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Structure of the Major *O*-Glycosidic Oligosaccharide of Monkey Erythrocyte Glycophorin

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Sialic acids and the major *O*-glycosidic oligosaccharide of glycophorin MK from monkey (Japanese monkey, *Macaca fuscata)* erythrocyte membranes were characterized. *N*-Glycolylneuraminic acid (Neu5Gc) was found as the major sialic acid, which was confirmed by gas-liquid chromatography-mass spectrometry as the trimethylsilyl methyl ester. Three *O*-glycosidic oligosaccharide units were obtained from a tryptic glycopeptide that contained all of the carbohydrate units in glycophorin MK by mild alkaline borohydride/borotritide treatment. Carbohydrate analyses of the oligosaccharides revealed that they were composed of Neu5Gc, galactose and *N*-acetylgalactosaminitol in the molar ratios of 1:1:1 (trisaccharide), 2:1:1 (tetrasaccharide) and 3:1:1 (pentasaccharide). The content of oligosaccharide units was estimated to be 1:12:5 for penta-, tetra- and trisaccharide, respectively, based on the yields, the molecular weight, and the number of oligosaccharide attachment sites in the amino-acid sequence. The tetrasaccharide was the major oligosaccharide and its structure was proposed to be Neu5Gcα2-3Galβ1-3[Neu5Gcα2-6]GalNAcol.

Glycophorin is the major sialoglycoprotein of erythrocyte membranes and has been found in various animal erythrocyte membranes. Of the glycophorin, the glycosylated portion which is exposed outside the membranes is thought to be the marker(s) for erythrocyte differentiation during erythropoiesis, erythrocyte clearance during circulation, and virus receptors [1-3]. Since the amino-acid sequences and carbohydrate structures of glycophorins are different from animal to animal [4-12], we have proposed that glycophorins might be species-specific antigens on erythrocyte membranes such as class I major histocompatibility antigens expressed on specific tissue cells.

We recently isolated and characterized glycophorin MK from Japanese monkey *(Macaca fuscata)* erythrocyte membranes. The chemical analysis of the glycophorin MK revealed that the preparation contained about 51% of protein and 49% of carbohydrate, which was

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Table 1. Carbohydrate compositions of glycopeptides T1 and T2 from glycophorin MK.

	mol%[a]		
	Gal	GalNAc	Neu5Gc
T1	36.2	23.4	40.3
T2	35.9	27.5	36.6

[a] Sugars were analyzed as their trimethylsilyl derivatives.

composed of galactose, *N*-acetylgalactosamine and sialic acid. The sequence study revealed that glycophorin MK consisted of 144 amino-acid residues and 18 *O*-glycosidic oligosaccharide chains, that the glycophorin contained no *N*-glycosidic oligosaccharide chains, and that the amino-acid sequence showed a striking sequence homology with that of human glycophorin A [13]. A partial amino terminal sequence of a sialoglycoprotein from gorilla erythrocyte membranes has also been determined [14]. These sequences suggest that the primary structure among primate animal glycophorins might be similar, indicating that the genes encoding glycophorins were derived from the same ancient origin. However, carbohydrate structures of glycophorins from primate erythrocyte membranes have not been studied so far, except for human glycophorin. We are now interested in the carbohydrate moiety of glycophorin MK.

In this paper, we report the characterization of the sialic acid and the structural study of the major *O*-glycosidic oligosaccharide from glycophorin MK.

Materials and Methods

Materials

Trypsin (TPCK-treated) was obtained from Worthington Biochemical Corp (Freehold, NJ, USA). Sodium borotritide (100 Ci/mmol) was purchased from Amersham International (Little Chalfont, Buckinghamshire, England). All other chemicals were analytical grade.

Preparation of Glycophorin MK and Its Tryptic Glycopeptide

Monkey (Japanese monkey, *Macaca fuscata*) erythrocyte membranes were prepared by the method of Dodge *et al.* [15], and glycophorin MK by the method of Marchesi and Andrews [16]. Two glycopeptides, T1 and T2 were obtained by digestion of glycophorin MK with trypsin. Carbohydrate compositions of the glycopeptides are shown in Table 1. Since the chemical characterization of the glycopeptides revealed that T1 and T2 were composed of 60 and 28 amino-acid residues, respectively, and that an amino-acid sequence of T2 was the same as a sequence from the amino terminus to residue 27 of T1, T1 possesses all of the carbohydrates in glycophorin MK [13]. The tryptic glycopeptide T1 was used as the source of *O*-glycosidic oligosaccharide units.

