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A CERAMIDE TETRASACCHARIDE OF HUMAN ERYTHROCYTE MEMBRANE REACTING WITH ANTI-TYPE XIV PNEUMOCOCCAL POLYSACCHARIDE ANTISERUM

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SUMMARY

A ceramide tetrasaccharide whose structure was identified as Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide was isolated from human erythrocyte membrane. The glycolipids react with anti-Type XIV pneumococcal polysaccharide antiserum. A ganglioside with an identical carbohydrate skeleton having *N*-acetylneuraminic acid substituted at the C3 hydroxyl of the terminal galactosyl residue was also characterized.

INTRODUCTION

Two ceramide tetrasaccharides have been previously isolated and characterized; they are "globoside" and " cytolipin R", and their structures were identified as GalNAcβ1→3Galαl→4Galβ1→4Glc→ceramide[2,3] and GalNAcβ1→3Galα1→3Galβ1 4Glc→ceramide[4,5], respectively. The third ceramide tetrasaccharide has now been isolated from human erythrocyte membrane and was characterized as Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide***,†. The isolation, structure, and immunological properties of this glycolipid are reported in this paper.

A similar ceramide tetrasaccharide of human muscle with probale carbohydrate sequence Gal→GlcNAc→Gal→Glc→ceramide was recently described by Svennerholm *et al.*[6]. Ganglioside with an identical carbohydrate skeleton was also isolated and characterized from human erythrocyte membrane. The same ganglioside, but with *N*-glycolylneuraminic acid, was isolated from bovine erythrocytes and spleen by Kuhn and Wiegandt[7], by Wiegandt[8], and by Wintzer and Uhlenbruck[9], and the

Abbreviations: GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid.

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*** A preliminary note of this structure was described in a previous paper (1).

† The term "paragloboside", as its R_F value is close to "globoside", has been used during the course of this study, and the term is tentatively used in the text to refer to this new ceramide tetrasaccharide.

presence of glucosamine-containing ganglioside with *N*-acetylneuraminic acid in human erythrocytes has been known since Wherrett and Brown[10] and Ando *et al.*[11,12] described the presence of this ganglioside. Two gangliosides with structures NeuAc2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide (GL_{ntet}1a) and NeuAc2→6Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide (GL_{ntet}1b) have been isolated and established by Wiegandt[13]. Recently, a ganglioside with the same structure as "GL_{ntet}1a" was described by Li *et al.*[14] as human extraneural ganglioside.

In these studies, however, the linkage of glucosamine was not chemically established. Complete identification of the structure of human erythrocyte ganglioside is also described in this paper.

EXPERIMENTAL PROCEDURE

Isolation of glycolipid

The sphingolipid fraction of human erythrocyte membrane was prepared according to the method described earlier[5], and was further fractionated on a column of "Anasil S" (a mixture of silicic acid and MgO: Analab Company, North Haven, Conn.). The Anasil was suspended in chloroform (analytical grade, containing 0.5–0.75% ethanol for stabilizer) and was packed into a column with outer dimensions

TABLE I

SEPARATION OF GLYCOLIPIDS ON AN ANASIL S COLUMN 35 cm × 3.5 cm FOR ABOUT 2 g OF SPHINGOLIPID MIXTURE OF ERYTHROCYTES

Solvent No.	*Solvent composition*				*Solvent vol.*	*Glycolipid eluted*
	Chloroform	*Methanol*	*Water*	*Acetone*		
1	90	10	1	0	700	Glucosylceramide
2	80	20	1	0	300	Lactosylceramide
					400	Trihexosylceramide
3	70	20	2	0	1000	Trihexosylceramide
4	65	25	3	0	1000	Globoside
5	60	30	3	0	500	Globoside
6	60	30	4	0	300	Globoside + unknown (paragloboside)
7	60	20	5	20	800	Globoside + unknown (paragloboside) + smal amount of H-glycolipic
8	60	25	5	25	1000	Blood group glycolipids I, II, H, Le^a, Le^b and ganglioside I
9	60	30	10	30	500	Blood group glycolipid III, ganglioside II, III
10	50	30	10	30	1000	Blood group glycolipid IV, ganglioside IV
11	50	40	10	30	500	None

