Lipid components of sialosylgalactosylceramide of human brain

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ABSTRACT A ganglioside, previously designated HG-B in our laboratory, was isolated from mixed human brain ganglioside preparations and shown to contain equimolar quantities of sialic acid, galactose, and sphingosine. Treatment of this material with neuraminidase yielded a galactosylceramide. The ganglioside, now referred to as sialosylgalactosylceramide, thus appears to be identical with G_{gal} reported by Kuhn and Wiegandt. The fatty acids and long-chain bases of this material were analyzed by gas–liquid chromatography. Approximately equal amounts of normal and hydroxy acids were found. Oleic, palmitic, and stearic acids were the only normal fatty acids present. In the hydroxy series, the C_{24} and C_{23} saturated acids were the major components. The ratio of C_{20} to C_{18} long-chain base was approximately 5:3.

These data suggest that sialosylgalactosylceramide has no direct metabolic relationship with either the major brain gangliosides or adult brain cerebroside.

KEY WORDS sialosylgalactosylceramide · ganglioside · cerebroside · hydroxy fatty acids · normal fatty acids · sphingosine · sialic acid · thin-layer chromatography · gas–liquid chromatography · di(2-ethylhexyl) phthalate · trimethylsilyl ether derivatives

KUHN AND WIEGANDT (1) and also Wiegandt and Baschang (2) isolated a minor component of brain gangliosides that yielded 3′-neuraminosylgalactose upon ozone degradation. This indicated that the parent ganglioside, G_{gal}, is unusual in that galactose is linked directly to sphingosine whereas the major brain gangliosides are derivatives of lactosylceramide, in which the glucose is the residue linked to sphingosine. Because sialosylgalactosylceramide (SGC) was present in such small amounts in the ganglioside mixture, we considered the possibility that it might be derived from adult brain cerebroside. The fatty acid and long-chain base compositions of brain gangliosides and cerebrosides are clearly distinct; cerebrosides contain a high proportion of hydroxy fatty acids (3) and no C_{20}-sphingosine (4-eicosasphingenine) (4) whereas gangliosides contain mostly stearic acid and approximately equal quantities of C_{18}- and C_{20}-sphingosine (4). Thus characterization of the ceramide moiety of SGC should contribute to an understanding of the metabolic relationship of this compound to the major brain gangliosides. We report here the isolation of small quantities of SGC [formerly designated HG-B (5)] from human brain and characterization of the lipid moieties.

MATERIALS AND METHODS

All solvents used were A.C.S. reagent grade; those used in analytical procedures were redistilled. Ratios of solvent mixtures are on a volume basis.

Thin-Layer Chromatography (TLC)

Thin-layer plates were prepared with Silica Gel G (E. Merck A.G., Darmstadt, West Germany). Unless stated otherwise, TLC procedures used were the same as those previously described (5). The solvent systems referred to in this paper have been numbered as follows: I, chloroform–methanol–2.5 N NH_4OH 60:35:8; II, chloroform–methanol–water 60:35:8; III, propanol–water 7:3; IV, propanol–concd NH_4OH 7:3; V, chloroform–methanol–water 65:25:4; VI, chloroform–methanol–water 65:30:5.

Galactosyl and glucosylceramides were distinguished on borate-impregnated plates developed with chloroform–methanol–water–15.0 N NH_4OH 280:70:6:1 as

Abbreviations: SGC, sialosylgalactosylceramide; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; EGS, ethylene glycol succinate polyester; TMSi, trimethylsilyl ether(s). Fatty acids are designated by chain length:number of double bonds.

described by Kean (6). "Beef brain cerebroside" (Light, Colnbrook, Essex, England), which contained theoretically correct amounts of galactose, was used as standard.

Preparation of Mixed Gangliosides of Human Brain

Mixed gangliosides were prepared from normal human adult brains as previously described (7), in a procedure which included a three-tube double withdrawal distribution with Folch solvents. The insoluble interphase material that forms during the distributions was collected and extracted with chloroform–methanol 2:1. This extract was examined by TLC and found to contain a significant quantity of the minor gangliosides as well as some nonganglioside lipids. The extract of the insoluble interphase material was added to all mixed human brain ganglioside preparations used for the isolation of SGC.