Isolation of Sialic Acids from Glycophorin MK

Preparation of sialic acids from glycophorin MK was performed by the method of Reuter *et al.* [17]: Glycophorin MK (5 mg by dry weight) was treated with 0.02 N formic acid (5 ml) at pH 2.0 and 70°C for 1 h. The hydrolyzate was fractionated on a Sephadex G-50 column (1.5 x 25 cm, Pharmacia, Uppsala, Sweden) equilibrated with water, and the fraction containing sialic acids was successively applied to a Dowex 50-X8 column (H^+, 3 ml) and Dowex 2-X8 column ($HCOO^-$, 3 ml). Elution of sialic acids from the Dowex 2-X8 column was carried out with 1 N formic acid (20 ml), and then the pooled eluate was lyophilized immediately. All procedures were performed at 4°C.

Isolation of the Reduced and Tritium-labeled Oligosaccharides

To the tryptic glycopeptide, T1 (15 mg dry weight), was added 0.6 ml of 0.8 M $NaBH_4$-$NaB[^3H]_4$ (7.5 mCi)/0.05 N NaOH and the mixture was incubated at 50°C for 17 h. The reaction mixture was acidified by addition of acetic acid in order to destroy excess borohydride, and then passed though a Dowex 50-X8 column (H^+ form, 1.5 x 5.0 cm). The column was washed with 100 ml of water. The eluate was concentrated with a rotary evaporator under reduced pressure, treated with methanol to remove boric acid as methyl borate, and applied to a Bio-Gel P-4 column (-400 mesh, 1.5 x 150 cm) which was previously equilibrated with 0.05 N acetic acid. Fractions (2 ml) were collected and analyzed for radioactivity.

The oligosaccharides were further fractionated on a DEAE-cellulose column (HCO_3^- form, 1.0 x 10 cm). The oligosaccharides were eluted with water (fractions 1-10), and with a linear gradient of 0-0.2 M ammonium bicarbonate (200 ml). Each fraction (5.8 ml) was collected and analyzed by radioactivity.

Analytical Procedures

Direct trimethylsilylation of the isolated sialic acids was performed by the method of Casals-Stenzel *et al.* [18]. The trimethylsilylated sialic acids were analyzed by a gas-liquid chromatography (GLC) using a 3% OV-17 column (3.0 mm x 1.5 m). For the methylation analysis of the sialic acids, the lyophilized sialic acid preparation (100 µg) was methylated with diazomethane and then trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane as described previously [19].

For the methylation analysis of oligosaccharides, the oligosaccharides (50-100 µg) were methylated, hydrolyzed, reduced and acetylated by the method of Stellner *et al.* [20]. The partially methylated alditol acetates were analyzed by both GLC and gas-liquid chromatography-mass spectrometry (GC-MS) using a Jeol-JMS-D-300 system under conditions described previously [21].

Carbohydrate analysis was performed on GLC as trimethylsilyl derivatives of the methyl glycosides which were released by methanolysis in 0.5 N methanolic HCl for 16 h at 65°C [22]. *N*-Acetylgalactosamine and *N*-acetylgalactosaminitol were also determined with a Hitachi amino-acid analyzer after hydrolysis of the sample in 4 N HCl at 100°C for 4 h.

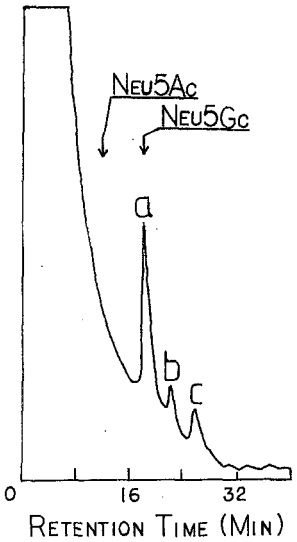

Figure 1. Gas-liquid chromatogram of trimethylsilyl derivatives of sialic acids from glycophorin MK. The purified sialic acids from glycophorin MK were directly trimethylsilylated with 20 µl of trimethylsilyl-imidazole (TMSI) reagent at 65°C for 5 min. Portions of 3 µl were applied on a 3% OV-17 column (3.0 mm x 1.5 m) with temperature increasing from 190°C to 250°C at 2°C/min. Sialic acids prepared from bovine submaxillary mucin glycoprotein were used as standards. Arrows denote the elution positions of the trimethylsilylated Neu5Ac and Neu5Gc on GLC:

Chromium Trioxide Oxidation

Chromium trioxide oxidation of acetylated oligosaccharides was performed using inositol as an internal standard [23].

