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Structural study of N-linked sugar chains of sheep erythrocyte membrane glycoproteins

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Our previous study showed that non-reducing terminal galactose residues of N-linked sugar chains present in sheep erythrocyte membrane glycoproteins are important for rosette formation with T lymphoblastic cells [Ogasawara *et al.* (1995) *Immunol Lett* 48: 35–38]. As a first step to elucidate the significant structures of sugar chains involved in rosette formation, we analysed N-linked sugar chains released from the membrane glycoproteins by hydrazinolysis. The oligosaccharides were labeled with NaB^3H_4 and fractionated using columns of *Aleuria aurantia* lectin-Sepharose, MonoQ and Bio-Gel P-4. Structural analyses of oligosaccharides by sequential exoglycosidase digestion in combination with methylation analysis revealed that the membrane glycoproteins contain bi- (19%), tri- (33%), and tetraantennary (44%) complex-type oligosaccharides and that the oligosaccharides having exposed galactose residues amount to 40% of the total.

***Keywords*: N-linked sugar chains, sheep erythrocyte, membrane glycoproteins**

Introduction

Sheep erythrocytes are known to form spontaneous aggregates termed 'rosette' with human T lymphocytes [1, 2]. This rosette formation has been shown to be mediated by the specific interaction between a glycoprotein called CD2 on T lymphocytes and its ligands on sheep erythrocytes [3, 4]. Rosette formation has been used as a model for CD2-mediated cell adhesion, because a physiological ligand, a glycoprotein called CD58, is broadly expressed on various human cells including erythrocytes, epithelial cells, endothelial cells, fibroblasts and most cells of hematopoietic origin [5], and the interaction of CD2 and CD58 functions not only in adhesion but also T cell activation [5, 6].

Human erythrocytes are known to be unable to form rosettes with autologous peripheral T lymphocytes [7]. This inability has been explained by the lower density of CD2 ligands on human erythrocytes than on sheep erythrocytes [8]. However, various human cells expressing the same level of CD2 ligands differ in the reactivity with CD2, indicating that the expression of ligand molecules on the cell surfaces does not necessarily correlate with the efficiency of the cells to interact with CD2 [9]. This suggests that the post-translational modification of the ligand molecules such as glycosylation may be another factor regulating the potential of ligands for CD2. Considering such a possibility and accumulating evidence indicating the presence of multiple ligand molecules for CD2 in human cells [9–11] and sheep erythrocytes [12], it is of interest to examine whether the CD2-mediated adhesion is affected by the carbohydrate moieties of the ligands. In a previous study [13], we have demonstrated that rosette formation of T lymphoblastic Molt-3 cells with sheep erythrocytes was inhibited by the addition of sheep erythrocyte membrane glycoproteins (sGP) and this inhibitory activity was reduced by diplococcal β-galactosidase digestion of sGP while it was enhanced by sialidase digestion. The inhibitory activity of sGP was also reduced by digestion with *N*-glycanase, but not with *O*-glycanase. These results suggest that terminal galactose residues of N-linked sugar chains of sGP are important for rosette formation. However, their structural information is not available. Therefore, in this paper we analysed the structures of N-linked sugar chains of sGP as a first step to finding a clue to the identification of functional sugar chains which may be involved in rosette formation with T lymphocytes.

Materials and methods

Materials

Sheep preserved bloods were obtained from Nippon Bio-Test Laboratories Inc., Tokyo. Lithium diiodosalicylate, Schiff's reagent, NaB^2H_4 and sialidase from *Arthrobacter ureafaciens* were purchased from Nacalai Tesque Inc., Kyoto. AG50 W-X12, AG3-X4A and the silver stain kit were obtained from Bio-Rad Laboratories Inc., New York.

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NaB^3H_4 (358 mCi mmol^{-1}) was purchased from New England Nuclear, Boston, MA. Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purchased from Boehringer Mannheim Yamanouchi, and β-*N*-acetylhexosaminidase from jack bean was purified from jack bean meal by the method of Li and Li [14]. *Aleuria aurantia* lectin (AAL)-Sepharose was kindly donated by Dr N. Kochibe, Gunma University, and *Sambucus sieboldiana* (SSA)-agarose was obtained from Honen Corporation, Tokyo.

Preparation of sheep erythrocyte membrane glycoproteins

Ghosts were obtained by hypotonic analysis of sheep erythrocytes. Membrane glycoproteins were solubilized from ghosts by the lithium diiodosalicylate extraction method [15], and termed sGP in this paper.

Analysis of glycoproteins and sugar composition

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [16]. Proteins and sugars on the gels were visualized by silver staining and periodic acid-Schiff (PAS) staining. sGP (50 μg) was heated in 1 ml of 4 N trifluoroacetic acid at 100 °C for 3 h for the composition analysis of neutral and amino sugars, or in 1 ml of 0.01 N HCl at 100 °C for 20 min for the composition analysis of sialic acid. The hydrolysates were freed from the acid by repeated evaporation with water, and were analysed by high pH anion exchange chromatography (HPAEC) using a Bio-LC (Dionex Corp., Sunnyvale, CA) equipped with a CarboPac PA-1 column (9 × 250 mm) and a pulsed amperometric detector [17]. Elutions were carried out isocratically using 22 mM NaOH for the neutral and amino sugar composition analysis and 100 mM NaOH/150 mM sodium acetate for the sialic acid composition analysis, respectively. Protein content was measured by the Lowry's method [18].

