con A–Sepharose were found to be larger than those of the bound fraction by 0.5 glucose unit (area c in Figure 2E). It was also noted that structures that contained the bisecting *N*-acetylglucosamine linked to digalactosylated biantennary sugar chains were not digested even by 5 units of jack bean β-*N*-acetylhexosaminidase, although the monogalactosylated and non-galactosylated biantennary sugar chains released this residue under the same conditions (d and e of Figure 2E).

Desialylated oligosaccharides obtained from rabbit IgG consist of 16 different biantennary complex-type sugar chains that have one of the four different core structures of Manα1→6(±GlcNAcβ1→4)(Manα1→3)Manβ1→-4GlcNAcβ1→4(±Fucα1→6)GlcNAc shown in Figure 3. These cores were also found in human IgG but were present in different amounts (Mizuochi et al., 1982b). A galactose residue in the monogalactosylated sugar chains is distributed on both α1→3 and α1→6 sides of the trimannosyl core. This lack of arm specificity is not unique, since it also occurs on bovine and human IgGs (T. Mizuochi et al., unpublished observation). It is possible that the structural heterogeneity of the sugar chains of rabbit IgG is caused because the IgG sample was obtained from pooled sera. However, our previous study indicated that the human IgGs purified from individual sera usually gave the same oligosaccharide pattern as pooled commercial IgG samples.

Papain digestion of IgG to give Fab and Fc fragments did not result in any preferential cleavage on the basis of the asparagine-linked oligosaccharides. However, study of the distribution of asparagine-linked sugar chains in the fragments confirmed that not only Fc but also Fab fragments contain sugar chains. The time-course study of hydrazinolysis confirmed that rabbit IgG contains approximately 2.3 mol of asparagine-linked sugar chains per molecule. If two sugar chains are located on the Fc portion, the $F(ab)'_2$ region should therefore contain about 0.3 mol of sugar chains. Interestingly, the sugar chains derived from the Fab fragments contained more sialic acid, galactose, and the bisecting *N*-acetylglucosamine residues than those from the Fc fragments. No disialylated structures were found on the the Fc fragments.

The presence and incidence of Fab glycosylation may be a conserved characteristic of IgG from any species. For ex-

ample, both human and bovine IgG also contain disialylated structures in essentially the same proportion as rabbit IgG. As human Fc fragments are devoid of disialylated carbohydrates and normal human serum IgG contains noninteger numbers of sugar chains per molecule (unpublished results), this suggests that the presence of disialylated oligosaccharides may be used as a marker for the presence of Fab glycosylation. At present, we have not determined whether asymmetrical IgG molecules are present (i.e., 3 mol of sugar chains/IgG) or whether the noninteger value represents an averaging of molecules with 2 and 4 mol of sugar chains, respectively. However, the presence of an odd number of sugar chains in one molecule in human IgG myeloma proteins (Mizuochi et al., 1982b) and the asymmetry of *O*-glycosylation (Fanger & Smyth 1972b) suggest that the former is biosynthetically possible (Fanger & Smyth 1972b). Another possibility is that some of the sites might be incompletely glycosylated as in the case of a myeloma IgM (Anderson et al., 1983).

Despite the suggestion that glycosylation of Fab for IgG was present in the variable domains (Abel et al., 1968; Spiegelberg et al., 1970; Sox & Hood, 1970), no role for the carbohydrate in antigen recognition has yet been established. However, preliminary studies suggest that the Fab sugars may mediate autoaggregation of IgG (unpublished results). Since both the L and H chains are exposed to the same biosynthetic enzymes (Bergman & Kuehl, 1978; Bergman & Kuehl, 1979), the differences between the oligosaccharide structures on the Fc and Fab fragments suggest that the glycosylation patterns not only result just from the availability of certain transferases but are determined by the structure of the immunoglobulin molecule itself.