Results

Characterization of Sialic Acids in Glycophorin MK

The gas-liquid chromatogram of the trimethylsilylated sialic acid derivatives revealed three peaks (Fig. 1): one was the major peak, labeled **a**; two were minor peaks, labeled **b** and **c**. The retention time of peak **a** corresponded to that of trimethylsilylated Neu5Gc which was obtained from commercial standard Neu5Gc, obtained from the terminal Neu5Gc residue in bovine submaxillary gland mucin. The retention times of peaks **b** and **c** suggested the presence of *O*-acetyl-Neu5Gc derivatives, but this could not be verified, because of the small amount.

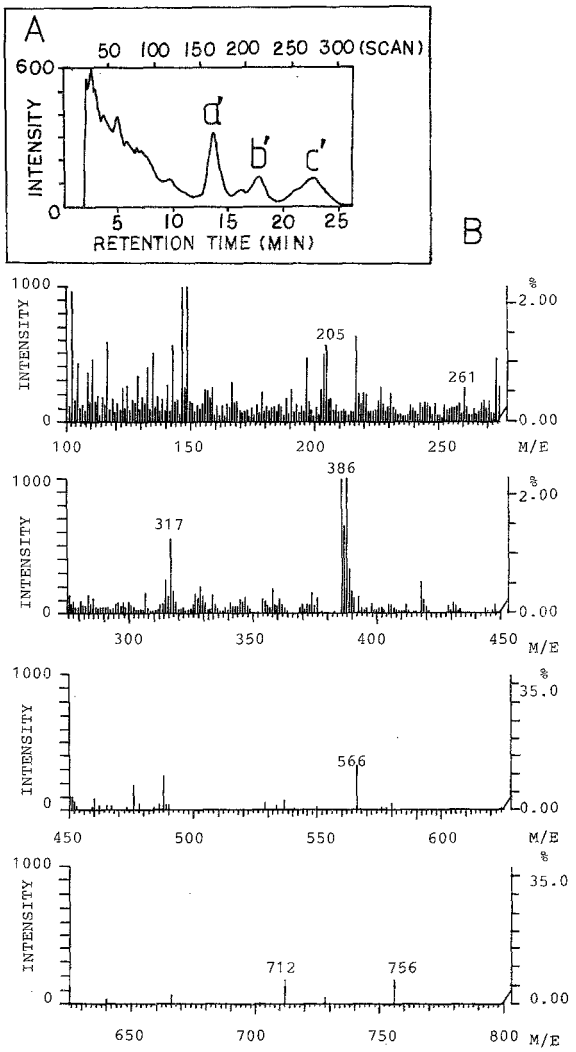

Figure 2. Electron ionization (EI) mass-spectrum (70 eV) of the trimethylsilylated methyl ester of the major sialic acid. A: RIC chromatogram. B: EI mass-spectrum of peak a'.

The RIC (Reconstituted Ion Chromatogram) of the trimethylsilyl derivatives of the methyl esters of the sialic acid showed three peaks: a', b', and c' (Fig. 2A). Peak a' was characterized by the ion peaks of m/z 756, 712, 566, 386, 317, 261, and 205, these ion peaks being identical to those of the trimethylsilyl derivative of the methyl ester of standard Neu5Gc (Fig. 2B). The ion peaks of peak b' were identical to those of peak a' (data not shown), suggesting that peak a' and b' were, respectively, the β-anomer and α-anomer of Neu5Gc methyl ester. The other minor peak, c', was characterized by ion peaks of 386 and 274, indicating that c' was an analogue of Neu5Gc [24].

