A NOVEL PENTAGLYCOSYL CERAMIDE CONTAINING DI-β-N-ACETYLGALACTOSAMINYL RESIDUE (PARA-FORSSMAN GLYCOLIPID) ISOLATED FROM HUMAN ERYTHROCYTE MEMBRANE

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Three different series of glycosphingolipids which all start from lactosyl ceramide have been known to exist in human erythrocyte membranes (1-3), as shown in Fig. 1. A series comprising Globoside I that has been well characterized for a long time (4) is most abundant (5). This series is extended in sheep and goat erythrocytes to Forssman hapten that has been proved to have an additional α-N-acetylgalactosaminyl residue to Globoside I (6). Forssman hapten has never been found in human erythrocytes, but a glycosphingolipid which is composed of the same saccharide composition, sequence and linkages as those of Forssman hapten has been isolated and characterized*. The glycosphingolipid, which was tentatively named Para-Forssman glycolipid, was further examined by nmr spectroscopy and enzyme digestion. The non-reducing terminal N-acetylgalactosamine was revealed to attach to the penultimate N-acetylgalactosamine via a β-configuration instead of α. Para-Forssman glycolipid showed a precipitin reaction with anti-Globoside I antibody, but no reaction with anti-Forssman antibody.

* A preliminary account of this work was presented at the 49th Meeting of the Japanese Biochemical Society (7).

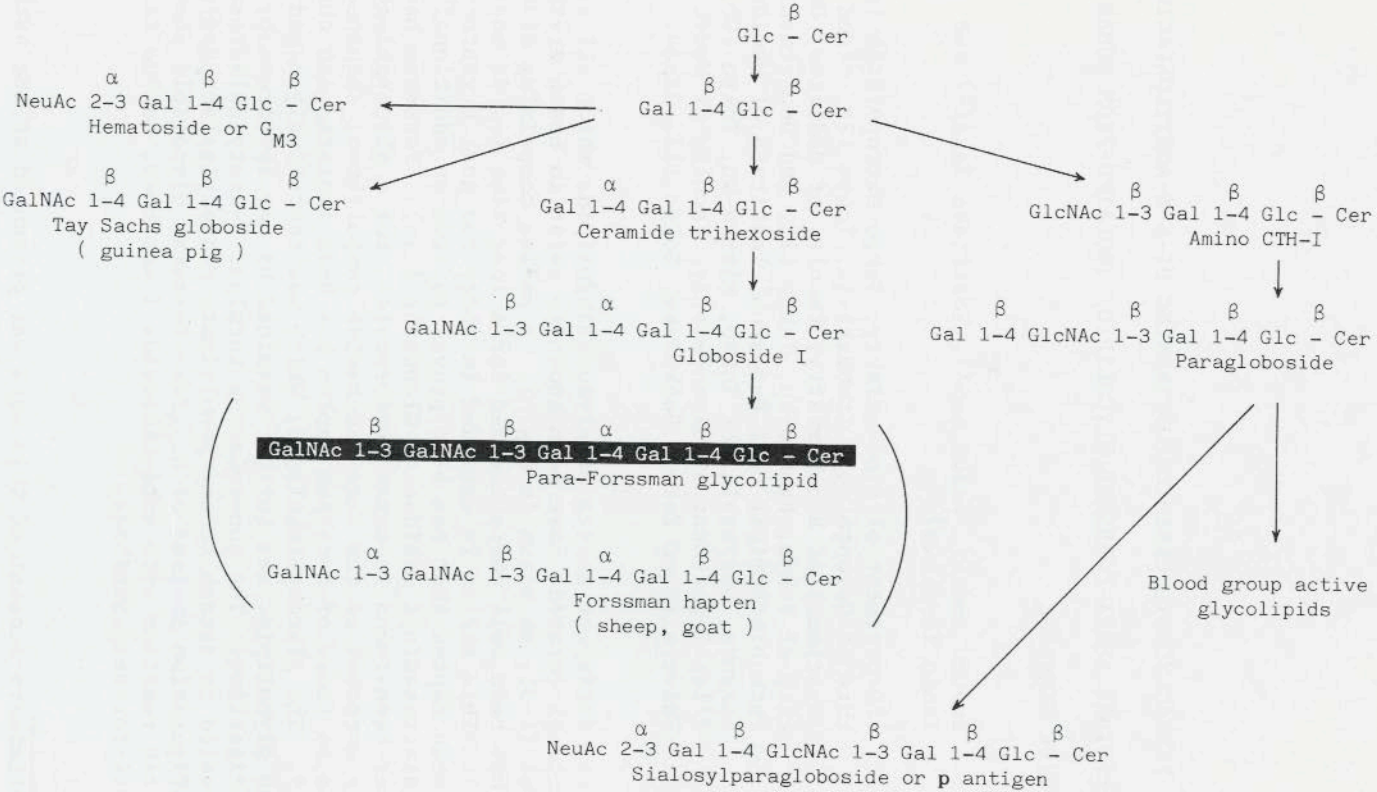
β
Glc – Cer
β β
Gal 1-4 Glc – Cer
α β β
NeuAc 2-3 Gal 1-4 Glc – Cer
Hematoside or G_{M3}
β β β
GalNAc 1-4 Gal 1-4 Glc – Cer
Tay Sachs globoside
(guinea pig)
α β β
Gal 1-4 Gal 1-4 Glc – Cer
Ceramide trihexoside
β β β
GlcNAc 1-3 Gal 1-4 Glc – Cer
Amino CTH-I
β α β β
GalNAc 1-3 Gal 1-4 Gal 1-4 Glc – Cer
Globoside I
β β β β
Gal 1-4 GlcNAc 1-3 Gal 1-4 Glc – Cer
Paragloboside
β β α β β
GalNAc 1-3 GalNAc 1-3 Gal 1-4 Gal 1-4 Glc – Cer
Para-Forssman glycolipid
α β α β β
GalNAc 1-3 GalNAc 1-3 Gal 1-4 Gal 1-4 Glc – Cer
Forssman hapten
(sheep, goat)
Blood group active
glycolipids
α β β β β
NeuAc 2-3 Gal 1-4 GlcNAc 1-3 Gal 1-4 Glc – Cer
Sialosylparagloboside or p antigen