Liberation of N-linked sugar chains from sGP

sGP (20 mg protein) dried thoroughly over P_2O_5 *in vacuo* was subjected to hydrazinolysis for 10 h as described previously [19]. After *N*-acetylation, one-fourth of the liberated oligosaccharides mixture from sGP was reduced with NaB^3H_4 to obtain tritium-labeled oligosaccharides for structural analysis, and the remainder was reduced with NaB^2H_4 to obtain deuterium-labeled oligosaccharides for methylation analysis. To facilitate detection of the deuterium-labeled oligosaccharides, one-tenth of the tritium-labeled oligosaccharides was added. The radioactive oligosaccharides were purified by paper chromatography using ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol) as a solvent.

Analytical methods

AAL-Sepharose column chromatography was carried out with modifications of the previous method [20] as follows. Oligosaccharides applied to the column (1 ml) were eluted with 10 mM Tris-acetate buffer, pH 7.4 and the bound oligosaccharides were eluted with the buffer containing 5 mM L-fucose. SSA-agarose column chromatography [21] was also modified as follows. The oligosaccharides were applied to the column (2 ml) and eluted with 50 mM Tris-acetate buffer, pH 7.4 and the bound oligosaccharides were eluted with the buffer containing 0.2 M lactose. MonoQ anion exchange column chromatography [22] and Bio-Gel P-4 column chromatography [23] were performed as described in the cited references. Methylation analysis of oligosaccharides was carried out as previously described [24].

Exoglycosidase digestion

Radioactive oligosaccharides were incubated with one of the following reaction mixtures at 37 °C for 18 h: 100 mU of *A. ureafaciens* sialidase in 0.1 M acetate buffer, pH 5.0 (50 μl); 10 mU of diplococcal β-galactosidase in 0.3 M citrate phosphate buffer, pH 6.0 (50 μl); 4 mU diplococcal β-*N*-acetylhexosaminidase in 0.3 M citrate phosphate buffer, pH 5.5 (50 μl); and 0.7 U of jack bean β-*N*-acetylhexosaminidase in 0.3 M citrate phosphate buffer, pH 5.0 (50 μl). A small amount of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Reactions were terminated by heating the reaction mixtures in boiling water for 3 min. The sialidase digest was applied to a small column (0.5 ml) of AG50W-X12 (H^+ form), and eluted with five bed volumes of water. The effluent was evaporated to dryness, and subjected to MonoQ anion exchange column chromatography. Each of the other digests was applied to a small column packed with 0.5 ml each of AG50W-X12 (H^+ form) in the upper layer and AG3-X4A (OH^- form) in the lower layer, and eluted with five bed volumes of water. The effluent was evaporated, and subjected to Bio-Gel P-4 column chromatography.

Results

Chemical nature of sGP obtained from sheep erythrocyte ghosts

When sGP solubilized from the ghosts by the lithium diiodosalicylate extraction method was subjected to SDS-PAGE, five bands with molecular masses of 95 kDa, 74 kDa, 53 kDa, 31 kDa, and 26 kDa were stained by periodic acid-Schiff reagent (Figure 1B). When increasing amounts of samples were applied, two additional bands with molecular masses of 43 kDa and 20 kDa became visible (data not shown). Monosaccharide composition analysis of sGP indicated that it contains much higher amounts of *N*-acetylgalactosamine compared with mannose (Table 1). Assuming that O- and N-linked sugar chains contain one *N*-acetylgalactosamine residue and three mannose residues per molecule respectively, it is roughly calculated that

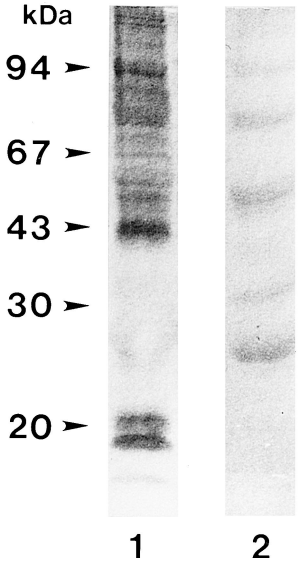

Figure 1. SDS-PAGE of sGP. Electrophoresis was performed as described in Materials and methods. Bands in lanes 1 and 2 were visualized by silver staining and PAS staining, respectively. Arrowheads in the left indicate the positions of standard proteins: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa).

Table 1. Monosaccharide composition of sGP

Monosaccharide	*Content ($\mu g\, mg^{-1}$ protein[a])*
Fuc	1.2
Man	5.0
Gal	91.7
GlcNAc	39.0
GalNAc	75.5
Neu5Ac	69.0
Neu5Gc	185.2

[a]The protein content was determined by the Lowry's method

O-linked sugar chains are thirty times as much as N-linked sugar chains. Both Neu5Ac and Neu5Gc were detected in a ratio 1:2.7. The carbohydrate content of sGP was approximately 30% (by weight).