3 cm × 35 cm, followed by washing with chloroform–methanol–water (90:10:1, by vol.) (Solvent 1). 2 g of the sphingolipid fraction[5] were suspended in Solvent 1 and introduced into the column. Elution was carried out by gravity flow, and the eluate was collected by a fraction collector. The elution schedule is shown in Table I. A glycolipid giving a spot slightly slower than globoside was co-eluted with globoside with Solvents 6 and 7. The major ganglioside was eluted by Solvent 8, and other gangliosides were eluted with more polar solvents (see Table I). The fractions eluted with Solvents 6 and 7, which contained globoside, "paragloboside", and a small amount of H-active glycolipid[12], were combined. This fraction (weight 170 mg), dissolved in Solvent 5 (Table I), was applied onto a long column of Anasil S (dimensions 80 cm × 1.5 cm) prepared in the same solvent, and was eluted with 360 ml of the same solvent. Each 6-ml aliquot was taken by a fraction collector (Fractions 1–60); then the column was eluted with 360 ml of Solvent 6 (Table I), and Fractions 61–120 were separated by a fraction collector. Finally the column was eluted with 120 ml of Solvent 7, and Fractions 121–141 were taken. Fractions were examined by thin-layer chromatography. Fractions 1–45 had no glycolipid; Fractions 45–80 contained globoside and a small amount of "paragloboside". Fractions 81–91 contained pure "paragloboside", and Fractions 93–100 had "paraglobiside" and a small amount of blood group H-active glycolipid.

The fractions enriched in paragloboside were again subjected to a third chromatography on a long column of Anasil S with the same dimensions (80 cm × 1.5 cm) using the same solvent system (Solvents 5 and 6); thus, a sufficient quantity of pure paragloboside was obtained. The yield of pure paragloboside was about 10 mg from 2 g of sphingolipid fraction. This yield is much greater that that of blood group ABH glycolipids.

Gangliosides I, II and III obtained from Anasil S chromatography (see Table I) were purified on DEAE-cellulose chromatography, and gangliosides were eluted with chloroform–methanol–NH_4OH–ammonium acetate (50 ml of a mixture of chloroform–methanol, (4:1, by vol.) containing 2 ml of concentrated NH_4OH and 0.47 g of ammonium acetate), according to Rouser *et al.*[15].

Analytical method

Amino sugars and neutral sugars were analyzed by gas chromatography by the method of Sawardeker *et al.*[16], as modified by Björndal *et al.*[17] and by Yang and Hakomori[18]. The sialic acid/neutral sugar ratio was determined according to Sweeley and Walker[19]. Fatty acids and sphingosines were analyzed by gas–liquid chromatography after methanolysis, according to the procedure described previously[18]. Homogeneity of the glycolipid was tested by thin-layer chromatography on Silica gel H, developed with chloroform–methanol–water (60:35:8, by vol., upper phase).

Methylation analysis

Glycolipids were methylated in dimethylsulfoxide, sodium hydride, and methyl iodide[20]. The degree of permethylation was checked with infrared spectra. Permethylated glycolipids were degraded in 0.5 ml of 95% acetic acid containing 0.25 M H_2SO_4 (ref. 21), followed by hydrolysis on addition of 0.5 ml water. The resulting partially O-methylated hexoses and hexosamines were converted to partially O-methylated hexosaminitols and partially O-methylated 2-deoxy-2-*N*-methyl-acetamidohexitols by

the method described[18]. The derivatives of neutral sugars were separated by gas chromatography and identified by mass spectrometry according to the procedure of Björndal *et al.*[22,23], and the derivatives of amino sugars were separated and identified by gas chromatography and mass spectrometry according to the method of Stellner *et al.*[21].

Degradation

The sequence of carbohydrates and their anomeric linkages were determined by enzymatic degradation. 100 μg of ganglioside were hydrolyzed in 100 μl of 0.05 M sodium acetate buffer, pH 5.5, containing 1% NaCl and 0.1% $CaCl_2$, and in 50 μl neuraminidase from *Clostridium perfringens*, Grade IV (Sigma Chemical Co.), which had 2 units in total dissolved in 1 ml. After 16–18 h at 37 °C, the incubation mixture was shaken with 6 vol. of chloroform–methanol (2:1, by vol.), and the glycolipids recovered in the lower phase were analyzed by thin-layer chromatography. On the other hand, glycolipids were hydrolyzed by various glycosidases: jack bean β-galactosidase[24], β-*N*-hexosaminidase[25], and fig α-galactosidase[26]. Conditions of enzymatic hydrolysis and determination of the degradation product by thin-layer chromatography were according to the method described previously[3].

Ganglioside was also hydrolyzed with 0.025 M H_2SO_4 at 80 °C for 90 min, and the degradation product was examined by thin-layer chromatography as described above.