Fig. 1.

ISOLATION OF PARA-FORSSMAN-GLYCOLIPID

Erythrocyte membranes were obtained from human red blood cells (blood group B, 104 l), and subjected to sequential lipid extractions using diethylether/methanol and chloroform/methanol (1:1 and 1:9) as previously described (5). Total lipids obtained (124 g) were applied to a silicic acid column(Mallinckrodt 100 mesh), and lipids were eluted with continuously increasing methanol in chloroform. Globoside I, paragloboside (lacto-N-neotetraosyl ceramide) (8,9) and less polar glycolipids were eluted with chloroform-methanol mixtures containing less than 40% methanol. Polar glycolipids obtained from the column with the solvent containing more than 40% methanol were applied to the second silicic acid column-(Mallinckrodt AR CC7, 200-325 mesh). Most of B-active glycolipid was eluted with 65-72% methanol in chloroform containing 4% of water, and Para-Forssman glycolipid was then eluted together with the tailing portion of B-active glycolipid with 72-75% methanol. The crude Para-Forssman glycolipid fraction was applied to an Iatrobeads column(Iatrobeads 6RS 8060, 60 μm, Iatron Lab., Inc., Tokyo; Ref. 10), and better separation of Para-Forssman glycolipid from B-active glycolipid was attained by gradient elution with a mixture of chloroform/methanol/water (80:15:2→10:85:5)(Fig. 2a). Para-Forssman glycolipid was ultimately purified by high performance liquid chromatography using finer Iatrobeads(6RS 8010, 10 μm; column size, 5 x 1000 mm, Ref. 1). Para-Forssman glycolipid was eluted after the peak of B-active glycolipid with chloroform/methanol/water (60:37:3), or before with *n*-propanol/2.5 N ammonia (70:30)(Fig. 2b and c). The Para-Forssman glycolipid preparation appeared as a single band on a thin-layer chromatogram that was located far below Forssman hapten and just below asialo G_{M1} (Fig. 3). The yield of purified material was 7.2 mg, which accounted for the low concentration (7 μg/100 ml) previously reported in erythrocytes (2).