Release of N-linked oligosaccharides from sGP and their fractionation by AAL-Sepharose chromatography and MonoQ anion exchange column chromatography

Because of the importance of N-linked sugar chains in rosette formation as previously observed [13], sGP was subjected to hydrazinolysis and the released oligosaccharides were labeled with NaB^3H_4. The radioactive

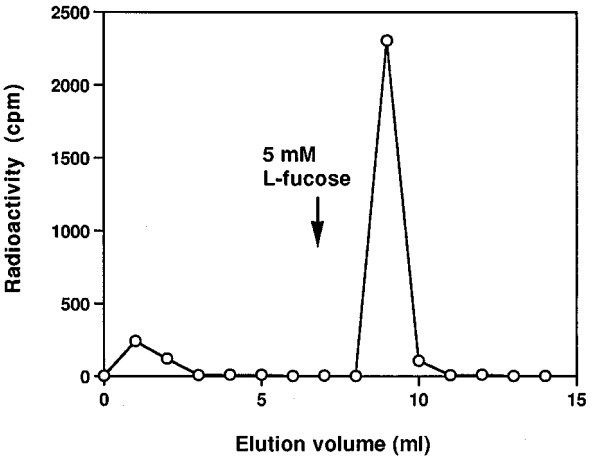

Figure 2. Fractionation of oligosaccharides by AAL-Sepharose column chromatography. The tritium-labeled oligosaccharide fraction obtained from sGP by hydrazinolysis was applied to an AAL-Sepharose column and eluted as described in Material and methods. Aliquots were taken for measurement of radioactivity.

oligosaccharides thus obtained were first fractionated using an immobilized AAL column which has an affinity to various fucosylated oligosaccharides [20]. As shown in Figure 2, 87% of the oligosaccharides as calculated from radioactivity were retained by the column and eluted with 5 mM L-fucose.

When both fractions unbound and bound to the AAL column were subjected to MonoQ anion exchange column chromatography, the AAL-unbound fraction was separated into a neutral (**N**) and three acidic fractions, **A1**, **A2**, and **A3** (Figure 3A), and the AAL-bound fraction to a neutral (**N**) and four acidic fractions, **A1**, **A2**, **A3**, and **A4** (Figure 3B). All of these acidic fractions were completely converted to neutral ones by exhaustive digestion with sialidase from *A. ureafaciens* (data not shown), and named **A1N**, **A2N**, **A3N** and **A4N**, respectively. Therefore, the acidic nature of these fractions was due to their sialic acid residues, and it was assumed that the fractions **A1**, **A2**, **A3** and **A4** are mono-, di-, tri- and tetrasialylated derivatives, respectively. On the basis of the radioactivities incorporated into the oligosaccharides, the percent molar ratio of **N**, **A1**, **A2**, and **A3** in the AAL-unbound fraction to the total was calculated as 2:4:6:1, and that of **N**, **A1**, **A2**, **A3** and **A4** in the AAL-bound fraction was 2:5:20:34:26, respectively.

Fractionation of asialo-oligosaccharides by Bio-Gel P-4 column chromatography

Asialo-oligosaccharides obtained by sialidase digestion of the acidic fractions as mentioned above were fractionated by Bio-Gel P-4 column chromatography. Among the AAL-unbound oligosaccharides, the fraction **A1N** (Figure 4A) and **A2N** (Figure 4B) were separated into three components

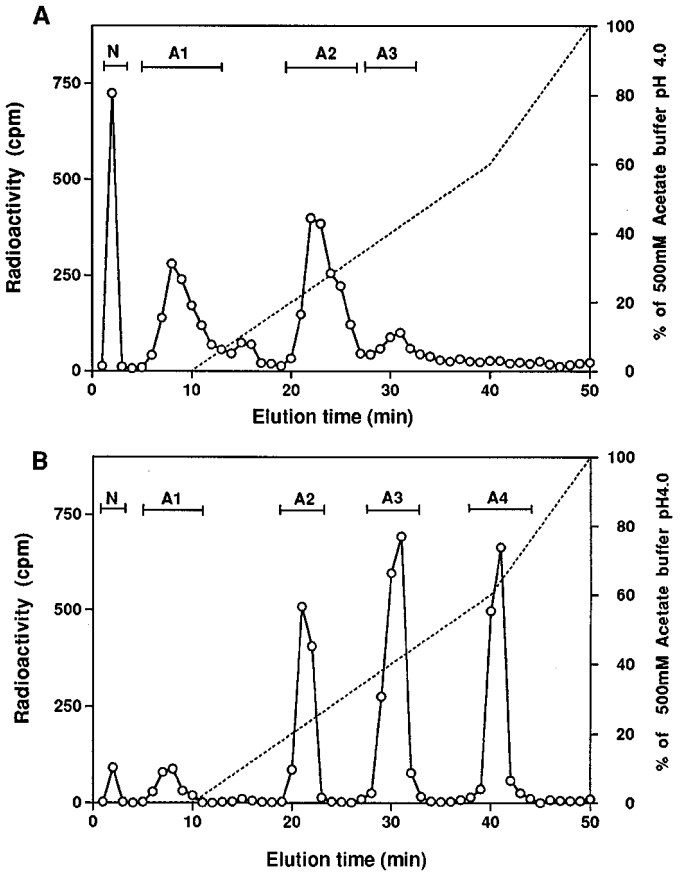

Figure 3. Fractionation of oligosaccharides unbound and bound to the AAL-Sepharose by MonoQ anion exchange column chromatography. (**A**) The AAL-unbound oligosaccharide fraction; (**B**) the AAL-bound oligosaccharide fraction. Chromatographic conditions are described in Materials and methods. Aliquots were taken for measurement of radioactivity.