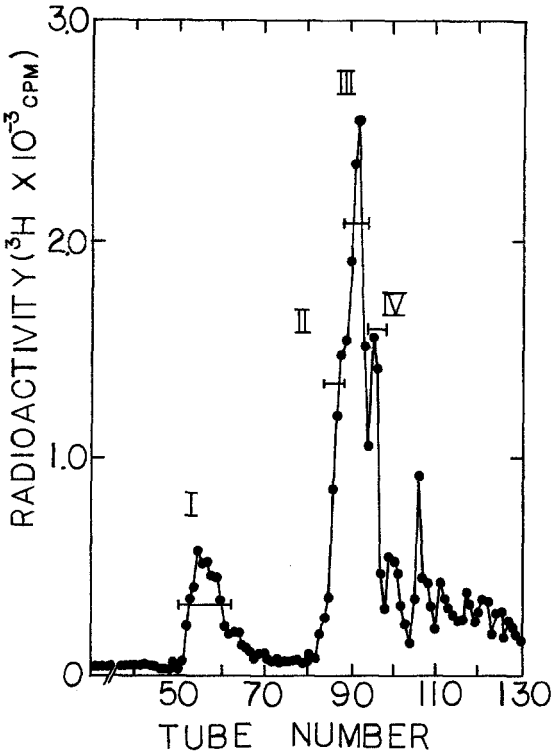

Figure 3. Fractionation of the reduced and [^{3}H]-labeled oligosaccharides. The eluate from a Dowex 50-X8 column was concentrated and fractionated on a Bio-Gel P-4 column (1.5 x 150 cm). Fractions (2.0 ml) were collected and 10 µl portions were used for detection of carbohydrate by radioactivity.

Isolation of the Reduced Oligosaccharides from theTryptic Glycopeptide

The *O*-glycosidic oligosaccharide units were separated into four pools, labeled I-IV (Fig. 3). Pool I contained a small amount of carbohydrates. A small peak was also detected at an elution position just behind peak IV (Fig. 3), and the labeled compound appeared to be a disaccharide composed of galactose and *N*-acetylgalactosaminitol in a molar ratio of 1:1, by carbohydrate analysis (data not shown). These fractions were not used for further structural examination. Each pool was eluted at the positions of penta-, tetra-, and trisaccharide from a Bio-Gel P-4 column (Fig. 3). Pools II, III, and IV were further fractionated by DEAE-cellulose column chromatography. The elution profiles of each fraction are shown in Fig. 4: A fraction, IVa, was from Pool IV (Fig. 4A); two fractions, IIIa and IIIb, were from Pool III (Fig. 4B); three fractions, labeled IIa, IIb and IIc, were obtained from Pool II (Fig. 4C). The carbohydrate composition of each fraction revealed that the composition of IIIb was identical to that of IIb (data not shown). To avoid confusion, the fractions IVa, IIIb, and IIc were renamed A1, A2, and A3, respectively, and used for further studies. The carbohydrate compositions of A1, A2, and A3 are shown in Table 2. The compositions revealed that A1,

