Abnormalities of glycosphingolipids in mucopolysaccharidosis Type III B

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Abstract Glycosphingolipids from brain, liver, and spleen of a patient with mucopolysaccharidosis type III B were quantitatively analyzed. Neutral glycosphingolipids containing glucosylceramide, lactosylceramide, globotriaosylceramide, globotetraosylceramide, and gangliotriaosylceramide were increased in the brain, while the contents of galactosylceramide and galactosylceramide I^3-sulfate were decreased. The total ganglioside levels were low in the grey matter (522 μg N-acetylneuraminic acid/g) and high in the white matter (342 μg N-acetylneuraminic acid/g), when compared with the normal values (744–918 μg/g in grey matter and 80–180 μg/g in white matter). The ganglioside compositions were characterized by a high proportion of II^3-N-acetylneuraminosylgangliotriaosylceramide (GM2), II^3-N-acetylneuraminosyllactosylceramide (GM3), and II^3-(N-acetylneuraminosyl)$_2$lactosylceramide (GD3). An unusual band of protein in place of an ordinary band of Wolfgram protein was detected as a major band by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The low levels of 4-eicosasphingenine in the brain gangliosides indicated that the disturbance of the sphingolipid metabolism already began at age 3 at the latest and that the brain remained immature. These abnormal glycosphingolipids and protein as well as the accumulation of heparan sulfate explain in part the severe progressive mental retardation which is most characteristic of the mucopolysaccharidosis III B. Abnormalities of glycosphingolipids in the liver and spleen are also found.—**Hara, A., N. Kitazawa, and T. Taketomi.** Abnormalities of glycosphingolipids in mucopolysaccharidosis Type III B. *J. Lipid Res.* 1984. **25:** 175–184.

Supplementary key words glycosphingolipids • gangliosides • sphingosine • globosides

Mucopolysaccharidosis III B (Sanfilippo syndrome type B) is a metabolic disease in which α-N-acetylglucosaminidase is hereditarily defective, and is characterized by severe progressive mental retardation and relatively mild somatic features (1). While the large amount of heparan sulfate accumulated in various organs, including brain, in mucopolysaccharidosis type III B is due to the defect of α-N-acetylglucosaminidase, some abnormal patterns of glycosphingolipids determined by thin-layer chromatography were reported in the brain (2). Glycosphingolipids are intrinsic constituents especially in central nervous system. It is well known that sphingolipidosis, in which a certain glycosphingolipid accumulates in the central nervous system due to the defect of the corresponding glycosphingolipid hydrolase, also shows severe progressive mental retardation which is characteristic of mucopolysaccharidosis type III. Thus, the abnormalities of glycosphingolipids in the mucopolysaccharidosis type III may be responsible in part for this characteristic clinical feature in the disease. The purpose of this report is to determine the abnormal glycosphingolipids quantitatively in the brain, liver, and spleen, and also to analyze sugar, sphingosine, and fatty acid compositions of the glycosphingolipids.

MATERIALS AND METHODS

Case report

The patient was a female and died at 18 years and 10 months of age. She was one of seven siblings; two brothers died at age 13 years and 15 years with similar symptoms. She began to have ataxia at age 3. Developmental retardation was noticed at age 4. Progressive mental retardation was observed at age 12. Enzyme assay revealed that α-N-acetylglucosaminidase was defective in both leukocytes and cultured skin fibroblasts of the patient, while the other lysosomal enzymes were normal. Uronic acid in urine was markedly increased (80.6–89.2 mg of uronic acid/g of creatinine). Glycosaminoglycan in the urine

Abbreviations: GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; $GbOse_3Cer$, globotriaosylceramide; $GbOse_4Cer$, globotetraosylceramide; $GgOse_3Cer$, gangliotriaosylceramide; GalCerI^3-sulfate, galactosylceramide I^3-sulfate; $nLcOse_4Cer$, neolactotetraosylceramide; II^3NeuAc-LacCer, II^3-N-acetylneuraminosyllactosylceramide; $II^3(NeuAc)_2$-LacCer, II^3-di-N-acetylneuraminosyllactosylceramide; II^3NeuAc-$GgOse_3Cer$, II^3-N-acetylneuraminosylgangliotriaosylceramide; II^3NeuAc-$GgOse_4Cer$, II^3-N-acetylneuraminosylgangliotetraosylceramide; $II^3(NeuAc)_2$-$GgOse_4Cer$, II^3-di-N-acetylneuraminosylgangliotetraosylceramide; IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$, IV^3,II^3-di-N-acetylneuraminosylgangliotetraosylceramide; IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4Cer$, IV^3-N-acetylneuraminosyl-II^3-di-N-acetylneuraminosylgangliotetraosylceramide; C–M, chloroform–methanol; GLC, gas–liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

consisted of heparan sulfate (92.5%) and chondroitin sulfate (7.5%). Thus, the patient was diagnosed as mucopolysaccharidosis type III B clinically and enzymatically.