Isolation of SGC

4.75 g of mixed human brain gangliosides was applied to an Anasil S column (900 g, 5.2 × 102 cm) which had been previously equilibrated with chloroform–methanol–water 65:30:5. Elution was begun with the same solvent system and 57-ml fractions were collected. Fractions were examined for gangliosides by TLC with solvent system I. Fractions 35–63, which contained SGC (HG-B) and other minor gangliosides, were pooled and evaporated to dryness to yield 325 mg of material. This mixture was applied to an Anasil S column (80 g, 2 × 61 cm) and eluted with chloroform–methanol–water 65:30:5; 8.5-ml fractions were collected. Although there was overlapping of the different gangliosides, and no attempt was made to recover all of the SGC, fractions 30–44 showed only a single resorcinol-positive spot in solvent system I. These fractions were pooled, concentrated, and examined by TLC in several solvent systems. Solvent systems III and V revealed the presence of a nonganglioside organic contaminant. The material was further purified on an Anasil S column (80 g, 2 × 60 cm) developed with chloroform–methanol–water 65:25:4. The fractions containing SGC were pooled and evaporated to dryness, and the material was rechromatographed with chloroform–methanol–water 65:25:4 as before. SGC fractions that were free of the nonganglioside contaminant were pooled, evaporated to dryness, and dissolved in 70% methanol. No attempt was made to obtain a dry weight of the sample, but we calculated from the total sialic acid present that 6 mg of SGC (calculated average mol wt 1089) was obtained.

SGC was treated for 24 hr with neuraminidase (EC 3.2.1.18) (*Vibrio cholerae*, General Biochemicals, Chagrin Falls, Ohio) in 0.05 M acetate buffer, pH 5.5, containing 1% NaCl and 0.1% $CaCl_2$. *N*-Acetylneuraminosyl lactose (General Biochemicals) was used as a test substrate.

Sialic acid was determined by the resorcinol method (8) as modified by Miettinen and Takki-Luukkainen (9). The sialic acid content was corrected for galactose interference as reported previously (10). Hexosamine was determined by the method of Svennerholm (11) and total hexose was determined by the phenol–sulfuric acid assay (12). Sphingosine was determined according to Lauter and Trams (13).

Characterization of Fatty Acids and Sphingosines

A 5000-series Barber-Colman unit equipped with a flame ionization detector was used for all GLC analyses. Helium was used as the carrier gas.

Samples of SGC were treated in 0.5 N anhydrous methanol–HCl as described for the analysis of glucose and galactose (14). The heptane extracts were concentrated and used for GLC or TLC analyses of the fatty acid methyl esters. The normal and hydroxy esters were separated by preparative TLC with diethyl ether–hexane 15:85 (15). After development of the plates, esters were made visible with iodine vapor. When all color had disappeared, appropriate areas of the silica gel were scraped off and fatty acid esters were eluted with diethyl ether.

Normal fatty acid esters were identified by GLC at 180°C on a 6 ft × 1/8 inch column packed with 3% SE-52 (methylpolysiloxy gum, General Electric) on Anakrom ABS, 110–120 mesh, (Analabs, Inc., Hamden, Conn.) with a helium flow rate of 30 ml/min. Identity of the esters was confirmed by analysis, before and after hydrogenation, on 15% ethylene glycol succinate polyester (EGS). Standard fatty acids were obtained from Applied Science Laboratories Inc., State College, Pa.

Hydroxy fatty acids were identified as their trimethylsilyl ether (TMSi) derivatives (16). The derivatives were prepared with Sweeley's (17) reagents and analyzed by GLC with the SE-52 column referred to above, operated at 202°C with a helium flow rate of 25 ml/min. Methyl-α-hydroxytetracosanoate and methyl-α-hydroxydocosanoate (Applied Science Laboratories) as well as the hydroxy fatty acid esters prepared from the "beef brain cerebroside" served as standards. Additional confirmation of the identity of TMSi derivatives of the hydroxy fatty acid methyl esters was provided by their mass spectra.