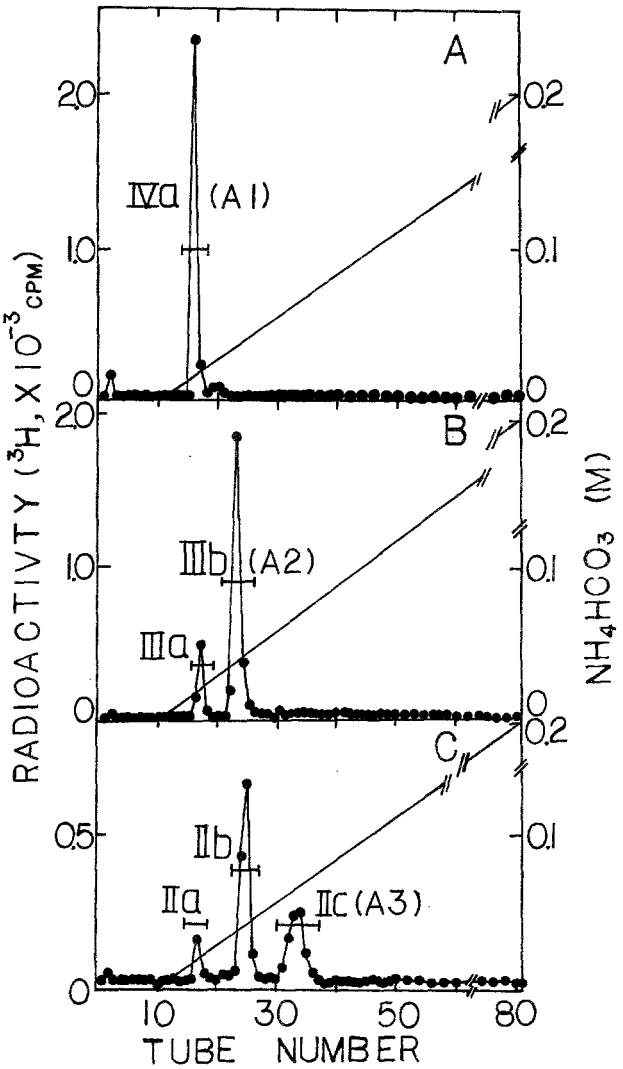

Figure 4. Fractionation of the reduced and [^{3}H]-labeled oligosaccharides.
The pooled fractions II-IV shown in Fig. 3 were fractionated by DEAE-cellulose DE-52 column chromatography as described in the Materials and Methods section. A: Pool IV; 30 µl portions were used to measure radioactivity. B: Pool III; 10 µl portions were used to measure radioactivity. C: Pool II; 20 µl portions were used to measure radioactivity.

A2, and A3 were composed of Neu5Gc, galactose, and *N*-acetylgalactosaminitol in the molar ratios of 1:1:1, 2:1:1, and 3:1:1, respectively. The carbohydrate compositions and elution positions of the oligosaccharides revealed that A1, A2, and A3 were tri-, tetra-, and pentasaccharides, respectively. The yields of the oligosaccharides demonstrated that tetrasaccharide A2 was the major oligosaccharide unit of glycophorin MK.

Table 2. Carbohydrate compositions of the reduced oligosaccharides.

	Molar ratios		
	A1	A2	A3
Gal	0.96 (1)[a]	0.92 (1)	1.09 (1)
GalNAcol	1.00 (1)	1.00 (1)	1.00 (1)
Neu5Gc	1.07 (1)	2.01 (2)	2.88 (3)
Yield (μg) [b,c]	152	590	69

[a] Values in parentheses indicate the integral number of the residues.
[b] Yield from 15 mg of glycopeptide, T1, from monkey glycophorin.
[c] The yield of oligosaccharides was obtained from the sum of the oligosaccharide with the same carbohydrate composition.

Structural Analysis of the Major O-Glycosidic Oligosaccharide (Tetrasaccharide)

To determine the position of glycosidic linkages, A2 and asialo-A2 were permethylated, hydrolyzed, reduced, and acetylated. The resulting monosaccharide derivatives were analyzed by GLC and GC-MS. The asialo-A2 was prepared by the treatment of A2 with 0.05 N sulfuric acid at 80°C for 1 h, and after neutralization with NaOH the reaction mixture was used for the methylation analysis without further separation of the released sialic acid.

The permethylated A2 was characterized by two peaks of 2,4,6-tri-*O*-methyl-D-galactitol acetate and 1,4,5-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxy-galactitol acetate (Table 3), which indicated the presence of a galactosyl residue substituted at C-3 and of an *N*-acetylgalactosaminitol substituted at C-3 and C-6. The derivatized alditol acetate of monosaccharides from the permethylated asialo-A2 were characterized by detection of 2,3,4,6-tetra-*O*-methyl-D-galactitol acetate and 1,4,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxy-galactitol acetate (Table 3), indicating that the sequence of asialo-A2 should be Gal1-3GalNAcol.

The permethylated asialo-A2 was directly analyzed by GC-MS. The permethylated saccharide produced two peaks with about 3 min difference in retention time (Fig. 5A). One peak (a) with a retention time of 5.6 min was characterized by the ion peaks of m/z 147, 201, 284, 328, 348 and 406, which were identical to those of permethylated *N*-glycolylneuraminic acid [25]. Another peak (b) with a retention time of 8.9 min was characterized by the ion peaks of m/z 187 and 219 from the non-reducing terminal galactosyl residue, and m/z 378, 422 and 466, derived from Gal1-3GalNAcol (Fig. 5B). The results strongly suggested that asialo-A2 was a disaccharide, Gal1-3GalNAcol. Thus, the structure of A2 was proposed to be Neu5Gc2-3Gal1-3[Neu5Gc2-6]GalNAcol.

To determine the anomeric configuration, asialo-A2 was oxidized with chromium trioxide. The treatment of asialo-A2 caused an 80% destruction of the galactosyl residue, indicating

Table 3. Methylation analysis of the major oligosaccharide unit, the tetrasaccharide.