Isolation of glycosphingolipids and glycosaminoglycan

Brain, liver (104 g), and spleen (101 g) were obtained at autopsy from the patient and kept frozen until use. The brain was separated into grey matter (115 g) and white matter (87 g). The procedure for isolation of glycosphingolipids is described in detail elsewhere (3). The tissue was homogenized with chloroform–methanol (C–M) according to Suzuki (4). The homogenate was filtered and separated into total lipid extract and a protein residue from which glycosaminoglycan was prepared as described below. The total lipid was treated with mild alkali to remove ester-lipids, then the alkali-stable lipids were separated into neutral and acidic sphingolipids (3). The neutral glycosphingolipid fraction was acetylated with pyridine-acetic anhydride 1:1 at 80°C for 2 hr. After evaporation of the solvent, the entire acetylated neutral glycosphingolipid fraction was dissolved in hexane–toluene 1:1 and applied to the column of silica gel 60 (60 g, Merck, West Germany). The column was washed with 1500 ml of hexane–toluene 1:1 to remove fatty acid methyl esters, cholesterol acetate, and a part of the cholesteryl esters. Acetylated glycosphingolipids were eluted with 400 ml of C–M 8:2 and acetylated sphingomyelin was eluted with 2,000 ml of C–M 4:6. The acetylated glycosphingolipids were evaporated to dryness and deacetylated with 12% ammonia in methanol at room temperature overnight. Particularly, for the isolation of brain lipids, the deacetylated glycosphingolipids were applied to a silica gel column and a large amount of cerebroside was separated from other glycosphingolipids. The former were eluted with C–M 9:1, while the latter were eluted with C–M 4:6. Acidic glycolipids were separated into galactosylceramide I^3-sulfate and ganglioside fractions according to Ledeen, Yu, and Eng (5). Each sphingolipid except brain cerebroside and sulfatide was separated by preparative thin-layer chromatography (TLC) on plates precoated with silica gel 60 (Merck, West Germany) and developed in C–M–water 65:25:4 (by volume) for neutral glycosphingolipids or with C–M–0.25% KCl 60:35:8 for acidic glycosphingolipids. The scraped lipids from TLC plates were further purified by silica gel column chromatography to remove nonlipid contaminants as follows. The silica gel powder was suspended in chloroform and applied to a column of silica gel 60 (10 g, 10 × 300 mm). The column was eluted with 100 ml each of chloroform, C–M 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and methanol. The 10-ml fractions were separated and checked by TLC. Purified lipids were subjected to chemical analyses.

Glycosaminoglycan was prepared as follows. To the protein C–M residue was added hexane–isopropanol 3:2 to obtain a fluffy powder (6). Hexane–isopropanol treatment should be done while the residue is wet with C–M. The mixture was filtered and the protein residue was dried in vacuo. Glycosaminoglycan was isolated from the residue according to Constantopoulos, McComb, and Dekaban (7).

Analytical procedures

The amount of glycosaminoglycan was determined by the carbazole borate method (8). Electrophoresis of glycosaminoglycan was performed on cellulose acetate membranes (Sartorius GmbH, West Germany) using 0.1 M barium acetate at a constant current of 1 mA/cm for 3 hr or 1 M acetate-pyridine buffer, pH 3.5, at a constant current of 0.5 mA/cm for 20 min (9). The bands of glycosaminoglycan were detected by Alcian blue (Wako Pure Chemical Industries, Japan). The cellulose acetate membrane was dried and analyzed by densitometry at 570 nm.

Fifty mg of white matter or grey matter was homogenized with hexane–isopropanol 3:2 containing 3% water to delipidate the tissue (6). The homogenate was centrifuged briefly and the supernatant was discarded. The pellet was dried under a stream of nitrogen and then stored in a vacuum desiccator. The dried residue was solubilized with 1% SDS and analyzed according to Laemmli (10). Electrophoresis was performed on 13% polyacrylamide gel containing 0.1% SDS (0.8-mm thickness) at 25 mA/14 cm for 2.5 hr using 0.02 M Tris-glycine buffer, pH 8.3. Bands were detected by Coomassie Brilliant Blue-R250. Molecular weight markers (BDH Co., England) were used as authentic samples.

Glycosphingolipid was methanolized with 3% HCl in dry methanol at 80° for 3 hr with or without mannitol as an internal standard. To the methanolyzate was added 3 × 3 ml of hexane and the two solvent layers (hexane and methanol) were analyzed by gas–liquid chromatography (GLC). The hexane layer which contained fatty acid methyl esters was analyzed by GLC using a silicone capillary column OV-101 (0.2 mm × 25 m) at 250°C. Methyl glycosides in the methanol layer were re-N-acetylated (11) and analyzed as trimethylsilyl derivatives by GLC (3). Sphingosine was analyzed as trimethylsilyl derivative by GLC as reported elsewhere (12). For the sphingosine composition of brain ganglioside, the ratio of 4-eicosasphingenine to 4-sphingenine was determined by ozonolysis (13). The amount of ganglioside was determined with resorcinol reagent (14) or estimated on TLC by densitometry at 570 nm. Cholesterol glucuronide was determined as described elsewhere (3). Sugar sequence of neolactotetraosylceramide was determined according to Svennerholm, Mansson, and Li (15) using jack bean β-galactosidase and jack bean β-N-acetylhexosamin-

idase (Seikagaku Kogyo Co., Japan). To ascertain the activity and specificity of each enzyme, gangliotetraosylceramide (GA1) prepared from bovine brain ganglioside by formic acid hydrolysis, globotriaosylceramide and globotetraosylceramide obtained from human erythrocytes, and Forssman globoside isolated from canine stomach were used as enzyme substrates.