The total fatty acid methyl ester fraction was treated with the TMSi reagents and analyzed on the 3% SE-52 column with a temperature program of 2°C/min from 135 to 202°C and at a helium flow rate of 25 ml/min. Mass spectra of the components of this total methyl ester fraction were obtained by Dr. Charles C. Sweeley (University of Pittsburgh) with an LKB 9000 instrument fitted with a 6 ft × 1/8 inch column packed with 3% OV-1 (polar silicone phase) (Supelco, Inc., Bellefonte, Pa.). These data provided confirmatory evidence of the

identity of the fatty acids and led to the identification of di(2-ethylhexyl) phthalate as a contaminant in this fraction. A sample of di(2-ethylhexyl) phthalate was obtained from Dr. P. P. Nair.

To characterize the sphingosine in SGC, we hydrolyzed the ganglioside in 1 N aqueous methanol–HCl at 75°C for 22 hr (18). The sphingosine was isolated and subjected to periodate oxidation according to the procedure of Sweeley and Moscatelli (19). The resulting aldehydes after purification on silicic acid were analyzed by GLC on a 6 ft × $^1/_8$ inch column packed with 15% EGS on Anakrom ABS, 110–120 mesh (Analabs). Sphingosine prepared from mixed gangliosides and "beef brain cerebroside" served as standards.

Peak areas were calculated by multiplication of height by width at half-height. Quantitative results with fatty acid standard RM-3 (Supelco) agreed with the stated composition data with a relative error less that 1% for major components (>10% of total mixture) and less than 0.5% for minor components (<10% of total mixture).

RESULTS

SGC (HG-B) was not observed on thin-layer plates when mixed human brain gangliosides were analyzed but was easily seen in mixtures of minor gangliosides eluted from Anasil S columns before galactosyl *N*-acetylgalactosaminyl (sialosyl) lactosylceramide (HG-1). The appearance of mixed human brain gangliosides and mixed minor gangliosides when analyzed in solvent system I is shown in plate A of Fig. 1. A thin-layer chromatogram of purified SGC and other minor gangliosides in solvent system I is shown in plate B of Fig. 1. The chromatographic mobilities of SGC relative to HG-1 (R_{HG-1}) in solvent systems II, III, IV, and V were 1.42, 1.13, 2.32, and 4.34, respectively. Only one spot was detected in all solvent systems with the purified SGC, whether the plate was sprayed with resorcinol reagent or 50% H_2SO_4. Analytical data, presented in Table 1, provided further evidence of purity and showed that the material contained equimolar ratios of sphingosine, galactose, and sialic acid.

The material was treated with *Vibrio cholerae* neuraminidase and the reaction products were examined in solvent systems I–V and in the Kean system (6). In all cases spots corresponding to the sialic acid and galactosylceramide standard were observed.

Examination of the fatty acid methyl esters obtained from SGC in the TLC system described by Vioque and Holman (15) revealed spots corresponding to hydroxy and normal fatty acid esters. Hydroxy and normal fatty acid ester fractions were obtained by preparative TLC and examined by GLC. The normal ester fraction contained palmitic, stearic, and oleic acids (Table 2). The composition of hydroxy fatty acid esters (Table 2) was similar to that reported for cerebrosides (3).

TABLE 1 COMPOSITION OF SIALOSYLGALACTOSYLCERAMIDE

Component	Content
	μmoles/μmole NANA
Total hexose*	1.12
Galactose†	1.05
Sphingosine	0.98
Hexosamine	0.08

NANA, *N*-acetylneuraminic acid.
* Phenol–sulfuric acid assay.
† GLC assay; no detectable glucose.

TABLE 2 COMPOSITION OF FATTY ACIDS OF SIALOSYLGALACTOSYLCERAMIDE

	Normal Fatty Acids	Hydroxy Fatty Acids
	% of total	
16:0	27.7	tr.
18:1	25.1	—
18:0	47.3	—
20:0		2.5
22:0		6.3
23:0		21.7
24:0		54.6
24:1		3.6
25:0		10.7
26:1		tr.

Fatty acids are designated by chain length:no. of double bonds.