ANALYSIS OF COMPOSITION, SUGAR SEQUENCE AND LINKAGES

Molar ratios of the constituents were determined as shown in Fig. 4 by the method previously described (11). In order to study the sugar sequence as well as the composition mass spectrometry was performed with the whole molecule of Para-Forssman glycolipid. Para-Forssman glycolipid was permethylated with methyl iodide in dimethylformamide in the presence of sodium hydride (12), and introduced into a Shimadzu-LKB 9000 mass spectrometer through a direct inlet to be measured by electron impact(70 eV). The fragmentation patterns of Para-Forssman glycolipid and Forssman hapten were very similar to each other (Fig. 5). In both the characteristic ions, m/z 260 for terminal GalNAc, m/z 505 for GalNAc-GalNAc, m/z 709 for GalNAc-GalNAc-Gal, and m/z 930 (913 + OH) for GalNAc-GalNAc-Gal-Gal, were detected, as well as a cluster of ions at 600-700 for ceramide portion and ions at 800-900 for -Glc-Cer.

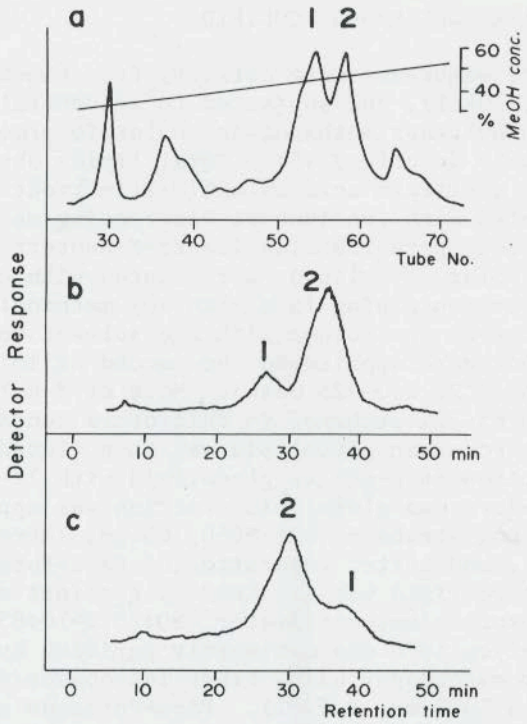

Fig. 2. Iatrobeads column chromatographies of Para-Forssman glycolipid.

a. Ordinary column chromatography with a linear gradient elution made by chloroform/methanol/water (85:15:2) and (10:85:5).

b. High performance liquid chromatography with chloroform/methanol/water (60:37:3).

c. High performance liquid chromatography with *n*-propanol/2.5 N ammonia (70:30).

Permethylated Para-Forssman glycolipid was subjected to acetolysis followed by reduction and acetylation for conversion to a mixture of alditol acetates (13). These partially methylated alditol acetates were analysed by gas chromatography and gas chromatography-mass spectrometry to determine linkages. The data are listed in Table 1. Thus the structure of the oligosaccharide portion (exclusive of anomeric configuration) was identified as GalNAc(1-3)-GalNAc(1-3)Gal(1-4)Gal(1-4)Glc-.

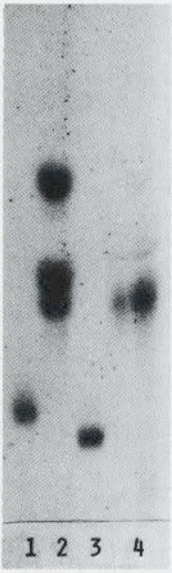

Fig. 3. Thin-layer chromatogram.

1. asialo G_{M1}; 2. paragloboside, Globoside I and ceramide trihexoside (from the bottom); 3. Para-Forssman glycolipid; 4. Forssman hapten. The plate was developed with chloroform/methanol/3.5 N ammonia (60:40:9).

