Structural Heterogeneity of Sugar Chains in Immunoglobulin G

CONFORMATION OF IMMUNOGLOBULIN G MOLECULE AND SUBSTRATE SPECIFICITIES OF GLYCOSYLTRANSFERASES

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The heterogeneous asparagine-linked sugar chains of bovine and human immunoglobulins G were separated into 12 components by reversed-phase high performance liquid chromatography, and their structures were determined by 1H NMR spectroscopy. Both immunoglobulin (Ig) G sources contained eight non-bisected biantennary complexes and four bisected biantennary complexes. In the non-bisected sugar chains of bovine IgG, galactosylation of the Mana1-3 branch predominated over that of the Mana1-6, whereas in the bisected complexes galactosylation of the Mana1-6 branch predominated. This difference can be explained by the substrate specificities of the galactosyltransferases and of the N-acetylglucosaminyltransferase III involved in their synthesis.

The sugar chains of human IgG1 differs in the distribution of its galactose residues from bovine IgG and human IgG2. The Mana1-6 branch of all IgG1s was more highly galactosylated than the Mana1-3 branch even in the non-bisected complexes. Such findings are in conflict with the substrate specificities of galactosyltransferases. Whereas these enzymes derivatized more of the Mana1-6 branch of native human IgG1, in denatured protein more of the Mana1-3 branch was galactosylated. Thus, protein conformation may influence the structure of its sugar chains.

Marked improvements in the methods for the structural analysis of sugar chains in proteins have been recently developed (1-6). An important advance was the introduction of a method for detecting glycolid chains by Hase et al. (7) based on derivatization of them with 2-aminopyridine to give UV absorbing or fluorescent products. 2-Aminopyridine exhibits some hydrophobicity, therefore, the modified sugar chains can be readily separated by HPLC on a reversed-phase column and their structures can be directly assigned from their 1H NMR spectral measurements.

The carbohydrate structures of IgG have been investigated in detail. It is known that they normally have one N-linked glycolid chain in each heavy subunit (8-11), and different molecules contain heterogeneous biantennary complexes (8, 11-15). Substrate specificities of glycosyltransferases are considered to play important roles in giving rise to these heterogeneities, and attempts have been made to explain this in terms of their branch specificities (16-21). These reports indicate that their specificities are different for non-bisected and bisected sugar chains. IgG has both such structures but the differences between the distributions of galactose in the non-bisected and the bisected sugar chains have not been elucidated.

This paper relates to the glycolid components of bovine and human IgGs. Our results indicate a difference in the structure of the major non-bisected sugar chain of bovine IgG reported by another group (13). The structural assignments are based on the 1H NMR spectra of sugar chains separated by reversed-phase HPLC following their derivatization with 2-aminopyridine (7). The substrate specificities of galactosyltransferase and of N-acetylglucosaminyltransferase clearly reveal their influences on the structures of the glycolid components of the major bovine antibody.

Structural studies on the sugar chains of human IgG have indicated that galactosylation of non-bisected, biantennary complexes at the Mana1-6 branch predominated over those at the Mana1-3 branch (8, 11, 12, 14, 15). These findings are contrary to a known substrate specificity of galactosyltransferases. Kobata et al. (22) suggested that there was a galactosyltransferase specific for IgG in human B-cells, and it appeared possible that the distribution of sugar chains was due to a unique branch specificity of this source enzyme.

In the present study, we also examined the substrate specificities of galactosyltransferase and of N-acetylglucosaminyltransferase III from B-cells of the bone marrow of myeloma patients. The specificity of this galactosyltransferase was found to be similar to those of other sources (17-21), and the structural characteristics of the sugar chains of human IgG noted above could not be explained in this manner. To determine the cause of the variation, the sugar chains of monoclonal IgG1 and IgG2 of myeloma patients were analyzed. IgG2 possessed the same kinds of biantennary complexes as the predominant IgG1 subclass but showed different galactose distributions. The results suggest that protein conformation may influence the structures of their glycolid chains. Variations in the specificity of galactosyltransferases for native and denatured IgG1 are also seen.

MATERIALS AND METHODS

Neuraminidase from Arthrobacter ureafaciens and a-L-fucosidase from bovine kidney were obtained from Nakarai Chemical Co., and Sigma, respectively. Bovine milk galactosyltransferase, UDP-Gal, and UDP-GlcNAc were obtained from Sigma. Anhydrous hydrazine, so-
dium cyanoborohydride, and 2-aminopyridine were purchased from Pierce Chemical Co., Aldrich, and Nakarai Chemical Co., respectively. 2-Aminopyridine was recrystallized from 1-hexane, but other chemicals were used without further purification. A crude N-acetylglucosaminyltransferase III was prepared from bovine kidney according to the method of Nishikawa et al. (23, 24). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad) (25).

