J. Biochem. 123, 487-491 (1998)

Further Characterization of Equine Brain Gangliosides: The Presence of GM3 Having *N*-Glycolyl Neuraminic Acid in the Central Nervous System[1]

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Received for publication, October 17, 1997

Equine brain gangliosides were isolated and their structures were characterized, to examine whether equine brain has *N*-glycolyl neuraminic acid in gangliosides, since other mammals predominantly possess *N*-acetyl neuraminic acid in brain gangliosides, and equine erythrocytes and organs except the brain have gangliosides exclusively containing *N*-glycolyl neuraminic acid. The gangliosides purified from the brain were identified by proton NMR spectroscopy and mass spectrometry, as well as GLC, resulting in their identification as GM4, GM3, GM2, GM1, GD1a, GD1b, and GT1b. Of these gangliosides, GM3 possessed *N*-glycolyl neuraminic acid as a minor component (18% of the total GM3), whereas other gangliosides exclusively contained *N*-acetyl neuraminic acid. The *N*-glycolyl neuraminic acid residue of the GM3 was confirmed by TLC immunostaining. The possibility of contamination of the GM3 by erythrocytes was eliminated based on the finding that the lipid compositions were characteristic of brain gangliosides. The presence, even as a minor component, of the *N*-glycolyl neuraminic acid in equine brain gangliosides is exceptional among the sialic acid species in mammalian central nervous system.

Key words: equine brain, ganglioside, horse brain, NMR, sialic acid.

Glycosphingolipids (GSLs) play a significant role in cells and tissues as mediators of cell-cell interaction, growth factors and substances inducing differentiation (*1-3*). They are widely distributed in mammalian tissues, cells, and body fluids, and gangliosides are particularly rich in brain (*4, 5*). Many studies for structural analysis of the mammalian brain gangliosides have been carried out and indicate the characteristic predominance of *N*-acetyl neuraminic acid (NeuAc) as a sialic acid species of the gangliosides. In contrast, equine gangliosides characterized from erythrocytes are GM3 containing *N*-glycolyl neuraminic acid (NeuGc), and its *O*-acetylated form at NeuGc (*6-8*). GM3, GM2, GM1, GD1a, and GalNAc-GM1 exclusively contain NeuGc in kidney and spleen (*9*). The equine brain gangliosides and their sialic acid species have been characterized by thin-layer chromatography (TLC) (*10*), revealing that GM1, GD1a, GD1b, and GT1 are the major components, GM3, GM2, GD3, and GQ1 are present in lesser amounts, and more than 97% of gangliosides have NeuAc. Here, we have examined the ganglioside structures by proton NMR, fast atom bombardment-mass spectrometry (FAB-MS), and GLC to determine whether NeuGc-linked gangliosides were present in the equine central nervous system.

MATERIALS AND METHODS

Materials—DEAE-Sephadex, A-25 was purchased from Pharmacia-LKB (Uppsala, Sweden), Iatrobeads from Iatron Laboratories (Tokyo), and precoated TLC-plates (Silica gel 60) and dimethylsulfoxide-D_6 from Merck (Germany). Avian antiserum against Hanganutziu and Deicher antigen (*11*) was kindly donated by Dr. M. Naiki (Department of Veterinary Science, National Institute of Health, Tokyo). Standard gangliosides were prepared in this laboratory. All other reagents were of analytical grade.

Extraction and Separation of Gangliosides—The ratio of solvent mixtures is expressed by volume. The whole equine brain (457 g wet weight) was homogenized with acetone (1 g/9 ml) to yield an acetone powder (104 g dry weight). The glycolipids were extracted three times from the powder with chloroform-methanol-water (CMW, 4:8:3) at room temperature. The acidic glycolipid fraction was isolated

[1] This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas No. 10142712 from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CMW, chloroform-methanol-water; FAB-MS, fast atom bombardment-mass spectrometry; NeuAc, *N*-acetyl neuraminic acid; NeuGc, *N*-glycolyl neuraminic acid; GM3 (NeuAc), GM3 containing NeuAc; GM3 (NeuGc), GM3 containing NeuGc; SGC, 3′-sulfogalactosyl ceramide; TLC, thin-layer chromatography. Nomenclature and abbreviations for gangliosides are used as recommended by Svennerholm [*Methods Carbohydr. Chem.* 4, 464-474 (1972)].