RESULTS

Accumulation of glycosaminoglycan in brain and visceral organs

Electrophoresis of glycosaminoglycan was performed on cellulose acetate membranes. The electrophoresis patterns were analyzed by densitometry and the accumulation of heparan sulfate was confirmed as described below. The samples from liver and spleen contained heparan sulfate exclusively, while the brain showed multiple bands which were comprised of heparan sulfate (53% in grey matter, 49% in white matter), a mixture of dermatan sulfate and hyaluronic acid (26% in grey matter, 31% in white matter), and unidentified materials (22% in grey matter, 21% in white matter). As the separation of dermatan sulfate and hyaluronic acid was poor in this buffer system, another buffer system, 1 M acetate-pyridine buffer, pH 3.5, was used to separate and identify dermatan sulfate and hyaluronic acid. The contents of uronic acid in various organs were determined by the carbazole method (7, 8). The following values were obtained: liver, 2.12%; spleen, 1.29%; grey matter, 0.40%; white matter, 0.48% (values are expressed as percent of C–M residue). Accumulation of glycosaminoglycan (mostly heparan sulfate) was observed in all the organs tested. Those values well agree with the data of Constantopoulos, Eiben, and Schafer (2).

Abnormal protein in brain

The cerebral protein fraction of the patient was analyzed by slab-SDS-PAGE. The strange PAGE pattern was obtained in the white matter. One major band which is not originally present in normal brain appeared as shown in **Fig. 1,** while the protein which has the same mobility as a high molecular weight protein, the so-called Wolfgram protein, was almost missing. The molecular mass of the abnormal protein was calculated to be approximately 54,000 based on molecular weight marker proteins. In addition, the relative content of myelin basic protein in the white matter was somewhat lower than normal. The grey matter showed a pattern similar to that of the white matter, but contained less abnormal protein. The abnormal SDS-PAGE pattern of brain protein may be related to the defect of α-N-acetylglucosaminidase,

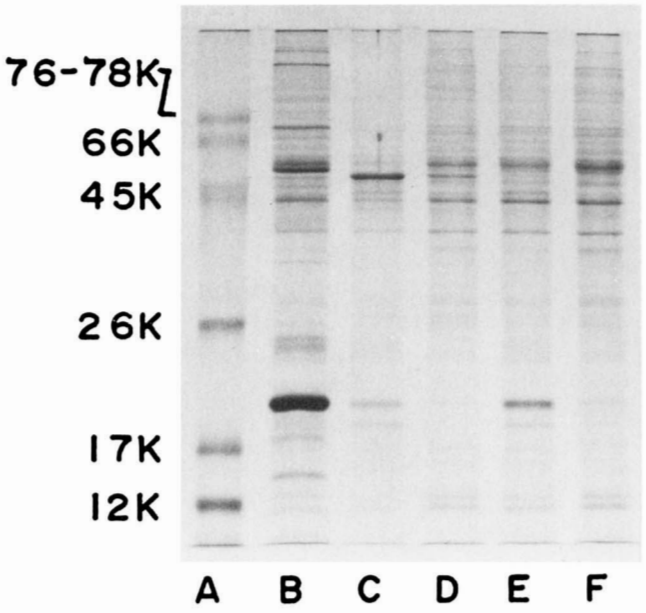

Fig. 1. Slab SDS-polyacrylamide gel electrophoresis of the brain protein. Electrophoresis was performed on 13% polyacrylamide gel containing 0.1% SDS (0.8-mm thickness) at 25 mA/14 cm for 2.5 hr using 0.02 M Tris-glycine buffer, pH 8.3. Bands were detected by Coomassie Brilliant Blue-R250. A, Molecular weight markers (from top to bottom: ovotransferrin, bovine serum albumin, ovalbumin, bovine chymotrypsinogen A, equine myoglobin, and equine cytochrome C); B, porcine spinal cord (control); C, white matter (patient); D, grey matter (patient); E, white matter (normal control); and F, grey matter (normal control).

but the abnormality in brain protein is now under investigation.

Abnormal glycosphingolipids in brain

Neutral glycosphingolipids, galactosylceramide I³-sulfate, and sphingomyelin. **Fig. 2** shows the TLC of neutral glycosphingolipids from which most of the galactosylceramide was first removed by silica gel column chromatography. The grey matter contained many bands, indicated by A, B, C, D, E and F in Fig. 2. Some of these bands were composed of double bands due to differences in the ceramide portions. Analytical results by GLC of trimethylsilyl (TMS) derivatives of methyl glycosides together with the R_f value of each glycosphingolipid band on TLC revealed that band B was lactosylceramide(glucose–galactose 1:1.1); band C was globotriaosylceramide(glucose–galactose 1:1.9); band D was a mixture of gangliotriaosylceramide(74%) and globotriaosylceramide(26%) which contained hydroxy fatty acid, as will be discussed later; and band E was globotetraosyl-ceramide(glucose – galactose – N - acetylgalactosamine 1:1.8:0.9). Band F was separated into two bands when the TLC plate was developed in C–M–water 60:35:8. Band F could not be determined due to its low sample concentration, although fucose, galactose, N-acetylgalactosamine, and glucose