**Purification of IgG—** Bovine IgG was prepared from plasma by precipitation with ammonium sulfate and DEAE-cellulose chromatography. The purity of the isolate was confirmed by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel in the presence of 2-mercaptoethanol.

Human serum or plasma were collected from healthy volunteers and from patients showing elevated levels of monoclonal IgG. Thirty IgGl and three IgG2 proteins were isolated from the latter sources. The IgG fraction was first precipitated with 18% sodium sulfate, the precipitate dissolved, and then reprecipitated at 14% saturated sodium sulfate. This IgG fraction was dialyzed against pH 8.0, 20 mM phosphate buffer, and applied to a DEAE-cellulose column equilibrated with the same buffer. Aliquots of the IgG fractions eluted were subjected to electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel containing 2-mercaptoethanol. Those fractions found to contain single components were used for HPLC analysis and galactosylation experiments.

**Preparation of Sugar Chains from IgG—** The proteins employed were denatured with guanidine HCl and 2 M EDTA and incubated in a nitrogen atmosphere at 50°C for 30 min. 51 mg of dithiothreitol was added and the reaction was allowed to proceed at room temperature for 20 min in the dark (26) and the iodoacetamide to maintain a pH of 8.1. The reaction was allowed to proceed for varying times and the reaction was stopped by heating at 100°C for 3 min. N-Acetylglucosaminyltransferase III from the bovine kidney and human B-cell were assayed in a pH 6.5, 0.125 M Mes buffer containing 20 mM UDP-GlcNAc, 80 mM substrate, 10 mM MnCl₂, and 0.5% (w/v) Triton X-100 (25, 26). Four kinds of pyridylaminated fucosylated biantennary complexes were used as substrates. The reaction was stopped after varying periods of incubation by heating at 100°C for 3 min. The structures of all of the sugar chains used as substrates for both enzyme assays were determined by 'H NMR spectroscopy.

**Galactosylation of Human IgG1—** A preparation of this protein isolated from the serum of a single myeloma patient was employed. The reaction was carried out in 0.5 ml of pH 7.2, 50 mM Hepes buffer containing 4 ng of the myeloma IgG1 (native or denatured form), 20 mM UDP-Gal, and 10 mM MnCl₂. The galactosyltransferases employed were commercial bovine milk enzyme (Sigma) and myeloma cell sonicates. After the reaction with bovine milk galactosyltransferase, the solution was dialyzed against distilled water and lyophilized. However, after use of the myeloma cell homogenate, the solution was adjusted to pH 8.2 and applied to an Affi-Gel Protein A column equilibrated with pH 8.2, 10 mM sodium phosphate buffer containing 0.15 M NaCl. After washing the column with the same buffer, the IgG fraction was eluted with pH 3.0, 0.1 M sodium citrate buffer (30, 31), dialyzed against pH 8.0, 20 mM phosphate buffer, and then applied to a DEAE-cellulose column equilibrated with the same buffer. The purified IgG fraction was collected, dialyzed against distilled water, and lyophilized. The sugar chains of these galactosylated IgG were isolated and analyzed.

**RESULTS**

**Revised Structures of Sugar Chains of Bovine IgG—** The fluorescence-labeled sugar chains were subjected to reversed-phase HPLC and found to contain minor amounts of sialylated materials. In order to simplify the HPLC elution pattern, the mixture of sugar chains was incubated with neuraminidase and lyophilized. The sugar chains of these galactosylated IgG were analyzed and the profile of which is shown in Fig. 1. The two sugar chain components VII and VIII were found to be the major glycoforms. The sugar chains of commercial bovine IgG (Sigma) gave the same result. Fractions VII and VIII were subjected to NMR measurements and gave the spectra shown in Fig. 2. The one for VIII (Fig. 2b) indicates that it is a digalactosylated biantennary complex whose structure is shown below.

\[
\text{Gal}β1\text{-4GlcNAcβ1-2ManO1-6}\text{Galβ1-4GlcNAcβ1-2ManO1-3}
\]

**Fig. 1. Result for the pyridylaminated sugar chains of bovine IgG.** The elution conditions are given in detail under "Materials and Methods."
The above numbering system of the sugar components will be employed in ongoing discussions of structure. The chemical shifts of the anomeric C-1, the C-2 and of the methyl protons of this sugar chain are summarized in Table I. These chemical shifts show good agreement with the data of Vliegenthart et al. (32). Since the 2-aminopyridine attached to the reducing end of the sugar chain contains an imino group, protonation at this point affected the chemical shifts of its adjacent residues, i.e. the chemical shifts of the 1H NMR signals due to GlcNAc 1, GlcNAc 2 and fucose were pH dependent but the others were not.7

In the NMR spectrum for component VII (Fig. 2a), the signal at 4.472 ppm is clearly due to a galactose and the one doublet signal due to GlcNAc 5 or 5' had shifted from 4.586 to 4.558 ppm. These results show that the sugar chain contains only one galactose. The chemical shift for Man 4 was almost the same as that for peak VIII. On the other hand, the chemical shift for Man 4' was significantly upfield upon elimination of a galactose. This result clearly indicates that the biantennary complex has a galactose on the Manol-3 branch but not on the Manol-6 branch (compare with chemical shifts for sugars V and VI in Table I and Fig. 3).