Partial methylated alditol acetates	Relative R.T. (min)[a]	Ratio of peak area[b]	
		A2	Asialo-A2
2,3,4,6-Tetra-*O*-Me-D-Gal (Gal1-)	1.00	0.04	1.02
2,4,6-Tri-*O*-Me-D-Gal (-3Gal1-)	1.28	1.13	0.13
1,4,5,6-Tetra-*O*-Me-*N*-Me-D-GalNAcol (-3GalNAcol)	1.66	0.12	1.00
1,4,5-Tri-*O*-Me-*N*-Me-D-GalNAcol (-3,6-GalNAcol)	2.17	1.00	0.02

[a] Relative retention time of the partially methylated alditol acetates were evaluated from that of 2,3,4,6-tetra-*O* Me-D-Gal.

[b] Ratios of peak area have not been corrected for difference in detector response for differential destruction or loss during processing. Thus, their quantitation does not represent the composition of the original samples.

a β-configuration for this galactosyl residue because of its significant lability. The sialyl residues were in the α-configuration, because they were released by neuraminidase (*Clostridium perfringens*) treatment. The proposed structure of A2 is shown in Fig. 6.

Discussion

Glycophorin MK contained Neu5Gc as the major sialic acid. Two minor peaks with absolute retention times at 20.0 min and 25.7 min were also detected on GLC, and might be 9-*O*-acetyl- and 7,9-di-*O*-acetyl-Neu5Gc, respectively, since the retention times were close to those reported previously [18, 26]. It has been reported that sialoglycoproteins of Rhesus monkey erythrocyte membranes contained Neu5Ac as the major sialic acid and 9-*O*-acetyl-Neu5Ac as the minor sialic acid [27]. Thus, sialic acid in the Japanese monkey erythrocyte membrane glycoprotein differed from that of the Rhesus monkey erythrocyte membrane sialoglycoproteins. Since the difference in sialic acid has been attributed to differences in the gene for mono-oxygenase between strains or species [28], the Japanese monkey might have evolved differently from the Rhesus monkey. 9-*O*-Acetyl-Neu5Ac in murine erythrocyte membranes has been known to activate the alternative complement pathway [29]. Thus, *O*-acetyl-Neu5Gc in glycophorin MK might be an immunological marker on the erythrocyte membranes.

The *O*-glycosidic oligosaccharides released by alkaline-borohydride treatment were tri-, tetra-, and pentasaccharides. A very small amount of disaccharide was also detected, which may be artificially produced during preparation of oligosaccharides from the tryptic