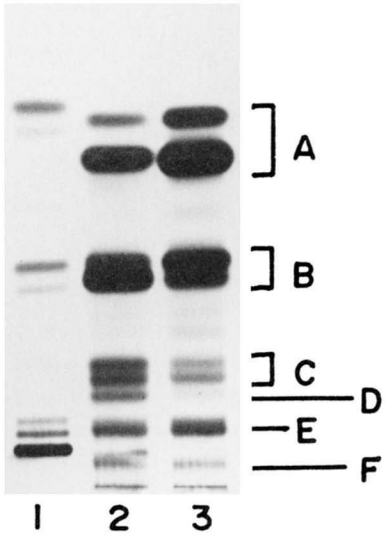

Fig. 2. Thin-layer chromatogram of neutral glycolipid fraction in brain. The plate was developed with chloroform–methanol–water (65:25:4). Bands were detected by cupric-phosphoric acid charring spray (28). 1, Glycosphingolipids from goat erythrocytes; 2, grey matter (patient); 3, white matter (patient).

were found as sugar constituents by GLC analysis when the two bands were analyzed as a mixture. The cerebroside fraction separated by silica gel column chromatography contained glucose and galactose at the ratio of 9.2% and 90.8%, respectively. The structures of glycosphingolipids in the grey matter were also confirmed by GLC of the partially methylated alditol acetates of sugars (16, 17). It should be noted that the brain of the patient contained not only galactosylceramide and galactosylceramide I^3-sulfate, but also glucosylceramide, lactosylceramide, gangliotriaosylceramide, globotriaosylceramide, and globotetraosylceramide. All of these glycolipids except galactosylceramide and galactosylceramide I^3-sulfate are usually undetectable in normal brain. The white matter showed a similar TLC pattern of glycosphingolipids. However, the cerebroside fraction contained only galactose, and gangliotriaosylceramide was present as a trace amount. Although lactosylceramide in extra-neural organ usually shows two bands on TLC due to the difference of fatty acid chain length, the lactosylceramide in the brain of the patient showed a high content of upper band in the white matter and, on the other hand, a high content of lower band in the grey matter. This difference of fatty acid chain length was confirmed by the analysis of fatty acid composition of lactosylceramide in both grey and white matter. This result suggested that the synthesis of lactosylceramide in the grey matter was somehow different from that in the white matter. The content of each glycosphingolipid in both the grey and white matter is shown in **Table 1.** The content of galactosylceramide in the grey matter (0.15% of fresh tissue) and the white matter (0.95% of fresh tissue) and the content of galactosylceramide I^3-sulfate in the grey matter (0.07% of fresh tissue) and the white matter (0.42% of fresh tissue) were markedly lower than the established values (18) in normal brain (0.3%, 3.1%, 0.1%, and 0.9%, respectively). The sphingosine compositions of neutral glycosphingolipids, galactosylceramide I^3-sulfate, and sphingomyelin were determined by GLC of the trimethylsilyl (TMS) derivatives of sphingosine (**Table 2**). Lactosylceramide contained not only 4-sphingenine and sphinganine but also 4-eicosasphingenine and eicosasphinganine. The 4-eicosasphingenine and eicosasphinganine are usually found only in gangliosides in normal brain. These eicosasphingosines were demonstrated in gangliosides of the patient's brain as described below in the section about the gangliosides. Thus, it was concluded that the lactosylceramide, which has 4-eicosasphingenine or eicosasphinganine, was at least an intermediate in the metabolic pathway of gangliosides, and, therefore, the metabolism of ganglioside might have been disturbed in the brain of the patient. Fatty acid compositions of the neutral glycosphingolipids, galactosylceramide I^3-sulfate, and sphingomyelin are shown in **Table 3.** The neutral glycosphingolipids in the globo-series contained a relatively high proportion of stearic acid. Another characteristic is the presence of hydroxy fatty acids in the neutral glycosphingolipids, although the amount is not large. Band D in Fig. 2 also contained hydroxy fatty acids, which indicated that band D was a mixture of nonhydroxy fatty acid-containing gangliotriaosylceramide and hydroxy fatty acid-containing globotriaosylceramide. No abnormalities were observed in the fatty acid compositions of cerebroside, galactosylceramide I^3-sulfate, and sphingomyelin.