**Structural Assignment of Other Sugar Chains—**After the above two sugar chains had been digested with fucosidase, they were analyzed by HPLC. Components VII and VIII shifted to the positions of III and IV, respectively (see Figs. 1 and 5). On treatment of components chains of peaks V and VI with fucosidase, their elution positions also shifted to those of III and IV, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5). These results indicate that the sugar chains giving rise to HPLC components V and VI with fucosidase, their elution positions also shifted to those of I and II, respectively (see Figs. 1 and 5).

### Table I

<table>
<thead>
<tr>
<th>No. of peak in Fig. 1</th>
<th>C-1 protons</th>
<th>Anomeric (C-1) protons</th>
<th>C-2 protons</th>
<th>Methyl protons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man1</td>
<td>Man4</td>
<td>Man4'</td>
<td>GlcNAc5</td>
</tr>
</tbody>
</table>

* These chemical shifts were pH dependent, due to the neighboring imino group.


![FIG. 2. 500 MHz 1H NMR spectra of the two major sugar chains of bovine IgG (corresponding to components VII and VIII in Fig. 1): component VII (a); component VIII (b). These spectra show the regions of 1H NMR signals due to anomeric (C-1) and methyl (Me) protons of sugar chains. The numbers and the letter F indicate the following assignments: 1, GlcNAc 1; 2, GlcNAc 2; 3, Man 3; 4, Man 4; 4', Man 4'; 5, GlcNAc 5; 5', GlcNAc 5'; 6, Gal 6; 6', Gal 6'; F, fucose. HDO and Ac indicate the signals due to the residual HDO in the 2H2O and the ammonium acetate used as elution buffer for HPLC, respectively.

2. 500 MHz 1H NMR spectra of the two major sugar chains of bovine IgG (corresponding to components VII and VIII in Fig. 1): component VII (a); component VIII (b). These spectra show the regions of 1H NMR signals due to anomeric (C-1) and methyl (Me) protons of sugar chains. The numbers and the letter F indicate the following assignments: 1, GlcNAc 1; 2, GlcNAc 2; 3, Man 3; 4, Man 4; 4', Man 4'; 5, GlcNAc 5; 5', GlcNAc 5'; 6, Gal 6; 6', Gal 6'; F, fucose. HDO and Ac indicate the signals due to the residual HDO in the 2H2O and the ammonium acetate used as elution buffer for HPLC, respectively.

3. These chemical shifts were pH dependent, due to the neighboring imino group.

4. Since the 1H NMR spectrum of component XI was observed with a mixture of components X and XI (Fig. 3e), these chemical shifts were obtained by a subtraction of the spectrum of Fig. 3 from that of Fig. 3e.
Regulation of Sugar Chain Synthesis

5.0 ..5 4.0 2.0

A Chemical Shift (ppm) to

FIG. 3. 500 MHz $^1$H NMR spectra of the sugar chains I, IV, V, VI, IX, X, XI, and XII in Fig. 1: a, I; b, IV; c, V; d, VI; e, IX; f, X; g, XI; h, XII. The $^1$H NMR spectrum for glycooid XI was obtained with a mixture of the sugar chains X and XI. The numbers and the letter F indicate the assignments: 1, GlcNAc 1; 2, GlcNAc 2; 3, Man 3; 4, Man 4; 4', Man 4'; 5, GlcNAc 5; 5', GlcNAc 5'; 6, Gal 6; 6', Gal 6'; 9, GlcNAc 9 (bisecting GlcNAc); F, fucose. HDO and Ac indicate the signals due to the residual HDO in the $^1$H_2O and the ammonium acetate used as elution buffer for HPLC, respectively.

The $^1$H NMR spectrum for glycooid VI (Fig. 3d) is similar to the spectrum shown in Fig. 2a. However, the signal of Man 4' showed a similar chemical shift to that seen in Fig. 2b and not to that of Fig. 2a. These results show that the sugar chain VI was a biantennary complex with a galactose only on the Man1-6 branch, as shown for VI in Fig. 4. Since the sugar chains I, II, III, and IV corresponded to the defucosylated ones of V, VI, VII, and VIII in Fig. 1, these are the structures shown for I, II, III, and IV, respectively, in Fig. 4. The $^1$H NMR spectra for components I and IV (Fig. 3, a and b) also indicated that these sugar chains had the structures given for I and IV in Fig. 4.

As shown in Fig. 1, the detector responses for components III and VII were larger than those of II and VI, respectively. These results show that in bovine IgG the amount of sugar chain galactosylated at the Man1-6 branch was greater than at the Man1-6 branch, irrespective of the presence or absence of a fucose.