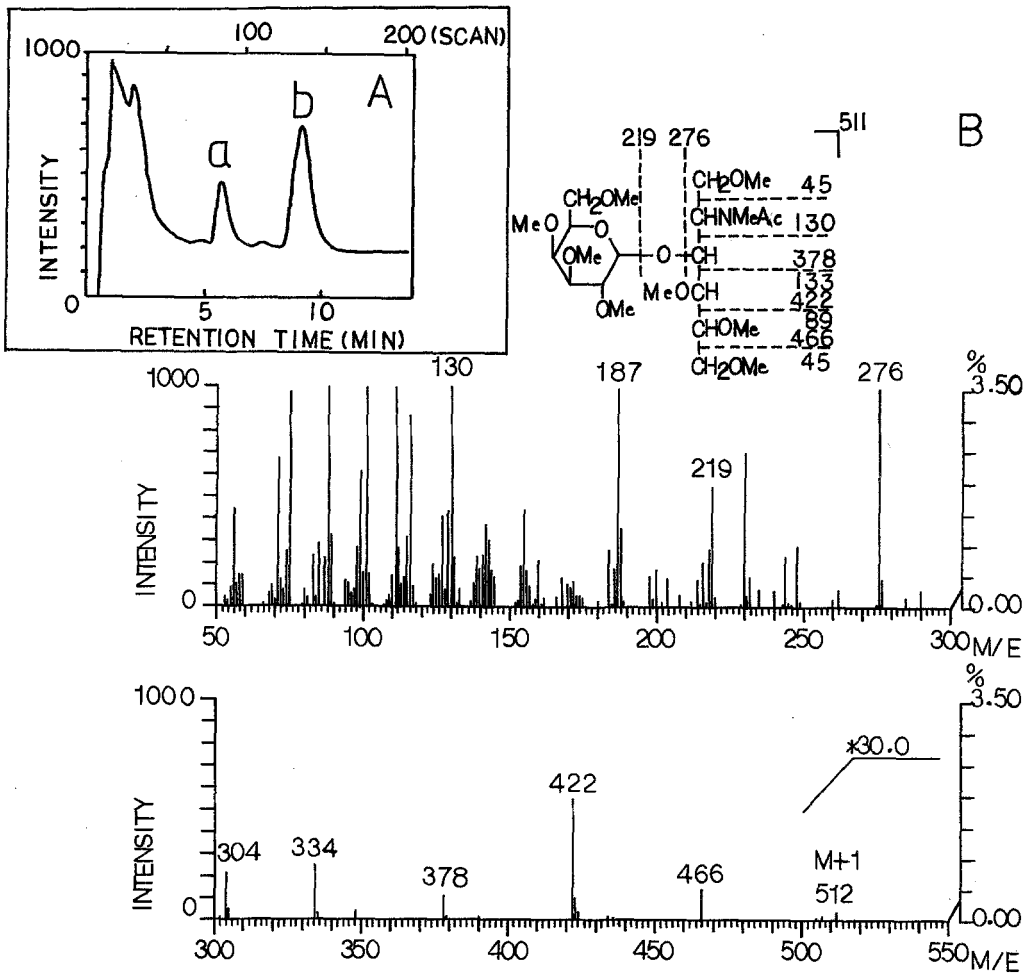

Figure 5. Electron ionization mass-spectrum of permethylated asialo-A2. A: RIC chromatogram. B: EI mass-spectrum of peak b.

glycopeptide. The carbohydrate compositions of the oligosaccharides indicated the presence of a common *O*-glycosidic oligosaccharide core structure, Gal-GalNAcol, and the differences in the content of sialic acid among the oligosaccharides. There were no complex *O*-glycosidic oligosaccharides containing branched or unbranched *N*-acetyllactosamine units found in bovine and porcine glycophorins [9, 10]. The content of oligosaccharide units was estimated to be 1:12:5 for penta-, tetra- and trisaccharide, respectively, based on the molecular weight and the yield of the oligosaccharides, and the number of oligosaccharide attachment sites in the amino-acid sequence [13].

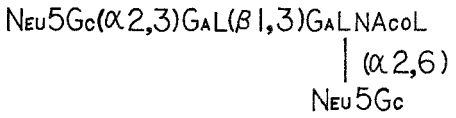

Figure 6. The proposed structure of the major oligosaccharide of glycophorin MK.

The tetrasaccharide was the major oligosaccharide, and its structure was confirmed as shown in Fig. 6. The structure was identical to that found in horse, dog, and human glycophorins [8, 11, 30]. The structure of the trisaccharide as one of the minor oligosaccharides was not determined. However, the carbohydrate composition of the trisaccharide was the same as that found in horse, dog, and human glycophorins, except for differences in the types of sialic acid. Thus, structures of the trisaccharide in glycophorin MK may be Neu5Gcα2-3Galβ1-3GalNAcol or Galβ1-3-[Neu5Gcα2-6]GalNAcol, or both. Another minor oligosaccharide, the pentasaccharide, was a novel oligosaccharide. Recently, Fukuda *et al.* have elucidated the structures of two pentasaccharides, which were trisialylated *O*-glycosidic oligosaccharide units, in human glycophorins [31]. Although the structural analysis of the pentasaccharide in glycophorin MK remains to be elucidated, it might include an additional Neu5Gc residue attached to the Neu5Gc residue located at the non-reducing terminal of the tetrasaccharide.

Both the tri- and tetrasaccharide found in glycophorin MK are typical oligosaccharides of animal glycophorins. It was thus less likely that glycophorins contain species-specific oligosaccharides. However, there are some differences in the distribution of various *O*-glycosidic oligosaccharides, types of sialic acid, and carbohydrate attachment sites in glycosylated portion of animal glycophorins [4-11]. Furthermore, *N*-glycosidic oligosaccharides were not found in monkey, horse, cow and dog glycophorins (unpublished results and [5, 7, 10]), whereas the saccharides were found in human and pig glycophorins [4, 6]. Taken together, the results indicate that the structural features of animal glycophorin may be different from species to species. This suggests that the amino-terminal portion exposed outside the erythrocyte may be important as a species-specific or self-recognizing antigen, just like a histocompatibility antigen.

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