from the combined and concentrated extracts with a DEAE-Sephadex, A-25 (acetate form) column (2.5×30 cm) by elution with CMW (40:60:10) containing 1 M ammonium acetate after removal of the unbound fraction from the column with CMW (40:60:10). Total acidic glycolipids were chromatographed on an Iatrobeads column (2.5×50 cm) by stepwise elution with 1,000-ml portions of CMW in ratios of 90:10:0.5, 80:20:2, 70:30:3, 60:40:4, 50:50: 5, and 40:60:6. The glycolipid fraction, which consisted of several glycolipid components, was further chromatographed on an Iatrobeads column of smaller size by stepwise elution with the above CMW, and the chromatography was repeated to obtain a homogenous glycolipid preparation. The purified glycolipids were chromatographed on a TLC-plate, developed with CM-2.5 M aqueous ammonia (60:40:9) and CM-2.5% $CaCl_2$ (60:40:9), and visualized with orcinol-sulfuric acid reagent or resorcinol-hydrochloric acid reagent under heating. The ganglioside due to GM3 was split into two bands on TLC developed with CM-aqueous ammonia, which were further separated into GL-2 and -2′ by preparative TLC.

Analyses of Lipid Moiety, Sialic Acid Species, and Sugar Linkage of Gangliosides—Fatty acids and long chain bases were separately analyzed from the methanolyzates of the purified glycolipid as a methyl ester and an *O,N*-trimethylsilyl ether, respectively, using a GLC apparatus (GC-14A, Shimadzu) equipped with a capillary column (0.25 mm×50 m) coated with 0.1% of DB-5 as reported previously (*8*). The sialic acid species of the purified gangliosides or of the whole ganglioside fraction were analyzed with the methanolyzates by the above GLC as per *O*-trimethylsilyl methyl ester methyl ketoside, according to the method of Yu and Ledeen (*12*). The methylation analysis of the glycolipids was performed by GC-MS on a mass spectrometer (see below) equipped with a capillary column (0.25 mm×50 m) coated with 0.1% of OV-1 as a partially methylated alditol acetate through permethylation, acetolysis/hydrolysis, reduction and acetylation, as described earlier (*8*).

NMR Spectroscopy—Proton NMR spectra of the glycolipids (approximately 1 mg) in 0.3 ml of dimethylsulfoxide-D_6 containing 2% D_2O were obtained at 90°C in the Fourier-transform mode on a Varian JNM-GX500 spectrometer equipped with a JEC-980B computer at the NMR-MS Laboratory of the Faculty of Agriculture, Hokkaido University, as described previously (*13*). Chemical shifts (d, ppm) were measured from tetramethylsilane as an internal standard.

FAB-MS—Negative ion FAB-MS was done on a JEOL JMS-HX100 mass spectrometer equipped with a JMA-DA500 Datalizer in the above laboratory, as described previously (*8, 13*). The sample was bombarded with Xe gas at 6 kV (20 mA) in a matrix of triethanolamine, and the fragments were accelerated at 5 kV.

TLC-Immunostaining—Immunostaining of purified GL-2′ and authentic gangliosides on a silica-gel thin-layer aluminum plate was performed as previously described (*14*) by a modification of the procedure of Magnani *et al.* (*15*). The purified GL-2′, 50 ng, was chromatographed on the TLC-plate developed with CM-$CaCl_2$ as described above, and the plate was treated with a diluted antiserum against Hanganutziu and Deicher antigen as a first antibody and with a peroxidase-conjugated rabbit antibody against avian immunoglobulin as a second antibody, followed by visualization of the band with a Konica-Immuno kit (Konica, Tokyo).