Table 1. Partially methylated alditol acetates derived from permethylated glycolipids

	2,3,6-0-Me 1,4,5-0-Ac glucitol	2,3,6-0-Me 1,4,5-0-Ac galactitol	2,4,6-0-Me 1,3,5-0-Ac galactitol	3,4,6-0-Me 1,5-0-Ac galacto-saminitol	4,6-0-Me 1,3,5-0-Ac galacto-saminitol
Globoside I	+	+	+	+	-
Forssman hapten	+	+	+	+	+
Para-Forssman glycolipid	+	+	+	+	+

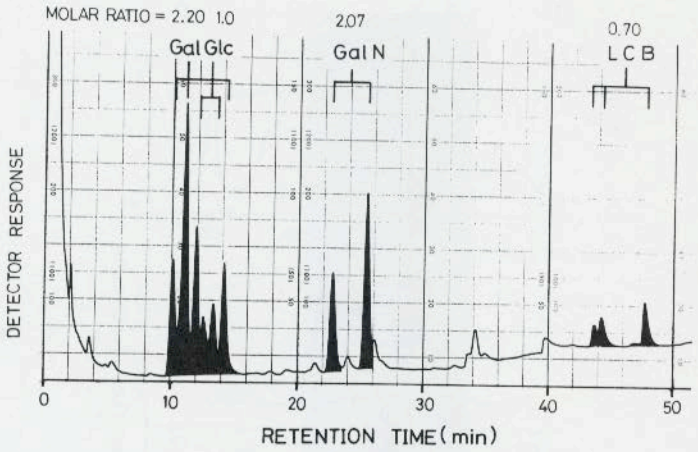

Fig. 4. Compositional analysis of Para-Forssman glycolipid by gas chromatography.

Saccharide components and long chain bases obtained by acid methanolysis were converted to their N,O-trifluoroacetyl derivatives, and then analysed by gas chromatography (1).

DETERMINATION OF ANOMERIC CONFIGURATION

Although Para-Forssman glycolipid had the same sugar sequence and linkages as Forssman hapten, it behaved differently on thin-layer chromatography (Fig. 3). To test the possibility that the anomeric configuration of the terminal N-acetylgalactosaminyl residue may be reversed, Para-Forssman glycolipid was reacted with β-hexosaminidase. The enzyme β-hexosaminidase that was isolated and purified from horse kidney by Dr. Y. Seyama (Department of Biochemistry, Faculty of Medicine, University of Tokyo)(14) completely degraded Globoside I to ceramide trihexoside (lane 4 in Fig. 6). Para-Forssman glycolipid was converted to ceramide trihexoside via Globoside I as seen in Fig. 6, lane 3. This gave strong evidence for the presence of β-hexosaminyl-β-hexosaminyl-structure, and at the same time proved that Para-Forssman glycolipid contained the same backbones in its structure as Globoside I and ceramide trihexoside.

Proton nmr spectrum was taken with the intact molecule of Para-Forssman glycolipid in order to confirm the anomeric configuration. Fig. 7 shows the spectrum of Para-Forssman glycolipid dissolved in pyridine-d_5 in comparison with the spectrum of Globoside I. One additional β-N-acetylgalactosaminyl residue was detected as indicated by the experiments described above. Two methyl signals