Ganglioside. Gangliosides in the grey and white matter showed abnormal patterns of TLC (**Fig. 3**). The relative amounts of II^3-N-acetylneuraminosylgangliotriaosylcer-

TABLE 1. Content of neutral glycosphingolipids and galactosylceramide I^3-sulfate in grey and white matter

	GalCer	GlcCer	LacCer	$GbOse_3Cer$	$GgOse_3Cer$	$GbOse_4Cer$	GalCer I^3-sulfate
				μmol/g fresh tissue			
Grey matter	1.884	0.192	0.142	0.048	0.010	0.020	0.753
White matter	11.656		0.171	0.025	±	0.051	4.692

TABLE 2. Sphingosine composition of neutral glycosphingolipids, galactosylceramide I^3-sulfate, and sphingomyelin

	Grey Matter				White Matter			
	d18:1[a]	d18:0[b]	d20:1[c]	d20:0[d]	d18:1	d18:0	d20:1	d20:0
	% of total sphingosine							
GalCer	99	1	—	—	89	11	—	—
LacCer	67	9	22	2	84	10	5	1
$GbOse_3Cer$	75	25	—	—	78	22	—	—
$GgOse_3Cer$	64	36	—	—				
$GbOse_4Cer$	79	21	—	—	85	15	—	—
GalCer I^3-sulfate	89	11	—	—	98	2	—	—
Sphingomyelin	90	10	—	—	98	2	—	—

[a] 4-Sphingenine.
[b] Sphinganine.
[c] 4-Eicosasphingenine.
[d] Eicosasphinganine.

amide (GM2-ganglioside), II^3-N-acetylneuraminosyllactosylceramide (GM3-ganglioside), and II^3-(N-acetylneuraminosyl)$_2$lactosylceramide (GD3-ganglioside) were increased (Fig. 3 and **Table 4**). Total gangliosides in the grey and white matter were determined with resorcinol reagent. The white matter contained 341.7 μg of lipid-bound N-acetylneuraminic acid per gram wet tissue [cf. normal value: 80–180 μg NANA/g wet tissue (18)], while the grey matter contained 522.3 μg of lipid-bound N-acetylneuraminic acid per gram of wet tissue [cf. normal value: 744–918 μg NANA/g wet tissue (18)]. The increased amount of ganglioside in the white matter may partially explain the contamination of the grey matter in the white matter fraction, because complete separation of white matter and grey matter is almost impossible. However, such contamination would be too small to explain the high value obtained in the white matter. This increase may be explained by the histological observation that revealed the presence of the demyelination and marked gliosis in the white matter, if the proliferated glia cell contained much more ganglioside than the myelin. On the other hand, the ganglioside content was low in the grey matter. This result was consistent with the histological data where a pseudolaminar spongy state was observed. The fatty acids of each ganglioside in the grey and white matter were analyzed. A small but significant increase in longer chain fatty acids (longer than C20) was observed in comparison with the established values obtained from normal brain, although the major fatty acid was stearic acid as usual.

The sphingosine composition was determined by ozonolysis. The ratio of 4-eicosasphingenine to 4-sphingenine in the total ganglioside was determined as 0.51 and 0.33 in grey and white matter, respectively. It is well known that the ratio of 4-eicosasphingenine to 4-sphingenine rapidly increases just after birth and approaches 1.0 around age 10 (19, 20). The brain of this patient showed low values which corresponded to that observed at about 3 years of age. This result strongly suggested that ganglioside metabolism in the brain of this patient was severely disturbed at an early age.

Glycosphingolipids in liver and spleen

Fig. 4 shows the TLC pattern of neutral glycosphingolipids in the liver and spleen of the patient. In both organs, bands corresponding to monohexosylceramide, dihexosylceramide, trihexosylceramide, and tetrahexosylceramide were detected. For the acidic lipids (**Fig. 5**), TLC showed one major band which corresponded to II^3-N-acetylneuraminosyllactosylceramide (GM3), and several minor bands. One of the minor bands had the same R_f value as that of cholesterol glucuronide. The TMS derivatives of methyl glycosides of each glycolipid were analyzed by GLC. The sugar compositions and the concentrations of glycosphingolipids in the liver and spleen are shown in **Table 5.** The tetrahexosylceramide in the spleen contained glucose, galactose, N-acetylgalactosamine, and N-acetylglucosamine. This indicated the presence of a glucosamine-containing glycolipid in addition to globotetraosylceramide in the tetrahexosylceramide fraction. It was difficult to separate these two glycolipids by TLC using the solvent system of C–M–water 65:25:4 or C–M–water 60:35:8. Thus, this fraction was acetylated by the method described in Materials and Methods. Preparative TLC was performed with the solvent system of C–M–water 95:5:0.3 and complete separation of two bands was obtained. Each band was scraped from the TLC plate, eluted from the silica powder, and deacetylated. The upper band was identified as globotetraosylceramide by GLC analysis of TMS derivatives, while the lower band was identified as lactoneotetraosylceramide by GLC analysis and enzymatic sequential degradation according to Svennerholm et al. (15).

Concentrations of glycolipids in the liver are shown in

TABLE 3. Fatty acid composition of neutral glycosphingolipids, galactosylceramide I^3-sulfate, and sphingomyelin