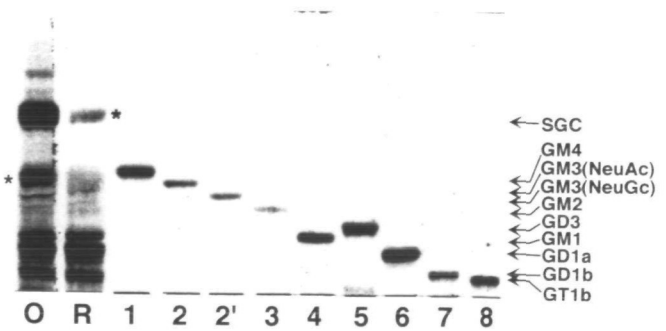

Fig. 1. **Thin-layer chromatography of equine brain gangliosides.** Purified gangliosides were chromatographed on a TLC-plate developed with CM-2.5 M aqueous ammonia=60:40:9 (by volume) and visualized with orcinol-sulfuric acid reagent for lane O and with resorcinol-hydrochloric acid reagent for the other lanes from R to 8. The arrows indicate the positions of respective authentic gangliosides. Asterisked bands are not specific for the staining reagents; SGC, sulfogalactosylceramide.

RESULTS

Extraction and Separation of Gangliosides—The gangliosides purified from equine brain are presented in Fig. 1. They were designated as GL-1, -2, -2′, -3, -4, -5, -6, -7, and -8 in order of increasing polarity, and they were confirmed to be gangliosides by their positive reaction with resorcinol reagent. The amounts of these gangliosides and 3′-sulfogalactosylceramide (SGC, II^3SO_3 GalCer) obtained from whole equine brain are summarized in Table I. On TLC with CM-ammonia or CM-$CaCl_2$ as a developing solvent, the mobilities of GL-2, -2′, -3, -4, -5, -6, -7, and -8 respectively were identical those of authentic GM3 containing NeuAc [GM3 (NeuAc), which was chemically synthesized from equine erythrocyte GM3 (NeuGc) (*16*)], GM3 (NeuGc), and GM2 (NeuAc) from Tay-Sachs brain (*17*), GM1 (NeuAc) from porcine brain, GD3 (NeuAc) from bovine milk, and GD1a (NeuAc), GD1b (NeuAc), and GT1b from bovine brain.

Analysis of Lipid Moiety—The fatty acid components of the purified gangliosides are summarized in Table II. Stearate (18:0) and arachidate (20:0) were the major acid

TABLE I. **Amounts of acidic glycolipids and sialic acid components of the gangliosides in equine brain.**[a]

GL-	Amount (mg)	Assignment	Sialic acid component (%)	
			NeuAc	NeuGc
	1,046	SGC[b]	—	—
1	13.2	GM4	99.9	trace
2	2.4	GM3	99.9	trace
2′	0.52	GM3	trace	99.9
3	2.0	GM2	99.9	trace
4	9.2	GM1	99.9	trace
5	2.5	GD3	99.9	trace
6	16.4	GD1a	99.9	trace
7	6.9	GD1b	99.9	trace
8	8.8	GT1	99.9	trace

[a]457 g wet weight; [b]sulfogalactosylceramide.

esters released from GL-2′, and the profiles of these acids in the other gangliosides except GL-1 were mostly similar. In GL-1, heterogeneous fatty acid esters with various chain lengths from palmitate (16:0) to cerotate (26:0) were detected. The long chain bases of the gangliosides were analyzed from the methanolyzates, revealing C_{18}-sphingenine (d18:1) and C_{20}-sphingenine (d20:1) in all purified gangliosides except GL-1, as shown in Table II. Other long chain bases, C_{18}- and C_{20}-sphinganine were detected in a lesser amount.

Analysis of Sugar Moiety—The methylation analysis of the respective gangliosides gave substitution sites of the

TABLE II. **Fatty acid components of equine brain gangliosides.**

GL	Fatty acid (%)																								UK[b]
	16[a]		17		18		19		20		22		23		24				25			26			
	:0[c]	:1[d]	:0	:1	:0	:1	:0	:1	:0	:1	:0	:1	:0	:0h[e]	:0	:0h	:1	:1h	:0	:0h	:1	:0	:1	:0h	
1	7	1			5	5					2		2	2	10	19	6	12		4	2	12	2	6	3
2	7		1		57		3		21		6				2										3
2′	6				61				17		8				5										3
3	10				57	8			12		3				6										4
4	3				66	3	1		17		3	1	1		1		1								4
5	8				61				11		3		4		4				3			3			2
6	1				64	1	1		27		1		1		1		2								3
7	10				51		1		21		5		1		2		4								4
8	9		3		59				8		9		1		7										3

GL	Long chain base (%)		
	d18:0	d18:1	d20:1
1	1	98	1
2	1	63	33
2′	2	71	27
3		71	29
4		51	49
5		52	48
6		64	36
7		56	44
8		55	45

[a]Carbon length, [b]unknown peak(s), [c]saturated, [d]unsaturated, [e]hydroxy.