Immunization and immunological assay

1 mg of "paragloboside" and 5 mg of bovine serum albumin dissolved in 1 ml water were mixed with 5 mg tubercule bacilli and 1 ml of Freud's adjuvant, were thoroughly emulsified, and the emulsion was then injected into a New Zealand white rabbit. After 2 weeks another booster injection was made, and after 45 days after the first injection, the rabbit was bled. The method employed was essentially the same as previously described[27]. Antisera were tested with a number of glycolipids (100 μg/100 μl) on double-diffusion agar plates according to the method described previously[28]. Anti-Type XIV pneumococcal antisera (obtained from Dr Donald Marcus) was reacted with "paragloboside" and a ceramide tetrasaccharide obtained by desialylation of ganglioside[1].

A complement fixation test of the total lipid fraction with anti-Type XIV pneumococcal antiserum was carried out according to the routine method carried out in this department[29].

RESULTS

Isolation and sugar composition of ceramide tetrasaccharide

The unknown ceramide tetrasaccharide (paragloboside) was separated from globoside and was eluted with chloroform–methanol–water (60:30:4, by vol.) from an Anasil S column immediately after the globoside was eluted, and it partially overlapped with globoside. The glycolipids bearing blood group H-, and Le^a activities were eluted immediately after paragloboside was eluted. Mutual separation of these three glycolipids was difficult[12]. The paragloboside contained 1 mole each of *N*-acetylglucosamine and glucose and 2 moles of galactose per ceramide. No galactosamine was detected in the hydrolysate by gas chromatography according to the method of Yang and Hakomori[18].

Sugar sequence and anomeric linkage of ceramide tetrasaccharide indicated by enzymatic hydrolysis

The glycolipid was hydrolyzed by β-galactosidase but not by β-*N*-acetylhexosaminidase, whereby the glycolipids were converted to a ceramide trisaccharide, suggesting that β-galactoside is located at the non-reducing terminal. The ceramide trisaccharide found in the hydrolysis product of paragloboside was then readily hydrolyzed by β-*N*-acetylhexosaminidase. The hydrolysis product was a lactosylceramide, which was further degraded by β-galactosidase to a glucosylceramide. The hydrolyzability by hydrolases indicated that the carbohydrate sequence is β-galactosyl-β-*N*-acetylglucosaminyl-β-galactosylglucosylceramide.

Methylation analysis

2,3,4,6-Tetra-*O*-methylgalactitol-1,5-diacetate, 2,4,6-tri-*O*-methylgalactitol-1,3,5-triacetate, and 2,3,6-tri-*O*-methylglucitol-1,4,5-triacetate were identified as partially *O*-methylated hexitols. The gas chromatography and mass spectrometry pattern was identical to that of published data[22,23]. 3,6-Di-*O*-methyl-2-*N*-methylacetamidoglucitol was identified as the partially methylated hexosamine component. The retention time on gas chromatography and the mass spectra of this compound were identical to the reference compound prepared through partially O-(1-methoxy)ethylated-2-deoxy-2-acetamidoglucose, as previously described[21]. No 4,6-di-O-methyl derivative was detected by gas chromatography or mass spectrometry (see Fig. 1). The results clearly indicated that "paragloboside" has the structure Galβ1→4GlcNAcβ1→