	Grey Matter							White Matter						
	GalCer	LacCer	$GbOse_3Cer$	$GgOse_3Cer$	$GbOse_4Cer$	$GalCerI^3$-sulfate	Sphingomyelin	GalCer	LacCer	$GbOse_3Cer$	$GgOse_3Cer$	$GbOse_4Cer$	$GalCerI^3$-sulfate	Sphingomyelin
	% of total fatty acid													
16	t[a]	2	4	5	7	1	8	t	2	8	9	8	t	6
18	5	70	20	67	42	4	61	3	23	37	45	48	2	34
19	t	t	t				t	t	t			1		t
20	1	7	4	5	4	1	3	t	2	4	4	3	t	1
21	t	t		t			t		t	t		t	t	t
22:1				1			t			2				1
22	1	2	21	3	13	1	2	1	3	15	3	8	1	2
23:1	t	t					t	t	1	1		1		1
23	1	1	4	t	2	2	1	1	4	4		5	2	2
24:1	11	8	17	1	17	16	14	13	29	11	5	18	22	30
24	3	4	24	1	10	6	3	4	10	12	5	6	8	6
25:1	4	2	t		t	7	3	4	10			1	8	7
25	1	1	1	1	1	3	1	2	4	t	2	1	3	2
26:1	3	2	t			7	2	4	10				4	6
26	t	t	t			1	t	t	1				1	1
27							t						t	t
18h[b]	t		t	1	1			t		t			t	
20h	t		t	1	1	t		t		t	4		t	
22h	4		t	3	t	2		4		t	8		2	
23h	10		t	2	t	7		10		1	4		6	
24h:1	14		1	2	t	9		13		1	t		9	
24h	20		1	7	1	17		22		2	11		16	
25h:1	5					4		4					4	
25h	5					5		5					5	
26h:1	7					5		6					6	
26h	1					1		1					1	
27h:1													t	
27h	t												t	
% of HFA	66	0	2	16	3	50	0	65	0	4	27	0	49	0

[a] Less than 1%.

[b] Hydroxy fatty acid.

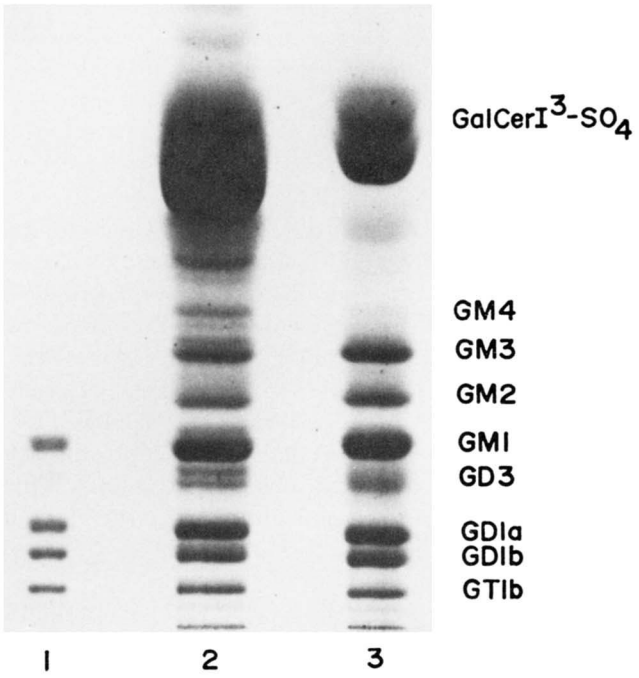

Fig. 3. Thin-layer chromatogram of gangliosides in brain. Developed with chloroform–methanol–0.25% KCl (60:35:8). Detected by cupric-phosphoric acid charring spray. 1, Control (from top to bottom, GM1, GD1a, GD1b, and GT1b); 2, white matter; 3, grey matter. Abbreviations for gangliosides are as follows: GM4, I^3NeuAc-GalCer; GM3, II^3NeuAc-LacCer; GM2, II^3NeuAc-$GgOse_3$Cer; GM1, II^3NeuAc-$GgOse_4$Cer; GD3, $II^3(NeuAc)_2$-LacCer; GD1a, IV^3NeuAc,II^3NeuAc-$GgOse_4$Cer; GD1b, $II^3(NeuAc)_2$-$GgOse_4$Cer; GT1b, IV^3NeuAc, $II^3(NeuAc)_2$-$GgOse_4$Cer.

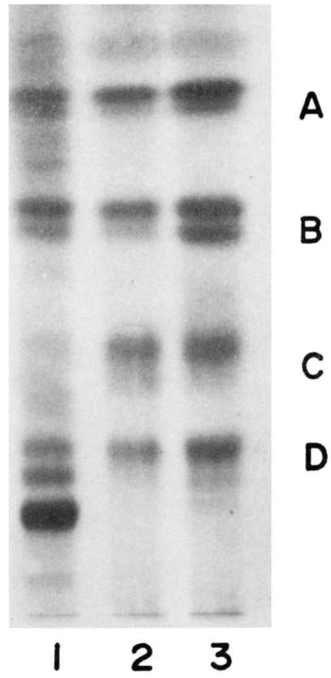

Fig. 4. Thin-layer chromatogram of neutral glycolipids in liver and spleen. Developed with chloroform–methanol–water (65:25:4). Detected by cupric-phosphoric acid charring spray. 1, Goat erythrocyte membrane (control); 2, liver; 3, spleen. A, B, C, and D represent mono-, di-, tri-, and tetra-hexosylceramides, respectively.