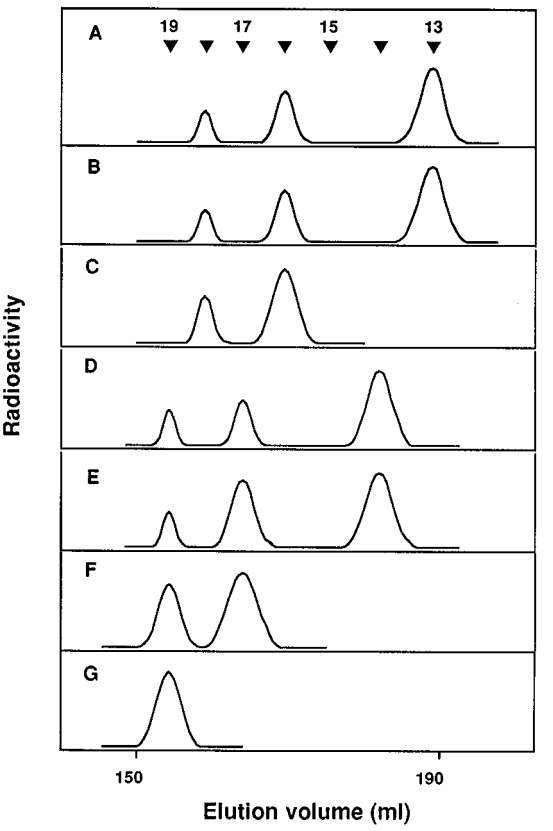

Figure 4. Bio-Gel P-4 column chromatography of asialo-oligosaccharide fractions. The acidic oligosaccharide fractions in Figure 3 were digested with sialidase, and then analysed by Bio-Gel P-4 column chromatography. (**A**), (**B**) and (**C**): **A1N**, **A2N** and **A3N** obtained from the AAL-unbound fractions, respectively. (**D**), (**E**), (**F**) and (**G**): **A1N**, **A2N**, **A3N** and **A4N** obtained from the AAL-bound fraction, respectively. Black arrowheads at the top of the figure indicate elution positions of glucose oligomers used as internal standards, and numbers indicate glucose units.

with effective sizes of 13, 16, and 18 glucose units and the fraction **A3N** (Figure 4C) was separated into two components with 16 and 18 glucose units. In the case of the oligosaccharides recovered in the AAL-bound fractions, the fractions **A1N** (Figure 4D) and **A2N** (Figure 4E) were separated into three components with 14, 17, 19 glucose units, the fraction **A3N** (Figure 4F) to two components with 17 and 19 glucose units, and the fraction **A4N** (Figure 4G) was eluted as a single component with 19 glucose units. The amounts of the fractions **N** were not enough to be analysed.

Exoglycosidase digestion

To determine the anomeric configurations and sequences of monosaccharides in the oligosaccharides, each oligosaccharide fraction separated by Bio-Gel P-4 column chromatography was subjected to sequential exoglycosidase digestion. The pooled oligosaccharides of 19 glucose units from the AAL-bound fraction (Figure 4 D–G) were digested with diplococcal β-galactosidase which can cleave the Galβ1 → 4GlcNAc linkage, but neither the Galβ1 → 3GlcNAc nor the Galβ1 → 6 GlcNAc linkage [25]. This digestion resulted in the removal of four galactose residues (Figure 5A). By the following digestion with diplococcal β-*N*-acetylhexosaminidase which can cleave the GlcNAcβ1 → 2Man linkage in the GlcNAcβ1 → 4 (GlcNAcβ1 → 2)Man group but not in the GlcNAcβ1 → 6 (GlcNAcβ1 → 2)Man group [26], one *N*-acetylglucosamine residues was removed (Figure 5B). The radioactive product in Figure 5B was then converted to a component with the same size (8.2 glucose units) as Manα 1 → 6(Manα 1 → 3)Manβ1 → 4GlcNAcβ1 → 4 (Fucα1 → 6) $GlcNAc_{OT}$ by digestion with jack bean β-*N*-acetylhexosaminidase (Figure 5C). Thus, it was evident that the 19 glucose unit oligosaccharide has a tetraantennary structure. When the pooled oligosaccharides of 17 glucose units from the AAL-bound fraction (Figure 4 D–F) were digested with diplococcal β-galactosidase, three galactose residues were removed (Figure 5D). The following digestion