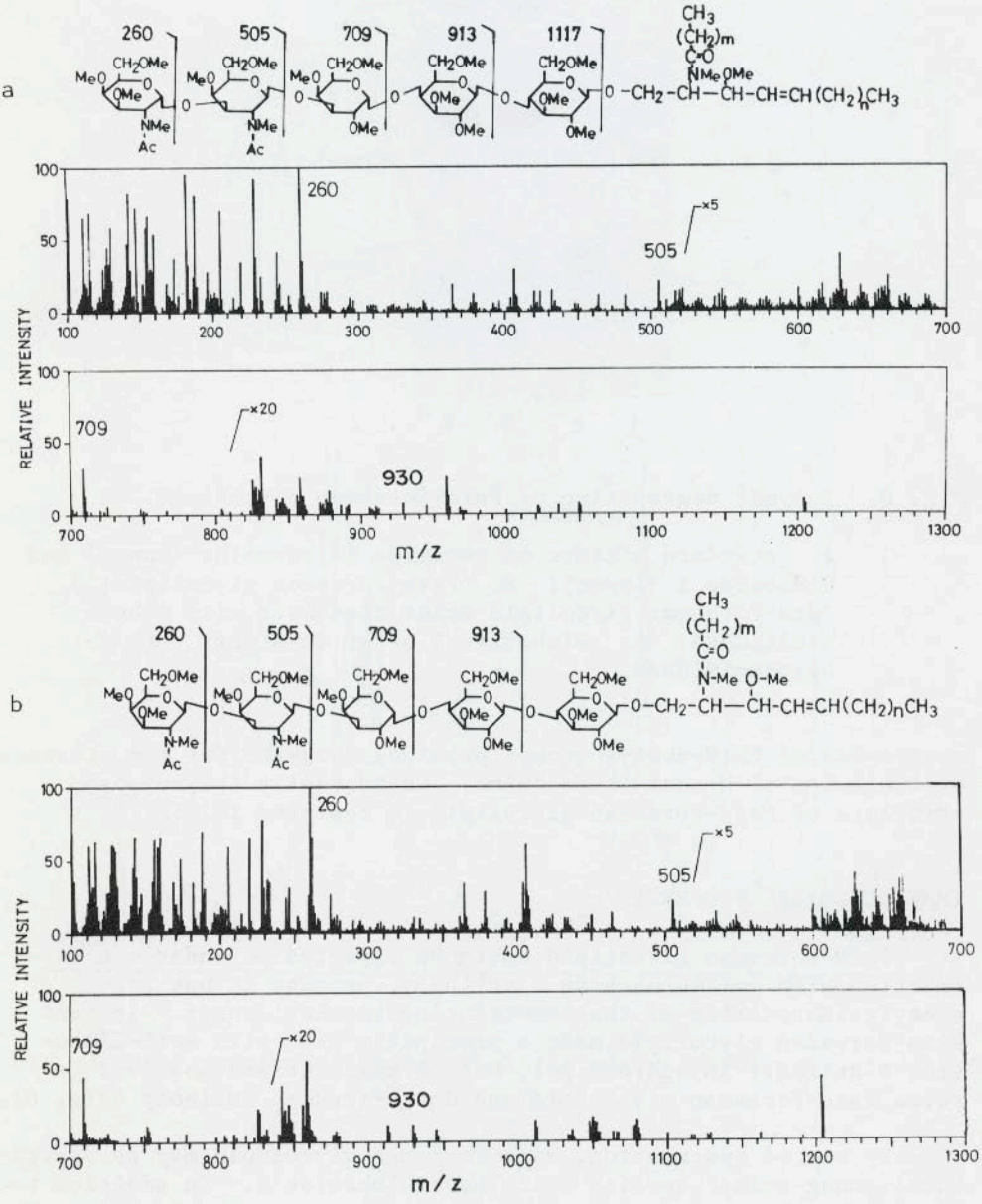

Fig. 5. Mass spectra of Forssman hapten (a) and Para-Forssman glycolipid (b).

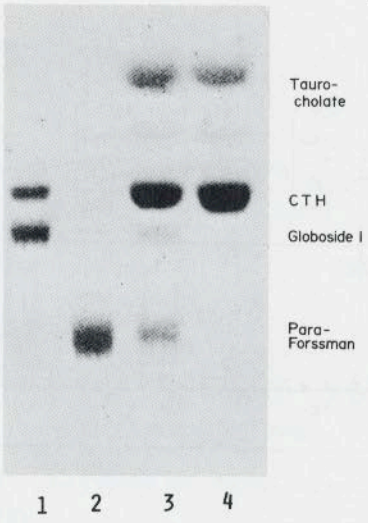

Fig. 6. Enzymic degradation of Para-Forssman glycolipid.

1. standard mixture of ceramide trihexoside (upper) and Globoside I (lower); 2. Para-Forssman glycolipid; 3. Para-Forssman glycolipid after treatment with β-hexosaminidase; 4. Globoside I after treatment with β-hexosaminidase.

corresponding to N-acetyl groups provided evidence for the presence of two moles of N-acetylhexosamine. Consequently the supposed structure of Para-Forssman glycolipid is depicted in Fig. 1.

IMMUNOCHEMICAL PROPERTY

Para-Forssman glycolipid would be expected to undergo cross-reaction with anti-Globoside I antibody, because it has β-N-acetylgalactosamine as the non-reducing terminal sugar. In fact Para-Forssman glycolipid made a precipitin line with anti-Globoside I antibody in agarose gel, but no reaction was observed between Para-Forssman glycolipid and anti-Forssman antibody (Fig. 8).