Table 5. The levels of monohexosylceramide and lactosylceramide were in the normal range, while those of globotriaosylceramide and globotetraosylceramide were three times higher than normal values established by others (21, 22). The spleen also showed a high level of globotetraosylceramide when compared with the normal level (23). Acidic lipids were composed 90.8% of II^3-N-acetylneuraminosyllactosylceramide, 3.1% of II^3-(N-acetylneuraminosyl)$_2$lactosylceramide, and 6% of four other minor bands in the liver. In the spleen, the major ganglioside was II^3-N-acetylneuraminosyllactosylceramide (76.1%). II^3-(N-acetylneuraminosyl)$_2$lactosylceramide was 9.9% of the total gangliosides and the six minor bands together were 14.0%. The minor gangliosides could not be determined because of low sample concentration. Levels of II^3-N-acetylneuraminosyllactosylceramide were in the normal range in both the liver and spleen.

TABLE 4. Ganglioside composition in brain

	Grey Matter		White Matter	
	NANA (%)	nmol Ganglioside/g Fresh	NANA (%)	nmol Ganglioside/g Fresh
GM4			4.3[b]	47.6
GM3	15.0 (<1)[a]	253.3	9.6 (<1)	106.1
GM2	12.2 (1.5–2)	206.1	8.9 (0.6–2.0)	98.4
GM1	17.7 (13.0–15.6)	299.0	18.8 (14.6–21.2)	207.8
GD3	10.1 (1.0–2.8)	85.5	13.0 (1.2–5.0)	71.9
GD1a	19.4 (29.1–43.7)	163.9	18.9 (30.0–38.2)	104.6
GD2	0.7 (1.2–4.2)	6.0	2.9 (1.2–3.1)	16.0
GD1b	12.6 (14.3–19.9)	106.5	10.8 (12.2–18.1)	59.7
GT1b	10.6 (15.8–25.7)	59.8	11.0 (14.1–21.2)	40.6
GQ	1.7 (3.2–4.8)	7.2	1.9 (2.8–6.1)	5.3

Abbreviations for gangliosides are shown in Fig. 3.
[a] Values in parentheses express normal levels of ganglioside in brain (18).
[b] Nine to eleven percent of total sialic acid (5).

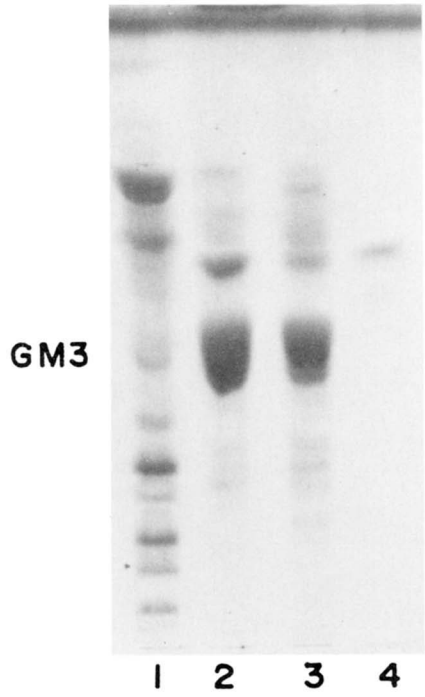

Fig. 5. Thin-layer chromatogram of acidic lipids in liver and spleen. Developed with chloroform–methanol–0.25% KCl (60:35:8). Detected by cupric-phosphoric acid charring spray. 1, Brain ganglioside (control); 2, liver; 3, spleen; 4, cholesterol glucuronide.

Cholesterol glucuronide contents were 15.9 nmol/g in the liver and 6.0 nmol/g in the spleen. No increase of cholesterol glucuronide was observed in the liver. On the other hand, a small amount of cholesterol glucuronide was detected in the spleen of this patient, although we could not detect any in the spleen of the patient with GM1-gangliosidosis type II (3). The fatty acid composition of each glycolipid in the liver and spleen was analyzed. All the glycolipids were composed of nonhydroxy fatty acids and their major chain lengths were C16, C22, and C24. The fatty acid composition of lactoneotetraosylceramide was C16(47%), C18(6%), C22:1(1%), C22(7%), C23(1%), C24:1(30%), and C24(9%). The composition was different from that of other glycosphingolipids and was characterized by a large proportion of C16. The fatty acid composition of IV^3-N-acetylneuraminosyllactoneotetraosylceramide from normal human erythrocytes was also determined for comparison. The values obtained were C16(1%), C22(11%), C22:1(1%), C23(2%), C24(41%), C24:1(37%), C25(1%), C26(2%), and C26:1(3%). The lactoneotetraosylceramide in the spleen is thought to originate probably from erythrocytes which contain a large amount of IV^3-N-acetylneuraminosyllactoneotetraosylceramide. However, lactoneotetraosylceramide in the spleen of this patient contained 47% of C16, while IV^3-N-acetylneuraminosyllactoneotetraosylceramide had only 1% of C16. When glucosylceramide obtained by enzymatic hydrolysis of the lactoneotetraosylceramide from the patient was analyzed by TLC, it corresponded to the lower half of the control glucosylceramide which was obtained from the spleen of the patient with Gaucher's disease and had a large amount of C24 fatty acid. This supports the analytical result of the fatty acid composition.

Sphingosine compositions of each glycolipid are shown in **Table 6.** Relatively high contents of sphinganine were observed in all the glycosphingolipids except for II^3-N-acetylneuraminosyllactosylceramide in the spleen.