The $^1$H NMR spectra of Fig. 3, c–h show the anomeric proton signals of fucose around 4.86 ppm and of the bisecting GlcNAc at 4.468 or 4.469 ppm. As the amount of the sugar chain XI in Fig. 1 was relatively small and its retention time was close to that of the sugar chain X, a mixture of these components was subjected to $^1$H NMR measurement.

In the $^1$H NMR spectrum for component IX (Fig. 3e), a doublet signal of galactose was not observed at 4.475 or 4.476 ppm. This result shows that IX has the structure indicated for it in Fig. 4. On the other hand, the $^1$H NMR spectrum of Fig. 3h showed that the doublet signals corresponded to two galactoses at 4.475 ppm. These results demonstrated that the structure of the sugar chain XII in Fig. 1 was that indicated in Fig. 4. The $^1$H NMR spectrum in Fig. 3f showed a midpoint 4.476-ppm doublet signal indicative of one galactose. The results of Fig. 3g indicate the presence of two glycooids, i.e. components X and XI of Fig. 1. It was confirmed by HPLC analysis that in this sample the amount of the sugar chain XI was about half of that of X. Therefore, the signal at 5.006 ppm in Fig. 3g was due to the same component as that in Fig. 3f. The peak at 4.996 ppm in Fig. 3g is due to sugar chain XI. The $^1$H NMR signal intensity of galactose at 4.476 ppm was almost the same as that of the bisecting GlcNAc at 4.468 ppm. These results confirmed that sugar chain XI contains one galactose. The chemical shifts of 5.006 ppm for component X and of 4.996 ppm for component XI corresponded to those due to Man 4' in Fig. 3, h and e, respectively. These results confirmed that sugar X was a bisected, biantennary complex with a galactose on the Man1-6 branch, and glycooid XI was a bisected, biantennary complex with a galactose on the other branch. Therefore, the sugar chains X and XI had the structures shown in Fig. 4. These results clearly indicated that in bovine IgG the bisected sugar chain with a galactose on the Man1-3 branch was a minor component.
compared with non-bisected sugar chains. To detect bisected non-fucosylated glycoids which may overlap with components V, VI, VII, and VIII in the HPLC elution pattern, a solution containing all of the sugar chains from bovine IgG was treated with fucosidase. The HPLC elution pattern is shown in Fig. 5. The four major components, designated I-IV, are the same as those in Fig. 1, respectively. The change in chromatographic profile upon removal of the fucose is clearly evident. Glycoids IX', X', XI', and XII' corresponded to the products resulting from defucosylation of sugar chains IX, X, XI, and XII shown in Fig. 1, respectively. Components IX', X', XI', and XII' (see Fig. 5) elute at the same positions as components which are unlabeled but can be noted at very low levels in Fig. 1. These results indicate that in bovine IgG most of the bisected, biantennary complexes are fucosylated.

The ratios of the 12 sugar chains (components I-XII) of bovine IgG are summarized in Table II on the basis of the areas of the peaks on the chromatograms. The data clearly show that the non-bisected sugar chains with a galactose on the Manol-3 branch, i.e. components III and VII, predominated over the ones with a galactose on the Manol-6 branch. The reverse is true for the bisected biantennary complexes, although these glycoids are present at low levels in bovine IgG.

On the other hand, in human normal IgG, the amounts of glycoids with a galactose on the Manol-6 branch, represented by components II, VI, and X are larger than those at the Manol-3 branch (components III, VII, and XII) (8, 12, 14). Results for the HPLC analysis of the sugar chains of myeloma IgG1 and IgG2 shown in Fig. 6 reveal marked differences which when referred to the structures of the different components shown in Fig. 4 indicate that these isolates were galactosylated to different degrees. We have found that such differences of galactosylation reflects the level of galactosyltransferase activity of the myeloma plasma cells producing IgG1 utilized. In all IgG1 glycid isolates (Fig. 6, a-d), Manol-6 branch was more highly galactosylated than the Manol-3 branch, irrespective of the degree of galactosylation. As anticipated these results were consistent with the sugar distributions in the IgG fraction of healthy individuals. However, as shown in Fig. 6e, in the glycid isolates of an IgG2 preparation, typical of the three different proteins assayed, the amounts of mono-galactosylation at the Manol-3 branch were larger than those at the Manol-6 branch (components II and VI). In the case of bisected sugar chains (components IX, X, XI, and XII), the amount of galactosylation at the Manol-3 branch (component XI) was very small in all IgG1 preparations but significantly elevated in that of IgG2. The distributions of the glycid components of myeloma IgG2 proteins were close to those seen in bovine IgG. These results may suggest that galactosyltransferases exist which are specific for each human IgG subclass.