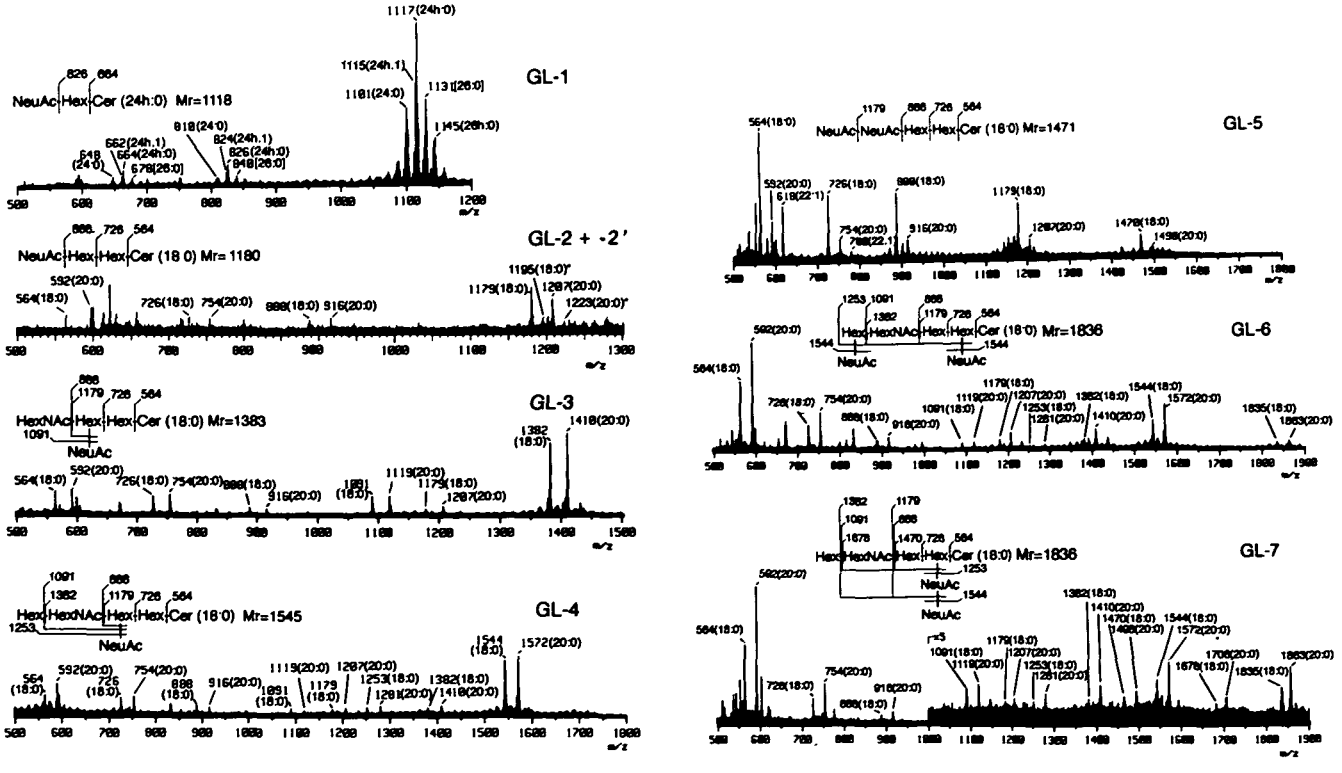

Fig. 2. **Partial negative ion FAB-MS spectra and assignment for ions of equine brain gangliosides.** Numbers in parenthesis indicate fatty acid chain length and saturation (:0) or unsaturation (:1). Asterisked ion indicates a pseudomolecular ion from ganglioside having NeuGc in the specrum of a mixture of GL-2 and -2′.