DISCUSSION

Glycosphingolipids in the brain, liver, and spleen of a patient with mucopolysaccharidosis type III B were analyzed. In the neutral glycolipid fraction, the presence of glycosphingolipids in the globo-series (globotriaosylceramide and globotetraosylceramide) and ganglio-series (gangliotriaosylceramide) in addition to glucosylceramide and lactosylceramide were identified. Constantopoulos et al. (2) reported the presence of bands which had the same mobilities with ceramidedihexoside, gal-gal-glc-cer, and galNAc-gal-glc-cer on the TLC plate. In the acidic glycolipid fraction, the proportion of II^3-N-acetylneuraminosylgangliotriaosylceramide, II^3-N-acetylneuraminosyllactosylceramide and II^3-(N-acetylneuraminosyl)$_2$lactosylceramide were also increased in the brain as observed by Constantopoulos et al. (2). They concluded that the increase of glycolipids which were the minor components

TABLE 5. Sugar composition and content of glycolipids in liver and spleen

	Liver	Spleen
	nmol/g fresh tissue	
GlcCer	50.7 (glc only)	102.6 (glc only)
LacCer	78.6 (glc:gal = 1:0.98)	175.0 (glc:gal = 1:1.0)
$GbOse_3Cer$	78.0 (glc:gal = 1:2.1)	50.7 (glc:gal = 1:1.9)
$GbOse_4Cer$	44.8 (glc:gal:galNAc = 1:1.7:0.9)	96.1 (glc:gal:galNAc = 1:1.88:0.99)
$nLcOse_4Cer$		30.3 (glc:gal:glcNAc = 1:1.80:1.00)
GM3	284.0 (glc:gal = 1:0.8)	235.2 (glc:gal = 1:0.9)

TABLE 6. Sphingosine composition of glycosphingolipids in liver and spleen

	Liver		Spleen	
	d18:1[a]	d18:0[b]	d18:1	d18:0
	% of total sphingosine base			
GlcCer	86.4	13.6	85.1	14.9
LacCer	77.8	22.2	89.9	10.1
$GbOse_3Cer$	84.3	15.7	85.4	14.6
$GbOse_4Cer$	73.8	26.2	84.3	15.7
$nLcOse_4Cer$			73.0	27.0
GM3	80.9	19.1	98.2	1.8

[a] 4-Sphingenine.
[b] Sphinganine.

in the brain was due to the low glycosidase activities as a result of the accumulation of heparan sulfate. In our report, some of the neutral glycosphingolipids in the brain of the patient were identified as those in globo-series. Apart from the pathogenesis of the disease, these glycosphingolipids may be present in normal brain, although the contents of those glycosphingolipids are too low to detect.

Ganglioside compositions in fetal brain are absolutely different from those of adult brain as reported by Irwin, Michael, and Irwin (24). They observed that II^3-(N-acetylneuraminosyl)$_2$lactosylceramide and II^3-N-acetylneuraminosyllactosylceramide comprised 77% of resorcinol-positive bands in the brain at 15 days of gestation, although their amounts decrease with age. Thus, the high proportion of II^3-(N-acetylneuraminosyl)$_2$lactosylceramide and II^3-N-acetylneuraminosyllactosylceramide may in part account for the immaturity of the brain of the patient with mucopolysaccharidosis III B. It is possible to conclude that ganglioside metabolism, particularly the biosynthetic pathway, in the brain of the patient was already disturbed at an early stage of life. This conclusion can be supported by the abnormal sphingosine composition of gangliosides. As a matter of fact, the ratio of 4-eicosasphingenine to 4-sphingenine of gangliosides in the brain (0.51 in grey matter and 0.33 in white matter) corresponded to that at age 3 or less. The lower level of the ratio indicated that the ganglioside synthesis in the brain of the patient was already disturbed at age 3 at the latest. The immaturity of the brain may account for the severe progressive mental retardation in mucopolysaccharidosis type III B.

The contents of galactosylceramide and galactosylceramide I^3-sulfate were significantly decreased. This result was consistent with histological observations which showed demyelination and marked gliosis in the white matter and pseudolaminar spongy state in the grey matter. The abnormal pattern of brain protein on slab SDS-PAGE may be related to the defect of α-N-acetylglucosaminidase in the brain.

The spleen of the patient contained a relatively large amount of lactoneotetraosylceramide which comprised one-fourth of the tetrahexosylceramide fraction. Lactoneotetraosylceramide in the spleen may be derived from erythrocytes which are trapped by the spleen, since human erythrocytes contain IV^3-N-acetylneuraminosyllactoneotetraosylceramide as a major ganglioside (25).

The analysis of monohexosylceramide in the liver showed that the sugar component is only glucose and that only nonhydroxy fatty acids are present. Recently Nilsson and Svennerholm (21) reported that the human liver contained a considerable amount of galactosylceramide. In our analyses of human liver from Japanese (26, 27), only glucosylceramide as monohexosylceramide was found. Recently, we found only glucosylceramide in the liver of a Japanese patient with GM1-gangliosidosis type II as monohexosylceramide (unpublished data). The composition of monohexosylceramide may vary with race.

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