**TABLE II**

<table>
<thead>
<tr>
<th>No. of peak in Fig 1</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>14</td>
</tr>
<tr>
<td>II</td>
<td>13</td>
</tr>
<tr>
<td>III</td>
<td>3.9</td>
</tr>
<tr>
<td>IV</td>
<td>4.8</td>
</tr>
<tr>
<td>V</td>
<td>11.8</td>
</tr>
<tr>
<td>VI</td>
<td>8.5</td>
</tr>
<tr>
<td>VII</td>
<td>27.1</td>
</tr>
<tr>
<td>VIII</td>
<td>32.5</td>
</tr>
<tr>
<td>IX</td>
<td>2.2</td>
</tr>
<tr>
<td>X</td>
<td>3.2</td>
</tr>
<tr>
<td>XI</td>
<td>0.5</td>
</tr>
<tr>
<td>XII</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*The structure of each sugar chain is shown in Fig. 4.

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*Fig. 5. Results for defucosylation of the sugar chains of bovine IgG with a-L-fucosidase. The components IX'-XII' corresponded to the products resulting from defucosylation of sugar chains IX-XII shown in Fig. 1, respectively.*

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*Fig. 6. Results for HPLC of the sugar chains of four IgG1 (a-d) and of one IgG2 (e) from myeloma proteins.*

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*Enzymatic Basis for Microheterogeneity—Normal IgG is known to have only one asparagine-linked sugar chain in each HC2 domain (8-10) which we have found to be heterogeneous in bovine and human IgGs. To explain this, the branch specificity of galactosyltransferase and the substrate specificity of N-acetylglucosaminyltransferase III which catalyzes the*
addition of bisecting GlcNAc to the β-linked mannose of the trimannosyl core of asparagine linked sugar chains was determined.

Branch Specificity of Galactosyltransferases—Since bovine plasma cells were not available, milk galactosyltransferase was employed. The substrate specificity of this enzyme was about the same as that from other bovine organs (liver, serum, kidney) and from human plasma cells.

Fig. 7 shows the branch specificity of the milk galactosyltransferase toward non-bisected biantennary complexes (components I and V). These glycan substrates were essentially single components. The amount of the mono-galactosylated sugar chain formed at the Manα1-3 branch (component III) was about three times larger than that at the Manα1-6 branch (component II). This result shows that in the first galactosylation the reaction rate toward the Manα1-3 branch is about three times greater than that toward the other branch. On using the agalactosyl, non-bisected, fucosylated biantennary complex (component V) as the substrate, the branch specificity was almost the same as the non-fucosylated chains. A similar distribution pattern of galactose is seen for the non-fucosylated, non-bisected sugar chains (components I-IV) and for the fucosylated, non-bisected sugar chains (components V-VIII) as shown by the data of Fig. 7, d and e.

The specificity of the galactosyltransferase toward the bisected biantennary complexes (components IX' and IX) is illustrated by the data of Fig. 8. The substrates employed were essentially pure components. In the early stage of the reaction (Fig. 8a), the amount of the mono-galactosylated glycan formed at the Manα1-3 branch (component XI') is slightly larger than that at the Manα1-6 branch (component X'). This result shows that in the first galactosylation of the bisected biantennary complex, the reaction rate toward the Manα1-3 branch is only somewhat greater than that toward the Manα1-6 branch. A similar substrate specificity was obtained with the fucosylated, bisected biantennary complex (component XI). The distribution of components after 20 min with the fucosylated substrate (see Fig. 8e) is similar to what is seen for the bisected sugar chains in bovine IgG (see Fig. 1). However, the amount of galactosylation at the Manα1-3 branch and the Manα1-6 branches was almost the same in the enzymatically formed products.

In the case of the non-bisected biantennary complexes, the galactosylation of the Manα1-3 branch was noted (see Fig. 7) to be higher than the Manα1-6 branch as reported by other groups (17-21, 26). However, in the case of the bisected biantennary complex the amounts of X' and XI' formed were comparable (see Fig. 8) showing that the activity toward the two branches was similar.

For human galactosyltransferase, bone marrow cells of myeloma patients producing either IgG1 or IgG2 were collected. The substrate specificity of the enzyme were also studied. In the galactosylation of non-bisected sugar chains (components I and V), the activity toward the Manα1-3 branch to form components III and VII was about three times higher than...
that at the Manα1–6 branch which gives components II and VI. However, in the galactosylation of the bisected sugar chains to form components X and XI, the activity toward the Manα1–3 derivative (XI) was only slightly more than at the Manα1–6 branch (X). This specificity was quite similar to bovine milk galactosyltransferase and was the same as those of all such myeloma cell enzyme preparations. If galactosyltransferases specific to the subclass of IgGs existed, differences in the specificities of enzyme from myeloma cells producing IgG1 and IgG2 would be expected. However, this was not observed in the present study. These results suggest that the component distributions in the sugar chains of human IgG are affected not by the property of galactosyltransferase but by differences in the proteins of the IgG subclasses.