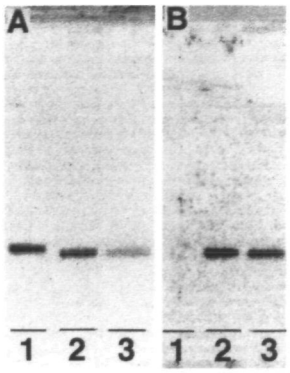

Fig. 3. **TLC-immunostaining of GL-2′.** Panel A, stained with resorcinol-hydrochloric acid; panel B, immunostained on TLC with Hanganutziu and Deicher antibody using 1/80 of the respective lipids in panel A. In A and B, lane 1 shows authentic GM3 (NeuAc); 2, authentic GM3 (NeuGc); 3, equine brain GL-2′.

sugar and sugar species as follows: GL-1 yielded 3-*O*-substituted galactitol; GL-2, -2′ and -5, 4-*O*-glucitol and 3-*O*-galactitol with equimolar ratio; GL-3, 4-*O*-glucitol, 3, 4-*O*-galactitol, and unsubstituted *N*-Ac galactosaminitol with equimolar ratio; GL-4 and -7, 4-*O*-glucitol, 3,4-*O*-galactitol, 3-*O*-*N*-Ac galactosaminitol, and unsubstituted galactitol with equimolar ratio; GL-6 and -8, 4-*O*-glucitol, 3, 4-*O*-galactitol, 3-*O*-*N*-Ac galactosaminitol, and 3-*O*-galactitol with equimolar ratio. The sialic acid species and molar ratio of the sialic acid of the isolated gangliosides were analyzed for the trimethylsilyl ketoside methyl ester derivatives (Table I for the former). The molar ratio of the sialic acid per mol of the ganglioside was obtained from the GLC analysis, and was 1 mol/mol for GL-1, -2, -2′, -3, and -4, 2 mol/mol for GL-5, -6, and -7, and 3 mol/mol for GL-8. The purified gangliosides except GL-2′ were exclusively composed of NeuAc, whereas GL-2′ contained only NeuGc. In addition, the ratio of NeuAc and NeuGc in the whole ganglioside fraction was observed to be 97.3:2.7.

NMR Study—The proton NMR spectra of the purified gangliosides were measured to identify sugar components and their anomeric configurations (data not shown). The anomeric protons of these gangliosides were all *b*-oriented, and *N*-acetyl methyl protons were detected at d1.8 ppm in these gangliosides except GL-2′. The chemical shifts and coupling constants of the anomeric proton as well as other ring protons from GL-1 to GL-8 were identical to those reported previously, as follows: GL-1 was GM4 (NeuAc); GL-2, GM3 (NeuAc); GL-2′, (NeuGc); GL-3, GM2 (NeuAc); GL-4, GM1 (NeuAc); GL-5, GD3 (NeuAc); GL-6, GD1a (NeuAc); GL-7, GD1b (NeuAc); and GL-8, GT1b (NeuAc). In particular, the spectrum of the GL-2′ was completely consistent with that of GM3 (NeuGc) from equine erythrocyte GM3 (NeuGc) (*9*).

FAB-MS Study—The negative FAB-MS spectra of the equine brain gangliosides other than GL-8 are summarized in Fig. 2, together with assignments of the fragment ions. A pseudomolecular ion and fragment ions due to sequentially deglycosidated fragments were observed in all spectra. A mixture of GL-2 and 2′ was measured without separation, and the ions at *m/z* 324 and 306 due to NeuGc and the dehydrated NeuGc, respectively, as well as the ions at *m/z* 1,195 and 1,223 due to $[M-H]^-$ having NeuGc with 18:0 and 20:0, were detected in the mixture (data not shown for the former ions), indicating the presence of the *N*-Gc form in the mixture. The sequences of sugar moieties assigned in the other spectra were sufficient for the structures to be identified by the above NMR study. The FAB- MS spectrum of GL-8 displayed mostly ions similar to those from GL-6 plus GL-7, and lacked pseudomolecular ions of GT1 (data not shown).

