COMPLEX GLYCOSPHINGOLIPIDS WITH BLOOD-GROUP A SPECIFICITY

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1. Introduction

The oligosaccharide chains present in blood-group ABH glycoproteins and glycolipids of glandular epithelial tissue exhibit a considerable degree of heterogeneity with respect to length of carbohydrate chains and degree of branching [1–6]. The sizes of the largest blood-group-active oligosaccharide chains from horse gastric mucosa are in the range of 8–12 sugar residues [7], whereas those from hog gastric mucosa [1,3,8] and human cyst fluid [2,4] are closer to 16–18 residues or even larger [2]. Glycolipids of unusual complexity with 30–50 sugar residues have recently been isolated from human erythrocyte membrane by Gardas and Koscielak [9,10]. So far the existence of glycolipids of comparable carbohydrate structures in glandular epithelial tissue have not been reported. In this report we describe the isolation and characterization of two blood-group A-active glycolipids, the carbohydrate portions of which consist of 12 and 18 sugar residues.

2. Experimental

2.1. *Materials*

Hog gastric mucosa scrapings, washed with hexane, were obtained from Wilson Laboratories (Chicago, Ill.). Human red cells A, B, O types, human blood-grouping serum anti-A, anti-B and anti-H (*Ulex europeous* extract) were from Schering Diagnostics (Port Reading, NJ). Ion-exchange cellulose Cellex-P and Bio-Gel P-10 (100–200 mesh) were supplied by Bio-Rad Laboratories (Richmond, Calif.). Florisil (100–200 mesh) was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Silica gel HR plates, 250 nm coating thickness were from Analtech, Inc. (Wilmington, Del.). Methyl ethers of neutral sugars were kindly provided by Drs H. Choi and K. Meyer (New York, NY). Partially methylated amino sugar standards were from the same source as reported in refs [3] and [6] and were also obtained from Dr P. A. J. Gorin (Saskatoon, Canada). Standard fatty acid methyl esters were from Applied Science Laboratory (State College, Pa.) and octadecasphingenine and octadecasphinganine were purchased from Miles Laboratories, Inc. (Elkhart, Ind.).

2.2. *Isolation of glycolipids*

The initial procedure for the extraction of the crude water-soluble glycolipids was essentially similar to that described previously [9,10]. Briefly the method included extraction of gastric mucosa with ethanol, filtration and solubilization of the dry residue in 0.02 M phosphate buffer pH 7.5. After 2 h an equal volume of *n*-butanol was added, the mixture was stirred at 4°C for 24 h and centrifuged in a Sorvall model RC2-B centrifuge at 15 000 × *g* for 20 min. The water phase was collected and the residue re-extracted as above. The combined aqueous phase was then extracted twice, each time for 24 h, with an equal volume of *n*-butanol, dialyzed and lyophilized. The lyophilizate was dissolved in 0.5 M NaOH at a concentration of 5 mg/ml and incubated at room temperature for 40 h. The products of alkaline degradation were dialyzed, concentrated to a

small volume, equilibrated with 0.02 M sodium citrate buffer saturated with *n*-butanol and applied in portions to a Cellex-P column (3.0 × 40 cm). The column was eluted with 0.02 M sodium citrate buffer, pH 3.6, saturated with *n*-butanol. Fractions (5 ml) were collected and monitored with the phenol–sulfuric acid reagent for carbohydrates [11]. The fraction containing glycolipids was dialyzed, lyophilized and acetylated [12]. Material recovered [12] from the acetylation mixture was dissolved in 1,2-dichloroethane–acetone (1:1), with the aid of sonication, and chromatographed on a Florisil column [5]. Glycolipid I was eluted from the column (2.5 × 40 cm) with 2 liters of 1,2-dichloroethane/methanol/water (2:8:1) and glycolipid II with 1.5 liters of chloroform/methanol/water (5:1:1). The final purification of the acetylated glycolipids I and II was accomplished by repeated preparative thin-layer chromatography in chloroform/methanol/water (65:25:4) and *n*-propanol/water (7:3). Gel filtration on a Bio-Gel P-10 column equilibrated with 0.5 M NaCl saturated with *n*-butanol was employed to study the molecular size and homogeneity of the deacylated glycolipids.