**Substrate Specificity of N-Acetylgalactosaminyltransferase III**—To explain the finding that component X is present in a much greater amount than XI in the bisected glycoforms of bovine and human IgGs, we examined the substrate specificity of N-acetylgalactosaminyltransferase which gives rise to bisected sugar chains. The data of Fig. 9 revealed that a non-galactosylated biantennary complex (component V) is the best substrate for the bovine kidney enzyme. Under our assay condition, no products were detected for biantennary complex mono-galactosylated on the Manα1–3 branch (component VII) and for the di-galactosylated one (component VIII). The enzyme from all human myeloma cells showed a quite similar specificity to the bovine kidney enzyme (data not shown).

These results indicate that component X was formed from component VI by this enzyme but that component XI was not formed from component VII. This supports the fact that in bovine IgG the glycoform X is present in greater amount than XI. These results agree with those of the previous studies on the substrate specificity of hen oviduct N-acetylgalactosaminyltransferase III (34–37).

The data of Table II for the non-bisected biantennary complexes of bovine IgG show greater amounts of the mono-galactosylated complexes on the Manα1–3 branch of both non-fucosylated and fucosylated chains (components III and VII, respectively) than on the Manα1–6 branch (components II and VI, respectively). These results agree with the branch specificity of bovine galactosyltransferase. In the case of the fucosylated, bisected, biantennary glycoforms, the amount of the mono-galactosylated derivative on the Manα1–3 branch (component XI) was much lower than X. This result can be explained not only by the specificity of the galactosyltransferase but by the substrate specificity of N-acetylgalactosaminyltransferase III.

However, these specificities cannot explain the result that in human IgG1 a non-bisected sugar chain galactosylated at the Manα1–6 branch is present in greater amounts than one galactosylated at the Manα1–3 branch. Human IgG1 and IgG2 subclasses differ in the distribution of their galactose residues as shown in Fig. 6. These results may suggest that the protein affects the distributions of components in the sugar chains.

**Galactosylation of Native Human IgG1 by Bovine Milk Galactosyltransferase**—The glycoform of one of IgG1 samples was found to be made up largely of the non-galactosylated biantennary material, component V, as shown in Fig. 6a. This IgG1 was reacted with bovine milk galactosyltransferase which possesses a similar substrate specificity for free sugar chains as the enzyme of human B-cells. The results obtained are shown in Fig. 10 and reveal that the products of the reaction resemble some of the glycoforms of the IgG1 myeloma proteins shown in Fig. 6. The degree of galactosylation increased with the amount of galactosyltransferase added. The amounts of non-bisected sugar chains showing major monogalactosylation at the Manα1–6 branch (component VI) were larger than that at the Manα1–3 branch (component VII). These results were consistent with the distribution of components in all of the myeloma IgG1 glycoform isoelectric points (see Fig. 6, a–d). The control experiment lacking UDP-Gal was same as the IgG1 with a low degree of galactosylation (Fig. 6a).

In contrast to the above results, galactosylation of free, non-bisected sugar chains with bovine milk galactosyltransferase produced more of the derivative at the Manα1–3 branch than at the Manα1–6 branch. These results also indicate that the distribution of sugar chains in human IgG is affected not only by the branch chain specificity of the galactosyltransferase but by the protein being galactosylated. To directly test this possibility, the experiments reported in the next section were performed.

**Galactosylation of Denatured IgG1 by Bovine Milk Galactosyltransferase**—The denatured IgG was prepared from the same myeloma IgG1 to provide the results shown in Figs. 6a and 10a. Galactosylation of the denatured protein was carried out in the same manner as previous. The HPLC profiles of its sugar chain components are shown in Fig. 11. It is apparent that the amounts of the mono-galactosylated derivatives of the non-bisected sugar chains at the Manα1–3 branch (components II and VI) are also higher than that at the Manα1–6 branch (components II and VI). It is apparent that components II and III are present at very low levels but the predominance of component III over II is readily apparent in Fig. 11b. Galactosylation of the bisected sugar chains in native IgG1 showed that only a small amount of the Manα1–3 branch was derivatized (see component XI in Fig. 10). However in

![Fig. 9. Substrate specificity of N-acetylgalactosaminyltransferase III of bovine kidney for a fucosylated non-galactosylated biantennary complex (a, component V), a fucosylated biantennary complex mono-galactosylated on the Manα1–6 branch (b, component VI), a fucosylated biantennary complex monogalactosylated on the Manα1–3 branch (c, component VII), a fucosylated di-galactosylated biantennary complex (d, component VIII), and a mixture of these sugar chains (e–g). The concentrations of substrates of a–d were 80 μM and in e–g were 20 μM of each of the sugar chains. The reactions were carried out for 3 h in a–d, 1 h in e, 2 h in f, and 4.5 h in g. Protein concentrations from the bovine kidney homogenates were 5.6 mg/ml in the reaction mixtures.](http://www.jbc.org/)
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Fig. 10. Results for HPLC of the sugar chains of a myeloma IgG1 (IgG1 shown in Fig. 6a) galactosylated for 60 min with various amounts of bovine mild galactosyltransferase: a, galactosylation without UDP-Gal; b–d, with UDP-Gal. The amount of galactosyltransferase used are as follows: a, 300 milliunits; b, 100 milliunits; c, 200 milliunits; d, 300 milliunits.