From the observations reported here on the asparagine-linked oligosaccharides, we conclude that in IgG with sugar chains on Fab in pooled rabbit serum most of the sialic acid is located on the Fab (more than 0.50 mol/IgG) and not on the Fc fragment (0.26 mol). In addition, since pooled rabbit serum consists mostly of the d_{12} IgG allotype, which is characterized by an *O*-linked hinge-region oligosaccharide, an extra contribution of 0.55 mol/IgG[2] will be made by this to the average sialic acid content of the molecule. Whether the *N*-glycolylneuraminic acid residues in rabbit IgG oligosaccharide are localized to a given glycosylation site or unique sugar structure is not known, but their presence must be taken into account when performing any analytical assay for sialic acid on rabbit IgG. The low level of Fc sialylation (and galactosylation) of rabbit IgG is inconsistent with the proposed role of desialylation in the clearance of IgG from serum (Winkelhake & Nicholson, 1976) and in experimental models of rheumatoid arthritis (Dodon & Quash, 1981). Moreover, studies using reporter groups attached to sialic acid to probe the Fc region in whole IgG (Winkelhake et al., 1984; Nezlin & Sykulev, 1982) must also be viewed with great caution due to the small proportion of Fc fragments actually containing sialic acid and the potential presence of non-Fc sialic acid moieties.

The low level of galactosylation on the arm is consistent with the recent X-ray crystallographic analysis of rabbit Fc, which predicts that at least 50% of the α1→3 arms must be devoid of galactose (Sutton & Phillips, 1983). In rabbit Fc, the opposing oligosaccharides in the C_H2 domains possess different primary sequences and conformations. In particular, one of the α1→3 arms is devoid of galactose, thereby exposing the GlcNAc residue, which then interacts directly with the core Manβ1→4GlcNAcβ1→ segment of the opposing carbohydrate chain. The α1→3 arm of this second (latter) oligosaccharide extends outward between the domains and can either be devoid of galactose or terminate in a single galactose residue (Sutton & Phillips, 1983). Considering the degree of outer chain heterogeneity built on the four types of core units present and given that each immunoglobulin molecule pairs two of these sugars together, the number of IgG molecules with unique carbohydrate compositions could extend into the hundreds if random oligosaccharide pairing were to occur. If, however, as suggested by the crystallographic studies only certain oligosaccharides "fit together", possible pairs would be limited to only those meeting the steric requirements of the protein–carbohydrate and carbohydrate–carbohydrate interactions within the C_H2 domains.

Some insight into the functional role of the sugar chains of IgG comes from recent studies using aglycosyl mouse IgG2a (Leatherbarrow & Dwek, 1983; Leatherbarrow et al., 1985) and aglycosyl mouse IgG2b (Nose & Wigzell, 1983). Both studies showed that the absence of carbohydrate did not affect antigen binding (Fab region) or protein A binding (C_H2:C_H3 interface). Leatherbarrow et al. (1985) reported that C1q binding constant was reduced very slightly, which resulted a consequent reduction in C1 activation. They suggested that this small effect would be very marked under the assay conditions for whole complement activation as used by Nose & Wigzell (1983), indicating that the oligosaccharides are at most indirectly involved in these events. By contrast, Leatherbarrow et al. (1985) found that there is an increase in the rate of proteolysis of aglycosyl IgG2a and reduction in binding to monocyte Fc receptors. For the aglycosyl IgG2b, Nose & Wigzell (1983) also reported the loss of ability to induce antibody-dependent cellular cytotoxicity, the failure of the antigen–antibody complexes to be eliminated rapidly from circulation, and the loss of ability to bind to macrophage Fc receptors.

The inability of aglycosyl IgG to bind to Fc receptor may suggest that the Fc receptor binding site is located across domains, i.e., involving C_H2–C_H2. Recent model building using the X-ray data suggests that the Fc carbohydrate is not required to maintain the gross C_H2–C_H2 cross-domain architecture (unpublished result). If so, such an arrangement might be expected to display more sensitivity to any slight structural perturbations in the molecular architecture of the Fc, which could arise in aglycosyl IgG. Alternatively, the essentially dimeric interaction between the oligosaccharides from each C_H2 domain could result in an oligosaccharide "surface" that may contribute to the Fc receptor binding site.

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Registry No. (Fuc)-I, 83816-32-4; I, 85188-33-6; (Fuc)-II (Gal,G1c,Man1→6), 97718-79-1; (Fuc)-II (Gal,Glc,Man1→3), 97718-80-4; II (Gal,Glc,Man1→6), 97673-94-4; II (Gal,Glc,-Man1→3), 97673-95-5; (Fuc)-III, 97718-81-5; III, 97718-82-6; (Fuc)-IV, 75805-07-1; IV, 75898-93-0; (Fuc)-V (Gal,Glc,Man1→6), 97673-96-6; (Fuc)-V (Gal,Glc,Man1→3), 83368-28-9; V (Gal,-Glc,Man1→6), 97673-97-7; V (Gal,Glc,Man1→3), 83368-27-8; (Fuc)-VI, 85100-22-7; VI, 85100-21-6.

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