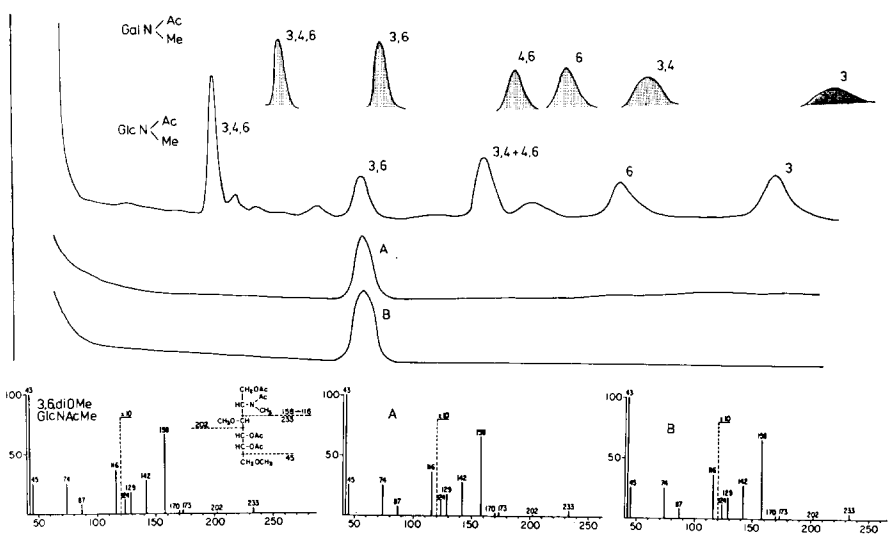

Fig. 1. Gas chromatography of partially O-methylated 2-deoxy-2-*N*-methylacetamidohexitols and that found in the hydrolysates of paragloboside and ganglioside of human erythrocyte membrane. Upper series of peaks (shaded): various derivatives of 2-deoxy-2-*N*-methylacetamidogalactitols; number indicates the position of O-methyl substitutions. Second series of peaks: various derivatives of 2-deoxy-2-*N*-methylacetamidoglucitols; numbers indicate the same as above. These data reproduced from ref. 21. A, peak obtained from paragloboside; B, peak obtained from ganglioside of human erythrocyte membrane. Mass spectra: left, reference 3,6-di-*O*-methyl-GlcNAcMe; middle, Peak A; right, Peak B.

3Galβ1→4Glc→ceramide. This carbohydrate structure is thus identical to lacto-*N*-neotetraose[30].

The oligosaccharide liberated from ceramide tetrasaccharide

A modified Wiegandt's degradation with osmium periodate and alkali[31] resulted in a liberation of oligosaccharide, which migrated on paper chromatography as lacto-*N*-*neo*-tetraose, but shortage of the substance did not permit further determination of the chemical properties of oligosaccharide liberated from this glycolipid. It is clear, however, that the results of methylation and enzymatic degradation agree completely to support this structure.

*Reactivity with Type XIV pneumococcal polysaccharide antiserum**

A remarkable reactivity was demonstrated by analysis with double-diffusion agar. The glycolipid had a line with anti-Type XIV pneumococcal polysaccharide antiserum and also gave a line with the antiserum prepared against the glycolipid (Fig. 2).

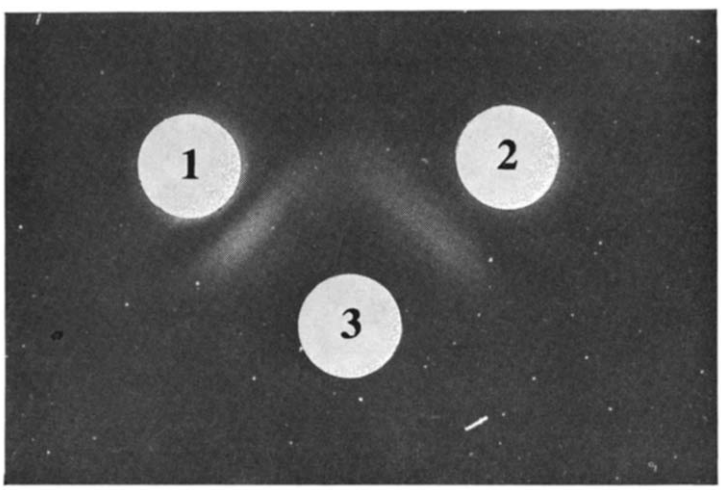

Fig. 2. Immunoprecipitin lines between anti-"paragloboside" (Well 1) and "paragloboside" (Well 3), and anti-Type XIV pneumococcal polysaccharide antisera (Well 2).

* Some "anti-Type XIV antiserum" obtained from a commercial source did not react or very weakly reacted with paragloboside, although the antiserum did react strongly with some preparations of so-called "Type XIV pneumococcal" polysaccharide. This polysaccharide antigen did not react with the antisera against paragloboside. Methylation study of such polysaccharide yielded only 3-*O*-methyl-GlcNAcMe as amino sugars and an excessive quantity of 2,3,4,6-tetra-*O*-methylgalactitol, indicating the terminal structure of:

$$\begin{matrix} \text{Gal}\beta \\ \qquad\searrow^{4} \\ \qquad\quad \text{GlcNAc}\rightarrow\text{R} \\ \qquad\nearrow_{6} \\ \text{Gal}\beta \end{matrix}$$

(Stellner, K., Saito, H. and Hakomori, S., unpublished work). In selection of antiserum, one should be careful for determination of Type XIV specificity.

Structure of ganglioside

The major ganglioside of human erythrocyte had 1 mole each of sialic acid, *N*-acetylglucosamine, and glucose, and 2 moles of galactose, in agreement with Wherrett and Brown[10] and Ando *et al.*[11]. The sialic acid of this ganglioside was easily hydrolyzed by neuraminidase to give a ceramide tetrasaccharide which had identical thin-layer chromatography behavior as "paragloboside". A ceramide tetrasaccharide obtained by desialylation of this ganglioside was readily hydrolyzed by β-galactosidase to give ceramide trisaccharide, which was further degraded by β-*N*-acetylhexosaminidase to give lactosylceramide.