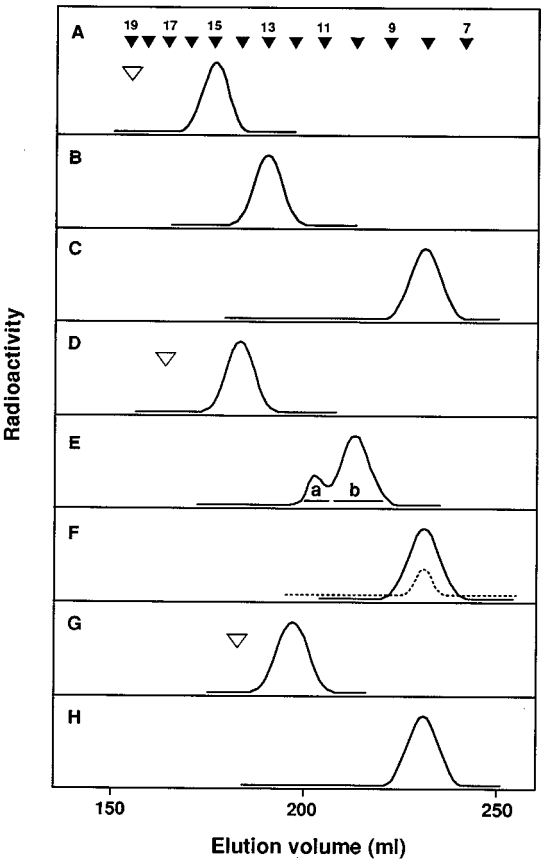

Figure 5. Sequential exoglycosidase digestion of asialo-oligosaccharides. The asialo-oligosaccharides digested with exoglycosidases were analysed by Bio-Gel P-4 column chromatography. (**A**) The pooled fractions of 19 glucose unit oligosaccharides (Figure 4 D–G) digested with diplococcal β-galactosidase; (**B**) the peak in (**A**) digested with diplococcal β-*N*-acetylhexosaminidase: (**C**) the peak in (**B**) digested with jack bean β-*N*-acetylhexosaminidase; (**D**) the pooled fraction of 17 glucose unit oligosaccharides (Figure 4 D–F) digested with diplococcal β-galactosidase; (**E**) the peak on (**D**) digested with diplococcal β-*N*-acetylhexosaminidase; (**F**) the peaks **a** (dotted line) and **b** (solid line) in (**E**) digested with jack bean β-*N*-acetylhexosaminidase; (**G**) the pooled fraction of 14 glucose unit oligosaccharides (Figure 4 D and E) digested with diplococcal β-galactosidase; (**H**) the peak in (**G**) digested with diplococcal β-*N*-acetylhexosaminidase. White triangles in (**A**), (**D**) and (**G**) indicate elution positions of 19, 17 and 14 glucose unit oligosaccharides before glycosidase digestion.

with diplococcal β-*N*-acetylhexosaminidase produced radioactive peaks **a** and **b** in a ratio of 1:5 with release of one and two *N*-acetylglucosamine residues, respectively (Figure 5E). By digestion with jack bean β-*N*-acetylhexosaminidase, two and one *N*-acetylglucosamine residues were removed from the peaks **a** and **b**, respectively, and the radioactive products with the size of 8.2 glucose units were obtained (Figure 5F). From the substrate specificity of diplococcal β-*N*-acetylhexosaminidase as described above, it was suggested that the oligosaccharide fraction eluted at the position of 17 glucose units is a mixture of two isomeric triantennary oligosaccharides, one having the GlcNAcβ1 → 4(GlcNAcβ1 → 2)Man group and the other having the GlcNAcβ1 → 6(GlcNAcβ1 → 2)Man group. The pooled oligosaccharides of 14 glucose units (Figure 4 D and E) were converted to the 8.2 glucose unit component with release of two residues each of galactose (Figure 5F) and *N*-acetylglucosamine (Figure 5G) by diplococcal β-galactosidase and β-*N*-acetylhexosaminidase digestions, respectively. All the products with 8.2 glucose units (Figure 5 C, F and H) were identified as Manα1 → (Manα1 →)Manβ1 → GlcNAcβ1 → (Fucα1 →)GlcNAc$_{OT}$ by further glycosidase digestion (data not shown).

From these results, it is assumed that the AAL-bound fraction is composed of tetraantennary, two isomeric triantennary, and biantennary complex-type oligosaccharides with fucosylated trimannosyl cores. Location of the branched groups, the GlcNAcβ1 → 4(GlcNAcβ1 → 2)Man group and the GlcNAcβ1 → 6(GlcNAcβ1 → 2)Man group, included in the isomeric triantennary oligosaccharides was estimated based on the following results (data not shown). When the peak **a** in Figure 5E was digested with jack bean α-mannosidase, one mannose residue was removed. The resulting radioactive product, GlcNAcβ1 → 6(GlcNAcβ1 → 2)Manα1 → 6 or 3 Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAc$_{OT}$ was then converted to a radioactive disaccharide, GlcNAcβ1 → 4GlcNAc$_{OT}$ by Smith degradation. Therefore, the presence of the GlcNAcβ1 → 6(GlcNAcβ1 → 2)Manα1 → 6Man sequence was proposed. On the other hand, the radioactive product, GlcNAcβ1 → 4Manα1 → 3 or 6Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAc$_{OT}$, which was obtained by jack bean α-mannosidase digestion of the peak **b** in Figure 5E, was converted to a radioactive trisaccharide, Manβ1 → 4GlcNAcβ1 → 4GlcNAc$_{OT}$. This indicates the presence of the GlcNAcβ1 → 4(GlcNAcβ1 → 2)Manα1 → 3Man sequence. The oligosaccharides in the AAL-unbound fraction were analysed in the same manner (data not shown), and were revealed to have the same structures as those in the AAL-bound fraction except for having non-fucosylated trimannosyl cores.

Methylation analysis

In order to confirm glycosidic linkage in the oligosaccharides, the NaB^2H_4-reduced acidic oligosaccharide mixture and that obtained after desialylation were subjected to methylation analysis, and the results are summarized in Table 2.

The difference found between the two samples is that both 2,4,6-tri-*O*-methyl and 2,3,4,6-tetra-*O*-methyl galactitol acetates were obtained in a ratio of 5:1 from the acidic oligosaccharides but only 2,3,4,6-tetra-*O*-methyl galactitol acetate was obtained from the desialylated oligosaccharides. This indicates that the Siaα2 → 3Gal linkage is included in the oligosaccharides. A trace amount of 2,3,4-tri-*O*-methyl galactitol acetate was also detected in the acidic

Table 2. Methylation analysis of the total acidic oligosaccharides before (A) and after (AN) sialidase digestion.