Fig. 11. Results for HPLC of the sugar chains of denatured myeloma IgG1 (IgG1 shown in Fig. 6a) galactosylated for 60 min with various amounts of bovine milk galactosyltransferase. The amount of galactosyltransferase used are as follows: a, 15 milliunits; b, 50 milliunits; c, 100 milliunits.

Fig. 12. Result for HPLC of the sugar chains of myeloma IgG1 (IgG1 shown in Fig. 6a) galactosylated for 3 h with 150 µl of a mixture of myeloma cell homogenates.

the case of the denatured IgG1, this derivative increased slightly (Fig. 11). This indicates that the native structure of IgG1 apparently interferes with the galactosylation of the Manα1-3 branch more than the Manα1-6 branch. It is apparent that the native conformation of human IgG influences the type of galactosylation of its sugar chains.

Galactosylation of Native IgG1 with Myeloma Cell Galactosyltransferase—The result for the galactosylation of the IgG1 protein whose sugar chain compositions are by the data shown in Figs. 6a and 10a is shown in Fig. 12. The degree and type of galactosylation was similar to that obtained with 100 milliunits of bovine milk galactosyltransferase in the experiment shown in Fig. 10a. These results indicate that the specificity of the galactosyltransferase from human myeloma cells for the glycoids of human IgG was similar to that of bovine milk galactosyltransferase.

DISCUSSION

Studies on the specificities of galactosyltransferases have shown that they have activity for both branches of biantennary sugar chains (17-21, 29) and that the ratio of the specific activities remains unchanged at each step during their purification (29). These results also suggested that the same enzyme galactosylated both branches. The galactosyltransferase activity toward the Manα1-3 branch was higher than toward the Manα1-6 branch in non-bisected biantennary complexes in the first galactosylation step (17-21, 29). We have also confirmed that galactosyltransferases from bovine milk and from human B-cells show higher activity toward the Manα1-3 branch in the first galactosylation of the biantennary complex. Blanken et al. (20) pointed out the discrepancy between the higher activity toward the Manα1-3 branch and the result of Tai et al. (13) that indicated the Manα1-6 branch to be more highly galactosylated in the glycoaid chains of bovine IgG. Another group (19, 21) reported that in the second galactosylation step of non-bisected biantennary complexes to form the di-galactosylated complexes, the activity toward the Manα1-6 branch was higher. Based on these results, they concluded that in the early stage of galactosylation the amount of the mono-galactosylated biantennary complex at the Manα1-3 branch was larger than that at the Manα1-6 branch but that the ratio of these two sugar chains would reverse due to a rapid increase of galactosylation of the Manα1-6 branch. These considerations are difficult to understand but support the result of Tai et al. (13). They are, however, in disagreement with our results which clearly show that the major mono-galactosylation site was the Manα1-3 branch. Moreover, we found that in the non-bisected biantennary complex the ratio of the mono-galactosylated Manα1-3 branch to that of the Manα1-6 branch did not reverse with time of reaction but increased slightly with degree of galactosylation. This result indicates that in the second galactosylation step of the non-bisected sugar chains the activity
toward the Man\(\alpha1-3\) branch was slightly higher than that toward the Man\(\alpha1-6\) branch. These results agree with the component distribution of the non-bisected sugar chains of bovine IgG found in this study.

However, we have found that in bisected, biantennary complexes of bovine IgG the amount of mono-galactosylated sugar chain on the Man\(\alpha1-6\) branch was greater than on the Man\(\alpha1-3\) branch. This result could not be explained by the specificity of the galactosyltransferase. The activity of this enzyme toward the bisected biantennary complex was lower than toward the non-bisected one. The large decrease of the activity toward the Man\(\alpha1-3\) branch could be due to the steric hindrance of the bisecting GlcNAc (38, 39). In the early stage of galactosylation, the amounts of both mono-galactosylated biantennary complexes was almost the same. The ratio of the mono-galactosylated Man\(\alpha1-3\) branch to that of the Man\(\alpha1-6\) branch gradually decreased with increasing galactosylation (Fig. 8). However, the branch specificity did not introduce a large difference in the amounts of the two mono-galactosylated bisected sugar chains of bovine IgG (components X and XI in Fig. 1). In the second galactosylation step to form the di-galactosylated derivatives, the activity toward the Man\(\alpha1-6\) branch is slightly higher than that toward the Man\(\alpha1-3\) branch.