2.3. *Analytical methods*

Gas–liquid chromatography was performed with a Perkin-Elmer model 801 apparatus. The alditol acetates, obtained from the glycolipids according to the procedure of Yang and Hakomori [13], were analyzed on 1% ECNSS-M columns programmed at 2°C/min. from 150°C to 210°C. The alditol acetates of partially methylated sugars derived from permethylated glycolipids were analyzed on the same columns at 140°C and 160°C for the neutral sugars, and at 190°C for hexosamines. Trimethylsilyl derivatives of long-chain bases and the methyl esters of fatty acids were analyzed on 3% SE-30 columns [5]. Hemagglutination and hemagglutination-inhibition assays were performed with the Takatsy microtitrator as descirbed previously [5]. Sialic acid was determined by the method of Miettinen and Takki-Luukkainen [14] and proteins by the Lowry procedure [15]. Chromatography of the long-chain bases isolated from the glycolipid hydrolysates [5] was performed on thin-layer plates [16]. Long-chain bases were detected with ninhydrin spray and glycolipids with orcinol reagent; iodine was used for preparative purposes.

2.4. *Structural studies*

Controlled periodate oxidation of the deacylated [12] glycolipids and I and II was performed in 0.015 M sodium metaperiodate in methanol–water at room temperature for 18 h in the dark [17]. After destruction of excess periodate and dialysis, the oxidized glycolipids were reduced with $NaBH_4$. The excess of borohydride was destroyed with a few drops of acetic acid, the mixture was dialyzed and subjected to completion of Smith degradation [3]. After the first periodate treatment, and following each completed cycle of Smith degradation an aliquot of the sample was analyzed for carbohydrates.

The glycolipids recovered after three consecutive Smith degradations of glycolipids I and II were subjected to partial acid hydrolysis in 1.0 M formic acid for 2 h at 100°C [5]. The glycolipid fragments recovered from the lower phase of the partition system were chromatographed on thin-layer plates developed in chloroform/methanol/water (65:35:8) and identified as described previously [6].

The isolated glycolipids were permethylated according to the procedure of Hakomori [18]. The permethylated glycolipids were purified by chromatography on thin-layer plates developed in benzene/methanol (85:15) and subjected to hydrolysis reduction and acetylation [19]. Partially methylated alditol acetates were analyzed by gas–liquid chromatography. The peaks were identified by comparing their retention times with those of standard compounds and with the reported [19] retention values.

3. Results

A procedure of Gardas and Koscielak [9,10] was applied for the extraction of water-soluble complex glycolipids from hog gastric mucosa. After alkaline degradation of the susceptible glycoproteins and dialysis, the proteins were separated from the glycolipids by chromatography on Cellex-P column. The glycolipids present in the recovered fraction, after acetylation, were freed of non-glycolipid contaminants and separated into two components on a Florisil column. The final purification of the individual, acetylated glycolipids was accomplished by repeated chromatography on thin-layer plates (fig.1). The purified glycolipids were obtained in a yield of 10.7

mg (glycolipid I) and 8.8 mg (glycolipid II) per 1000 g of dry stomach mucosa.

Deacylated glycolipids I and II gave single, slightly included peaks when chromatographed on Bio-Gel P-10 in *n*-butanol-saturated NaCl solution. The extent of A activity of the isolated glycolipid I was 0.2 μg/0.1 ml and glycolipid II 0.25–0.5 μg/0.1 ml. Gas–liquid chromatography of the alditol acetates, formed from the carbohydrate portion of glycolipid I (table 1), established the presence of fucose, glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine in a molar ratio of 2:1:5:8:2, respectively. Fucose, glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine in the respective molar ratio of 1:1:3:6:1 were