Methylated sugar	*Molar ratio*[a]	
	A	*AN*
Fucitol		
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.8	0.8
Galactitol		
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.5	2.9
2,3,4-Tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	tr.[b]	0
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	2.5	0
Mannitol		
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	0.9	1.0
3,6-Di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.6	0.6
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0.4	0.3
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0
2-N-Methylacetamido-2-deoxyglucitol		
3,6-Di-*O*-methyl (1,4,5-tri-*O*-acetyl)	3.2	3.0
1,3,5-Tri-*O*-methyl (4,6-di-*O*-acetyl)	0.7	0.8
1,3,5,6-Tetra-*O*-methyl (4-*O*-mono-acetyl)	0.1	0.1

[a]Numbers in the Table were calculated by using the underlined values as 1.0.
[b]Less than 0.05.

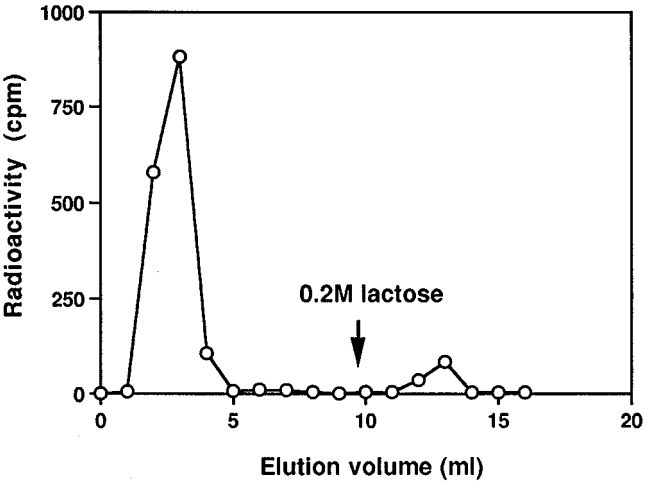

Figure 6. SSA-Sepharose column chromatography of acidic oligosaccharides. The mixture of the acidic oligosaccharides was applied to the SSA-Sepharose column, and eluted as described in Materials and methods.

oligosaccharides, suggesting that the Siaα2 → 6Gal linkage is included. This was further examined in a different way as described later. Other methylated sugar derivatives detected in both samples were as described below. The fucose residue was detected as 2,3,4-tri-*O*-methyl fucitol acetate. Both 1,3,5-tri- and 1,3,5,6-tetra-*O*-methyl 2-*N*-methylacetamido-2-deoxyglucitol acetates were obtained from the reducing terminal *N*-acetylglucosaminitol, confirming the presence of fucosylated and non-fucosylated trimannosyl cores. The inner *N*-acetylglucosamine residues were all detected as 3,6-di-*O*-methyl 2-*N*-methylacetamido-2-deoxyglucitol acetate. This also supports the presence of the Galβ1 → 4GlcNAc linkage in the outer chain moieties which was suggested by the results of diplococcal β-galactosidase digestion (Figure 5). In accordance with the bi- to tetraantennary structures of the oligosaccharides, mannose residues were detected as 2,4-di-*O*-methyl, 3,6-di-*O*-methyl, 3,4-di-*O*-methyl, and 3,4,6-tri-*O*-methyl mannitol acetates.

Analysis of sialyl linkage of oligosaccharides by SSA-agarose chromatography

Occurrence of a trace amount of the Siaα2 → 6Galβ1 → sequence in the oligosaccharides suggested by methylation analysis was further examined using an SSA-agarose column which has been shown to bind the sialylated oligosaccharides with the Neu5Acα2 → 6Gal or GalNAc group but not those with the Neu5Acα2 → 3Gal group [21]. As shown in Figure 6, 7% of the acidic oligosaccharides bound to the column. On the other hand, authentic oligosaccharides, Neu5Acα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 or 3 (Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3 or 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc$_{OT}$ and Neu5Acα2 → 3 and 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6[Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 4(Neu5Acα2 → 6Galβ1 → GlcNAcβ1 → 2)Manα1 → 3]Manβ1 → 4GlcNAcβ1 → 4GlcNAc$_{OT}$ all bound to the column (data not shown). Thus, this result suggests that N-linked oligosaccharides with sialic acid residues linked at the C-6 position of galactose residues also exist to small extent in sGP. No further analysis of the SSA-bound oligosaccharides could be done because of their low amounts.

Based on the results obtained by sequential exoglycosidase digestion, methylation analysis and SSA-agarose chromatography, structures of N-linked sugar chains of sGP are proposed as summarized in Figure 7. The released oligosaccharides are not suitable for the identification of neuraminic acid species because of conversion of Neu5Gc to Neu5Ac by hydrazinolysis. However, it is likely from the composition analysis shown in Table 1 that both Neu5Gc and Neu5Ac are included in N-linked sugar chains.

Discussion

Rosette formation of T lymphocytes with sheep erythrocytes has been used as a convenient experimental model for CD2-mediated cell adhesion, because erythrocytes express ligand molecules for CD2 but not other molecules called ICAM-1, LFA-1, and major histocompatibility antigens which are involved in T cell-mediated cell adhesion. Its simplicity is evident from the complete inhibition of rosetting by the addition of a monoclonal antibody to CD2.