The substrate specificities of N-acetylglucosaminyltransferase III and of galactosyltransferase explain the distribution of the bisected sugar chains of bovine IgG. The N-acetylglucosaminyltransferase III from bovine kidney showed very different activities toward some substrates (Fig. 9). It appeared to have a high specificity for a non-galactosylated sugar chain and its activity toward the mono-galactosylated Man\(\alpha1-6\) branch decreased about 3-fold. No activity was noted for sugar chains galactosylated at the Man\(\alpha1-3\) branch. This substrate specificity was similar to that of N-acetylglucosaminyltransferase III from hen oviduct (34–37) and from human bone marrow myeloma cells. However, the non-bisected chain mono-galactosylated at the Man\(\alpha1-6\) branch was a good substrate for N-acetylglucosaminyltransferase III.

In the second galactosylation steps of both non-bisected and bisected biantennary complexes, the activities toward both branches were essentially the same. We summarized the branch specificity of galactosyltransferase and substrate specificity of N-acetylglucosaminyltransferase in Scheme 1 to explain the component distribution of the mono-galactosylated non-bisected and bisected sugar chains of bovine IgG. This projection indicates that the fractional ratio of the two mono-galactosylated biantennary complexes was mainly due to the branch specificity of galactosyltransferase, and the ratio of the two mono-galactosylated bisected biantennary complexes resulted not only from this specificity but also from the substrate specificity of N-acetylglucosaminyltransferase III. Thus, the component distribution of sugar chains of bovine IgG can be explained by the properties of these two glycosyltransferases when free sugar chains are employed as substrates. These results suggest that in bovine IgG one branch of a biantennary complex was not more deeply buried in the protein molecule than the other branch.

Human IgG1 and bovine IgG have the same kinds of sugar chains but differed in the distributions of their components (see Figs. 1 and 6). We attempted to interpret these differences in terms of possible unique substrate specificities of the galactosyltransferases from myeloma cells but find them to be similar to the bovine milk enzyme. A difference in the type of galactosylations for free sugar chains and those attached to proteins have been found for the same galactosyltransferase. Thus, when free sugar chains are employed as substrates, the amount of non-bisected biantennary complex galactosylated at the Man\(\alpha1-3\) branch was larger than that at the Man\(\alpha1-6\) branch. However, this ratio was reversed in the glycochain chains of human IgG. As anticipated, the ratio of such derivatives in monoclonal IgG1 was about the same as that in normal IgG. However, their ratios in IgG2 were different from those of normal IgG and monoclonal IgG1. Since the specificity of galactosyltransferase from the myeloma cells producing IgG1 and from those producing IgG2 are the same, these differences in the distribution of sugars in their glycochain chains would appear to be due to differences in the structures of these proteins.

When native IgG1 was galactosylated, little derivatization of the Man\(\alpha1-3\) branch was observed in contrast to denatured protein. These results suggest that the Man\(\alpha1-3\) branch of the glycochain chains are more deeply buried in the native IgG1 than in IgG1 but are exposed upon denaturation. Thus, in the bisected sugar chains of human IgG1, the mono-galactosylated complex at the Man\(\alpha1-3\) branch was present at a low level in comparison with human IgG2 and bovine IgG. These results suggest that galactosylation of the sugar chain of human IgG results in changes in its conformation or that differences in the conformation affect the types of glycochain.

Deisenhofer et al. (9, 40, 41) have showed by crystallographic studies that the sugar chain in human IgG is buried in the protein. Our galactosylation results suggest that in human IgG1 the Man\(\alpha1-3\) branch is less accessible than the Man\(\alpha1-6\) branch. It is possible that the crystal structures of different subclasses of IgG will show that the environments around the sugar chains are different.

We have analyzed the substrate specificities of galactosyltransferase from many myeloma cells which produced either IgG1 or IgG2 but have found them to be similar to the enzymes from other sources (17–21). No evidence was obtained that would suggest the existence of galactosyltransferases specific for human IgG as reported by Kobata et al. (22). Many glycosyltransferases have been purified and their properties analyzed (17–21, 33, 42–44). Further studies should reveal whether these enzymes are responsible for the unique distribution of sugar chains in different proteins. Our study clearly supports the likelihood that the structure of a protein will affect the synthesis and type of sugar distributions in the glycoc chain chains. The results for galactosylation experiments performed on native and denatured IgG and on free sugar chains which reveal different degrees of derivatizations at the Man\(\alpha1-3\) and \(\alpha1-6\) branches supports such considerations.

In conclusion, (i) substrate specificities and activities of glycosyltransferases predominantly affect the synthesis and type of sugar distributions in the exposed carbohydrate structure of the protein, (ii) the levels of sugar derivatizations on the Man\(\alpha1-3\) branch increase more than on the Man\(\alpha1-6\) branch, and (iii) the substrate specificities and activities of glycosyltransferases are not unique to any one protein but are subject to change during the synthesis of the protein.
chains, and (ii) the structure of the protein may influence the availability of its sugar chains for reactions with various glycosyltransferases and thus affect the structures of its carbohydrate chains.

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