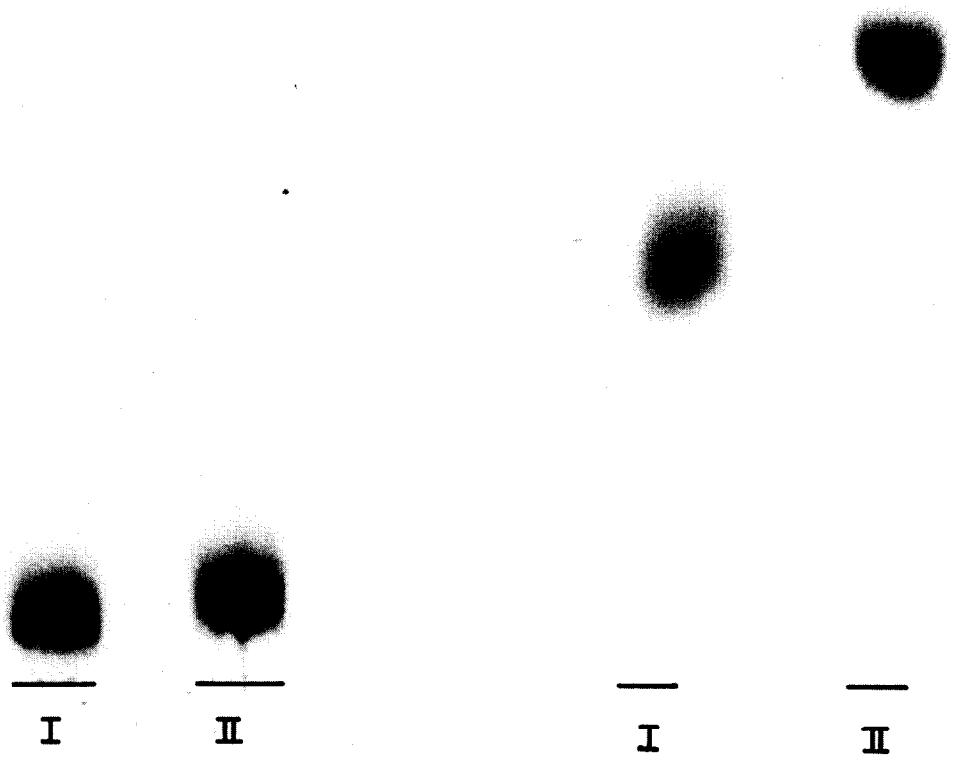


Fig.1. Thin-layer chromatography of the acetylated complex glycosphingolipids from hog gastric mucosa. (1) Glycolipid I. (2) Glycolipid II. Left plate developed in chloroform/methanol/water (65:25:4). Right plate developed in *n*-propanol/water (7:3).

Table 1
Effect of serial periodate oxidation on the monosaccharide compositions of glycolipids I and II

Glycolipid		Molar ratios				
		Fuc	Gal	Glc	Glc*N*Ac	Gal*N*Ac
Native	I	2.05	5.01	1.0	7.96	2.08
	II	1.06	2.97	1.0	6.10	1.02
Initial periodate oxidation	I	–	4.03	1.0	6.02	–
	II	–	3.0	1.0	4.01	–
First Smith degradation	I	–	3.98	1.0	6.04	–
	II	–	2.97	1.0	3.96	–
Second Smith degradation	I	–	2.03	1.0	4.0	–
	II	–	2.01	1.0	1.98	–
Third Smith degradation	I	–	2.03	1.0	1.98	–
	II	–	2.02	1.0	1.97	–

found in glycolipid II (table 1). Gas–liquid chromatography of the fatty acid methyl esters showed that hexadecanoate, octadecanoate and octadecenoate were the major fatty acids of both glycolipids. Thin-layer chromatography of long-chain bases revealed that C_{18}-sphingenine is the only base present in these glycolipids. The molar ratio of sphingosine to glucose was about 0.9:1 for both glycolipids. Protein and sialic acid were not detected.

The results of three sequential Smith degradations performed under controlled conditions [17] on glycolipids I and II are shown in table 1. Analysis of the glycolipid fragments recovered after three complete steps of Smith degradation of glycolipids I and II showed, in both glycolipids, the presence of glucose, galactose and *N*-acetylglucosamine in the molar ratios of 1:1:2. Partial acid hydrolysis of these fragments resulted mainly in the formation of Gal→Glc→ceramide (major product), Glc*N*Ac→Gal→Glc→ceramide and Glc*N*Ac→Glc*N*Ac→Gal→Glc→ceramide (minor products). These results suggest that the sequential arrangement of the sugar units in the saccharide chains adjacent to the ceramide core in both glycolipids I and II is Gal→Glc*N*Ac→Glc*N*Ac→Gal→Glc→ceramide.

Table 2
Molar ratios of the alditol acetates found in the hydrolyzates of permethylated glycolipids I and II

Methylated sugars	Molar ratios	
	Glycolipid I	Glycolipid II
2,3,4-Tri-*O*-methylfucositol	1.7	0.8
2,3,6-Tri-*O*-methylgalactitol	0.9	–
2,3,6-Tri-*O*-methylglucitol	1.0	1.0
4,6-Di-*O*-methylgalactitol	1.9	0.9
2,4-Di-*O*-methylgalactitol	0.8	1.8
2-Mono-*O*-methylgalactitol	0.9	–
3,4,6-Tri-*O*-methyl-*N*-methylacetamidogalactitol	2.0	1.1
3,4,6-Tri-*O*-methyl-*N*-methylacetamidoglucitol	2.1	1.9
3,6-Di-*O*-methyl-*N*-methylacetamidoglucitol	5.8	3.7