Acid hydrolysis with a mild acid (0.025 M H_2SO_4) of the ganglioside also gave a ceramide tetrasaccharide identical to "paragloboside" in R_F value on thin-layer chromatography and in immunological activity. Permethylated ganglioside on acetolysis followed by hydrolysis gave 2,4,6-tri-*O*-methylgalactitol-1,3,5-triacetate, 2,3,6-tri-*O*-methylglucitol-1,4,5-triacetate. The results of amino sugar identification are shown in Fig. 1. A ceramide tetrasaccharide obtained by removal of the sialyl residue from this ganglioside gave on permethylation, followed by acetolysis and hydrolysis, 2,3,4,6-tetramethylgalactitol-1,5-diacetate and 2,4,6-tri-*O*-methylgalactitol-1,3,5-triacetate, 2,3,6-tri-*O*-methylglucitol-1,4,5-triacetate and 3,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidoglucitol-1,4,5-triacetate. All these sugars were identified by gas chromatography and mass spectrometry. These results indicate that the structure of a major ganglioside of human erythrocyte is sialyl(2→3)galactosylβ(1→4)*N*-acetylglucosaminylβ(1→3)galactosylβ(1→4) glucosylceramide (see Fig. 3).

Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide
Neu Acα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide

Fig. 3. Structures of two glycolipids isolated from human erythrocytes.

Fatty acid and sphingosines of paragloboside and ganglioside

The results of analysis are shown in Table II.

DISCUSSION

Glycolipids bearing blood group A and H specificities all belong to the Type 2 chain[32], *i.e.* Galβ1→4GlcNAcβ1→3Gal→R, rather than to the Type 1 chain, discussed in recent structural studies on variants of blood group A (ref. 1) and blood group H (ref. 33) glycolipids. It is highly possible that this glycolipid is a precursor of ABH blood group glycolipids. The cross-reactivity with anti-Type XIV pneumococcal polysaccharide demonstrated by this glycolipid further supports the idea that it could be the precursor of blood group glycolipids.

This glycolipid could also be a precursor of the major ganglioside of human erythrocytes, whose structure was identified as a sialyl group substituted at C3 of the terminal galactose of this ceramide tetrasaccharide. The terminal sialyl–galactose linkage seems to be very easily hydrolyzed by acid and sialidase. A question arises, therefore, whether lacto-*N*-*neo*-tetraosylceramide is the artifact resulting from the hydrolysis of sialosyllacto-*N*-*neo*-tetraosyl ceramide produced by the action of serum neuraminidase during storage of blood. Therefore, fresh blood was extracted, and the lipid fraction was tested by complement fixation to determine whether any reactive

TABLE II

FATTY ACIDS AND SPHINGOSINES OF PARAGLOBOSIDE AND GANGLIOSIDE

	Paragloboside		*Ganglioside*	
	Fatty acid	*Base*	*Fatty acid*	*Base*
C16:0	6		10	
C18:0	11	0	23	0
C18 (3-OH; phytosphingosine)		0		0
C18:1	6	+ (over 90%)	9	+ (over 90%)
C20:0	3	0	2	0
C20:1		0		0
C22:0	15	0	4	0
C22:1	2	0	3	0
C24:0	33		27	
C24:1	23		21	
Unidentified	1	+	1	+

lipid is present with anti-Type XIV pneumococcal antisera. The reactive lipid is present with anti-Type XIV pneumococcal antisera. The results indicated that the lipid fraction from 10 ml of fresh blood can fix the complement up to a dilution of 1:200, and the lipid hapten able to react with anti-Type XIV pneumococcal polysaccharide is indeed present.

The presence of a ganglioside having lacto-*N*-*neo*-tetraosylceramide as a backbone structure was demonstrated by Kuhn and Wiegandt[7] 10 years ago. Recently, the structure of spleen and kidney gangliosides with sialyl substitution at C3 and C6 positions of terminal galactosyl residue of lacto-*N*-*neo*-tetraosylceramide was alleged by Wiegandt[13], although location of the sialyl residue at the terminal galactose has been predicted for many years by susceptibility of sialic acid to neuraminidase treatment (refs 10, 12, and personal communication from Dr J. Wherrett, University of Toronto). As recently shown by Li *et al.*[11], this type of ganglioside can be a major ganglioside of extraneural tissue of humans.

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Dr Klaus Stellner of this laboratory aided in methylation analysis, and β-galactosidase and β-*N*-acetylhexosaminidase of jack bean were donated by Dr Y.-T. Li of Tulane University, to whom we are gratefully indebted.

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