By way of speculation, Para-Forssman glycolipid may be distributed among animal species which have Globoside I. In addition to distribution, its biosynthesis and biological roles now need to be elucidated.

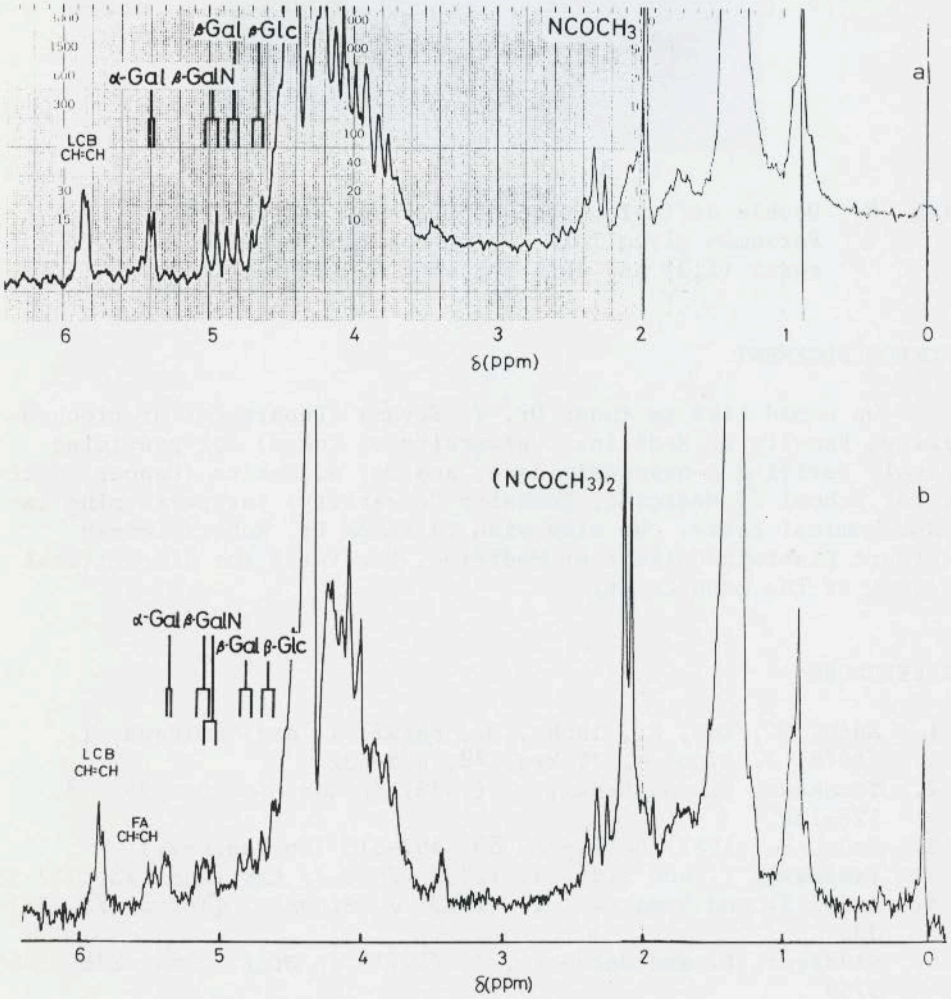

Fig. 7. Nmr spectra of Globoside I (a) and Para-Forssman glycolipid (b).

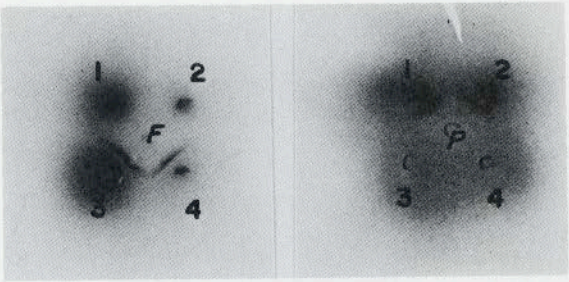

Fig. 8. Double diffusion test of Forssman hapten (F) and Para-Forssman glycolipid (P) with anti-Globoside I rabbit serum (1,2) and anti-Forssman hapten rabbit serum (3,4).

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