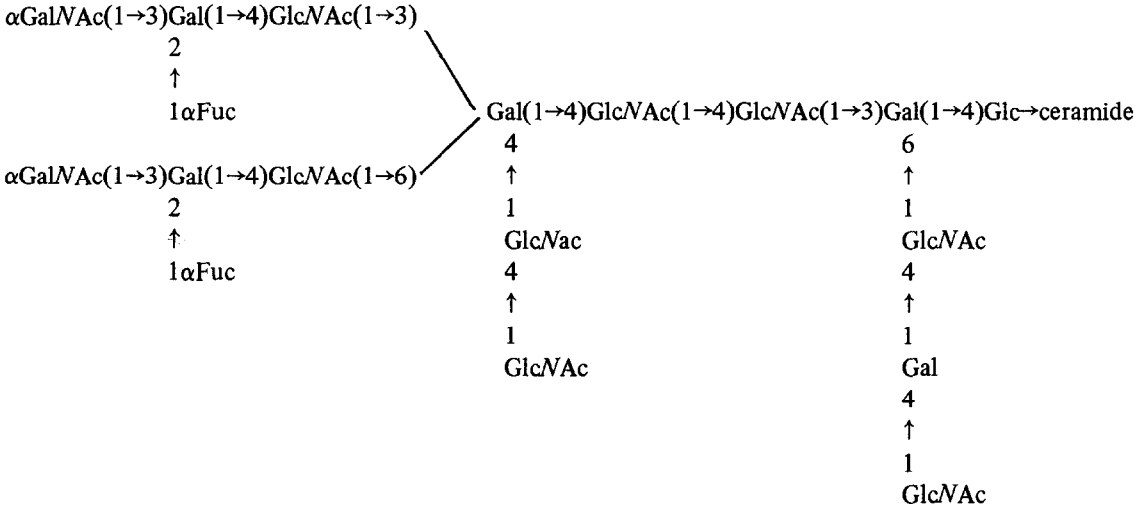
The ratios are expressed relative to 2,3,6-tri-*O*-methylglucitol.

The molar ratios of partially methylated alditol acetates found in the hydrolysates of the permethylated glycolipids I and II are given in table 2. Results of analysis indicated that two residues of fucose, two residues of *N*-acetylgalactosamine and two out of eight *N*-acetylglucosamine residues in glycolipid I are located at non-reducing termini. Analysis of the partially methylated alditol acetates from hydrolysates of glycolipid II revealed that fucose, *N*-acetylgalactosamine and two out of six *N*-acetylglucosamine residues in this glycolipid were terminal.

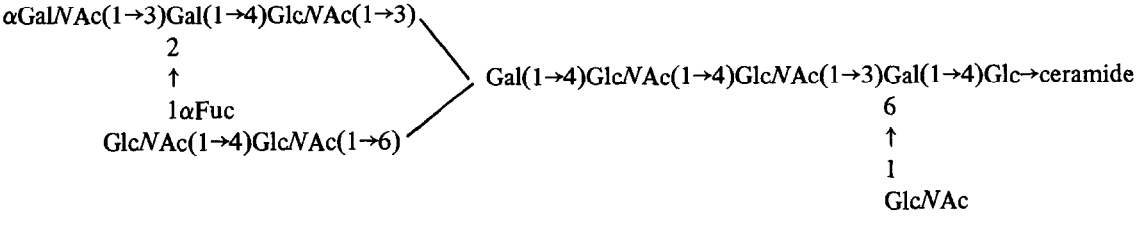
4. Discussion

Two glycolipids (I and II) consisting of 18 and 12 sugar units, respectively, and exhibiting blood-group A-activity have been purified from the water-soluble glycolipid fraction of hog gastric mucosa. From the results of analysis of sugar composition, Smith degradation, partial acid hydrolysis and permethylation studies, the structures of these glycolipids are proposed to be:

I.



II.



The assignment of the α-configuration to the terminal *N*-acetylgalactosamine and fucose is based on the type-A antigenic properties of the glycolipids. The proposed structures show considerable similarity to the structures of the oligosaccharide chains found previously only in the blood-group-active glycoproteins of the same tissue [1–4,7,8,20]. The backbone of the suggested structures consists of ceramide pentasaccharide which is common to both glycolipids. The ceramide pentasaccharide consists of two adjacent *N*-acetylglucosamine residues and two residues of galactose, both of which are involved in branching. The side chains attached to the galactose at branching points, apparently exhibit a considerable degree of variance with respect to the number and type of sugar residues. This probably accounts for the great heterogeneity commonly found among blood-group-active glycoproteins and glycolipids. The existence of glycolipids with carbohydrate structures identical to those found in oligosaccharides of glycoprotein origin, substantiates further the existence of a common pathway for the biosynthesis of blood-group-active glycoproteins and glycolipids.

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