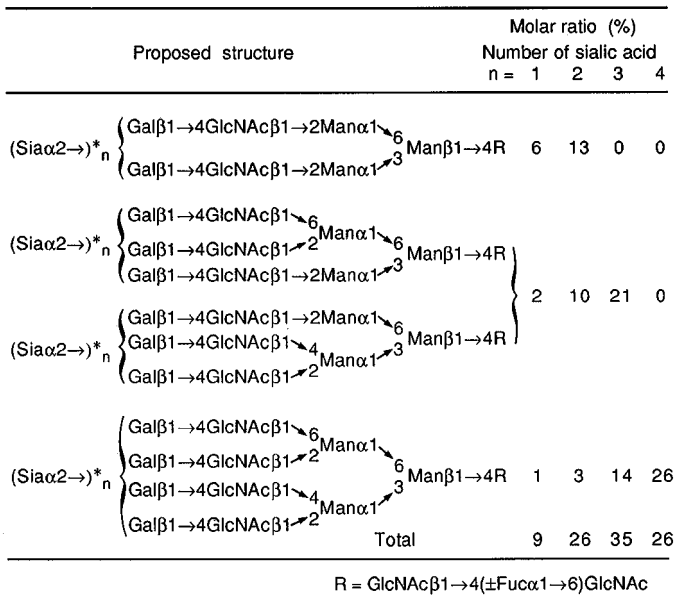

Figure 7. Proposed structures and contents of N-linked sugar chains included in sGP. Most of oligosaccharides contain the Siaα2 → 3Gal group, but 7% of them contain the Siaα → 6Gal group. Neutral oligosaccharides (4%) was too low to be analysed.

By using this model system, we showed that sGP, glycoproteins solubilized from sheep erythrocyte ghosts, inhibit rosette formation of T lymphoblastic Molt-3 cells with sheep erythrocytes, and suggested that exposed galactose residues of N-linked sugar chains included in sGP may play an important role in rosette formation [13].

In this study, we have analysed the carbohydrate moiety of sGP. The composition analysis (Table 1) suggests that the sGP used in this study contains much smaller amounts of N-linked sugar chains compared with O-linked sugar chains which are composed of Siaα2 → 3Galβ1 → 3(Siaα2 → 6)GalNAc and its asialo form [27]. However, our previous results [13] indicated that removal of the Galβ1 → 3GalNAc moiety by *O*-glycanase does not affect the rosetting inhibitory activity of sGP. We have, therefore, analysed the structures of N-linked sugar chains of sGP and elucidated that their structures are of bi-, tri-, and tetraantennary complex-type containing different numbers of sialic acid residues. In view of the proposed role of exposed galactose residues, it should be noted that the *N*-acetyllactosamine antennae of the oligosaccharides are not fully sialylated. On the basis of the proposed structures and contents of oligosaccharides (Figure 7), it is evident that the N-linked oligosaccharides containing the galactose residue(s) exposed at the nonreducing termini amount to approximately 40% of the total, and the ratio of exposed galactose residues to sialylated galactose residues is approximately 1:6. From the contents of tetra-*O*-methyl galactitol and tri-*O*-methyl galactitol derivatives detected in the methylation analysis (Table 2), the ratio of exposed and sialylated galactose residues is also calculated to be 1:5. Thus, it is noted that significant amounts of oligosaccharides contain exposed galactose residues.

Unlike sheep erythrocytes, human and mouse erythrocytes cannot form rosettes with human peripheral blood lymphocytes [28]. The difference must be considered from at least two distinct viewpoints. The first is the quantitative difference in ligands for CD2, i.e. both human and sheep erythrocytes express comparable CD2 binding sites/cell, but the ligand density is three times lower on the former than the latter because of their different surface areas [7]. The second is the qualitative difference in the interaction of CD2 and its ligands. In this respect, it is of interest to compare the structures of sugar chains present on these erythrocyte membrane glycoproteins which may affect affinity of the interaction between CD2 and its ligands. Mouse erythrocyte glycoproteins are similar to sGP in that both glycoproteins contain multiantennary sugar chains, but are different in that the mouse sugar chains are highly sialylated compared with those included in sGP [28]. On the other hand, human erythrocytes contain glycoproteins predominantly expressing biantennary sugar chains with bisecting *N*-acetylglucosamine residues as already shown by the analysis of three intrinsic glycoproteins, glycophorin A [29], Band 3 [30–32] and glucose transporter [33]. Judging from the reported structures, there are exposed galactose residues. For example, monosialyl, monogalactosylated biantennary oligosaccharides present on glycophorin A amount to 54% of the total [29]. Thus, the different rosetting ability is not simply explained by the degree of galactose exposure of oligosaccharides, suggesting that some other factors are involved in regulation of rosette formation. To be noted in this respect is the fact that the oligosaccharides in human erythrocyte glycoproteins express a significant number of bisecting *N*-acetylglucosamine residues in their oligosaccharide moieties. It has been shown that bisected and nonbisected oligosaccharides have different affinities to certain lectins as shown in the cases of *Ricinus communis* agglutinin I and concanavalin A [34, 35]. It is also known that the addition of bisecting *N*-acetylglucosamine residues inhibits further formation of multiantennary structures [36]. Therefore, it may be possible to assume that the steric effect of bisecting *N*-acetylglucosamine residues found in sugar chains of human erythrocyte glycoproteins negatively regulates rosette formation. Localization (clustering) of the exposed galactose residues in the polypeptide chains and their spacial arrangement on the cell surfaces should also be considered as other factors regulating rosetting efficiency. Further effort will be needed to solve this issue.