TLC-Immunostaining—A small amount of purified GL-2′ specifically reacted with avian antibody against Hanganutziu and Deicher antigen, which recognizes the NeuGc residue in glycoconjugates (*11*), whereas GL-2 (data not shown) and authentic GM3 (NeuAc) did not react with the antibody, as demonstrated in Fig. 3. This reactivity of GL-2′ toward the antibody confirmed the presence of a NeuGc residue in the molecule.

Thus, the combined data from GLC, GC-MS, NMR, and FAB-MS, as well as TLC-immunostaining, identified GL-2′ purified from equine brain as GM3 containing NeuGc, and the other gangliosides from GL-1 to -8 as GM4, GM3, GM2, GM1, GD3, GD1a, GD1b, and GT1b containing NeuAc, respectively, as summarized in Table I.

DISCUSSION

Reports on mammalian brain gangliosides (*4, 5*) indicate that the ganglioside patterns of such mammals as man, cattle, and pigs are similar and have NeuAc as the common major sialic acid component. In tissues and fluids, these mammals have NeuAc or both NeuAc and NeuGc linked to the gangliosides. The horse, on the other hand, has exclusively NeuGc bound to the gangliosides in its erythrocytes (*6, 7, 9*), kidney and spleen (*18*). Equine brain gangliosides have previously been characterized through the identification of structures and sialic acid species by TLC-analysis (*10*), which indicated the presence of GM3, GM2, GM1, GD3, GD1a, GD1b, GT1, and GQ1, and the predominance of NeuAc-gangliosides, which accounted for 97% of the gangliosides. The predominance of the NeuAc-conjugates agreed with our observations as to total gangliosides; however, among the individual gangliosides, GM3 clearly contained NeuGc in our study. Moreover, GQ1 was hardly detected in our study, even though the polarity of the running solvent used for TLC-analysis was elevated; and GM4 was detected in our study by not in the previous work.

Since equine erythrocytes are rich in GM3 exclusively containing NeuGc, the GM3 (NeuGc) isolated from brain gangliosides might derive from contamination by erythrocytes. This possibility was eliminated for the following reasons: (i) erythrocyte GM3 was largely *O*-acetylated at C-4-*O* on NeuGc (4-*O*-Ac GM3) in 72% of the total ganglioside amount (*7, 9*); (ii) the fatty acid composition of non-*O*-acetylated GM3 (NeuGc) (24% of total gangliosides) or 4-*O*-Ac GM3 (NeuGc) in erythrocytes was significantly different from brain GM3 (NeuGc), *i.e.*, 24:0 was a major acid accounting for 65% in the former two lipids (*7*), 18:0 for 61% and 20:0 for 17%, but 24:0 accounted for only 5% in the latter; and similarly, (iii) the long chain base of erythrocyte GM3 (NeuGc) accounted for 98% with C_{18}-sphingenine (*7*), while brain GM3 (NeuGc) was composed of C_{18}- and C_{20}-sphingenine at 71% and 27%, respectively (Table II). The majority of 18:0 as fatty acid and C_{18}- and C_{20}-sphingenine as long chain bases in brain gangliosides

has already been reported for several mammals (*19-25*), these lipid compositions being characteristic of brain gangliosides. Another possibility is that GM3 (NeuGc) is derived from blood vessels and other cells of mesodermal origin. Although gangliosides from these tissues and cells in the horse were not characterized, this possibility might be also negated by the difference in lipid compositions of the GM3 compared to those from, for example human aorta (intima), in which GM3 contains 16:0, 18:0, 18:1, 22:0, and 24:0 as the major fatty acids (*26*). Similarly, gangliosides from other tissues of mesodermal origin such as skeletal muscles are reported to be composed of 16:0 and 18:0 (*27*) or 18:0 (*28*) in rabbit GM3 and 16:0 to 24:1 in pig disialoganglioside (*29*) and human gangliosides (*30*) as the major fatty acids and C_{18}-sphingenine as a major base, which are quite different from equine brain GM3 (Gc).

We are deeply indebted to Mr. Kim Barrymore for his help in the preparation of this article, Drs. Masahiko Chiba and Yoshihiro Maeda for their technical help in the preparation of glycolipids and the Hokkaido Ebetsu Meat Inspection Office for donation of equine brains.

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