The present work provides an additional example of species-specific glycosylation. As already mentioned, sGP is similar to mouse [28] and rat [37] erythrocyte glycoproteins in the expression of non-bisected multiantennary sugar chains, but is different from human erythrocyte glycoproteins [29–33] which express bisected biantennary sugar

chains. sGP is unique in that it does not contain oligosaccharides with polylactosamine structures which are widely found in membrane glycoproteins from human, rat and mouse erythrocytes. The sialic acid residues in N-linked sugar chains of sGP are mostly linked to the C-3 position of galactose residues. Similarly, a mouse glycophorin has only the Neu5Acα2 → 3Gal linkage [28]. On the other hand, rat erythrocyte membrane glycoproteins contain both Neu5Acα2 → 3Gal and Neu5Acα2 → 6Gal linkages [37]. In the case of human erythrocyte membrane glycoproteins, expression of sialyl linkages in N-linked sugar chains differ between glycoproteins; glycophorin A expresses only the Neu5Acα2 → 6Gal linkage [29] while Band 3 and glucose transport expresses both linkages [31–33]. Thus, expression of sialyl linkages is also species specific.

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References

1 Lay WH, Mendes NF, Bianco C, Nussenzweig V (1971) *Nature* **230**: 531–32.
2 Jondal M, Holm G, Wigzell H (1972) *J Exp Med* **136**: 207–15.
3 Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB, Herzenberg LA (1981) *J Immunol* **126**: 2117–22.
4 Hünig T (1985) *J Exp Med* **162**: 890–901.
5 Springer TA, Dustin ML, Kishimoto TK, Marlin SD (1987) *Ann Rev Immunol* **5**: 223–52.
6 Dustin ML, Springer TA (1991) *Annu Rev Immunol* **9**: 27–66.
7 Johansen KS, Johansen TS, Talmage DW (1974) *J Allergy Clin Immunol* **54**: 86–93.
8 Selvaraj PM, Dustin ML, Mitnacht R, Hünig T, Springer TA, Plunkett ML (1987) *J Immunol* **139**: 2690–95.
9 Parish CR, Recny MA, Knoppers MH, Waldron JC, Warren HS (1993) *J Immunol* **150**: 4833–43.
10 Deckert M, Kuber J, Zuccola D, Bernard-Pomier G, Angelisova P, Horejsi V, Bernard A (1992) *Eur J Immunol* **22**: 2943–47.
11 Sandrin MS, Mouhtouris E, Vaughan HA, Warren HS, Parish CR (1993) *J Immunol* **151**: 4606–13.
12 Bernard A, Tran HC, Boumsell L (1987) *J Immunol* **139**: 18–23.
13 Ogasawara H, Kusui K, Takasaki S (1995) *Immunology Lett* **48**: 35–38.
14 Li Y-T, Li S-C (1972) *Methods Enzymol* **28**: 702–13.
15 Marchesi VT (1972) *Methods Enzymol* **8**: 252–54.
16 Laemmli UK (1970) *Nature* **227**: 680–85.
17 Hardy MR, Townsent RR, Lee YC (1988) *Anal Biochem* **170**: 54–62.
18 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) *J Biol Chem* **193**: 265–75.
19 Takasaki S, Mizuochi T, Kobata A (1982) *Methods Enzymol* **83**: 263–68.
20 Yamashita K, Kochibe N, Ohkura T, Ueda I, Kobata A (1985) *J Biol Chem* **260**: 4688–93.
21 Shibuya N, Tazaki K, Song Z, Tarr GE, Golgstein IJ, Peumans WJ (1989) *J Biochem* (*Tokyo*) **106**: 1098–103.
22 Takamoto M, Endo T, Isemura M, Yamaguchi Y, Okamura K, Kochibe N, Kobata A (1989) *J Biochem* (*Tokyo*) **106**: 228–35.
23 Yamashita K, Mizuochi T, Kobata A (1982) *Methods Enzymol* **83**: 105–26.
24 Phillips LR, Fraser BA (1981) *Carbohydr Res* **90**: 149–52.
25 Paulson JC, Prieels J-P, Glasgow LR, Hill RL (1978) *J Biol Chem* **253**: 5617–24.
26 Yamashita K, Ohkura T, Yoshima H, Kobata A (1981) *Biochem Biophys Res Commun* **100**: 226–32.
27 Glöckner WM, Newman RA, Dahr W, Uhlenbruck G (1976) *Biochem Biophys Acta* **443**: 402–13.
28 Angel A-S, Grönberg G, Krotkiewski H, Lisowska E, Nilsson B (1991) *Arch Biochem Biophys* **291**: 76–88.
29 Yoshima H, Furthmayr H, Kobata A (1980) *J Biol Chem* **255**: 9713–18.
30 Tsuji T, Irimura T, Osawa T (1980) *Biochem J* **187**: 677–86.
31 Tsuji T, Irimura T, Osawa T (1981) *J Biol Chem J* **256**: 10497–502.
32 Fukuda M, Dell A, Oates JE, Fukuda MN (1984) *J Biol Chem* **259**: 8260–73.
33 Endo T, Kasahara M, Kobata A (1990) *Biochemistry* **29**: 9126–34.
34 Narasimhan S, Freed JC, Schachter H (1986) *Carbohydr Res* **149**: 65–83.
35 Harada H, Kamei M, Tokumoto Y, Yui S, Koyama F, Kochibe N, Endo T, Kobata A (1987) *Anal Biochem* **164**: 374–81.
36 Brockhausen I, Narasimhan S, Schachter H (1988) *Biochimie* **70**: 1521–33.
37 Matsumoto A, Yoshima H, Shiraishi N, Maeda S, Kobata A (1982) *Arch Biochem Biophys* **217**: 696–709.

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