In a determination of the relative amounts of hydroxy and nonhydroxy fatty acids, the total fatty acid methyl ester fraction was trimethylsilylated and subjected to GLC. The ratio of hydroxy to nonhydroxy fatty acids was estimated as 0.9 by comparison of the total peak areas corresponding to the fatty acids listed in Table 2; no correction for the contribution of the trimethylsilyl groups to the GLC response was made.

In addition to the fatty acids listed in Table 2, the total methyl ester fraction gave on GLC analysis an additional peak not observed in the hydroxy or normal fatty acid ester fractions obtained by preparative TLC. This peak had an equivalent chain length of 22.1 on 3% SE-52 and 23.9 on 15% EGS. The mass spectra and chromatographic behavior of this material were similar to that reported by Dr. Nair[1] for di(2-ethylhexyl) phthalate. A sample of the phthalate was obtained from Dr. Nair and was found to have the same retention times as the unknown on 1% SE-52 and 6% OV-1. Since this substance is a commonly used plasticizer it was considered to be a contaminant, not a natural fatty ester.

[1] Nair, P. P. Personal communication. Sinai Hospital of Baltimore, Inc., Belvedere Avenue at Greenspring, Baltimore, Md. 21215.

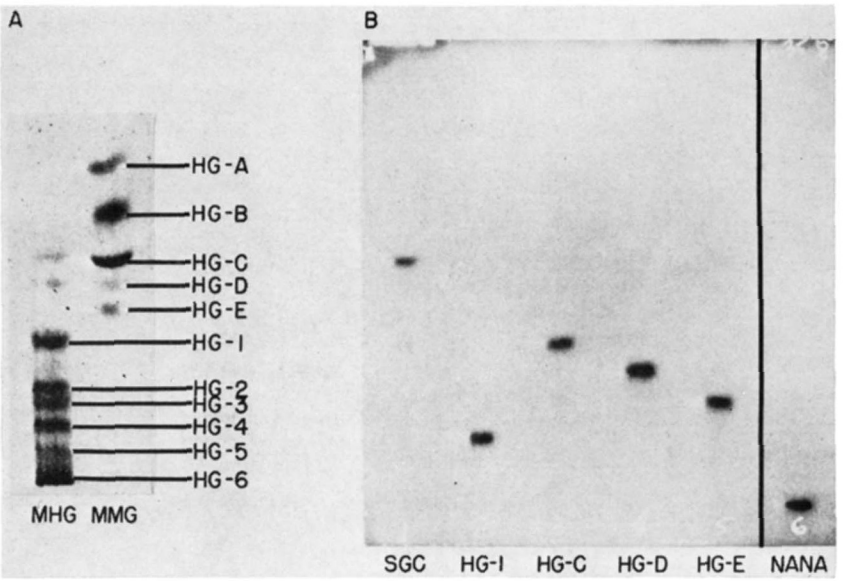

FIG. 1. TLC of mixtures of human brain gangliosides developed with chloroform–methanol–2.5 N NH_4OH 60:35:8. Plate A: MHG, mixed human gangliosides; MMG, mixed minor gangliosides. Plate B, purified preparations of minor gangliosides, HG-1, and NANA (*N*-acetylneuraminic acid). Spots were made visible with resorcinol reagent. See references 5 and 7 for explanation of ganglioside nomenclature.

Analysis of the long-chain base fraction by periodate oxidation and GLC analysis of the resulting aldehydes showed that more C_{20}- than C_{18}-sphingosine was present (Table 3).

DISCUSSION

The SGC reported here was isolated from a large sample of mixed gangliosides of normal adult brain prepared by a procedure involving a Folch solvent distribution, and no attempt was made to isolate all the SGC from brain. In fact, we have evidence that some of this ganglioside remains in the lower phase of the solvent partitions. It is possible, therefore, that there may have been selective losses based on solubility properties and that the lipid composition reported here does not truly represent that of total brain SGC. Nevertheless, the fatty acid and long-chain base compositions found are sufficiently distinctive to be significant.

TABLE 3 GLC ANALYSIS OF THE LONG-CHAIN BASES AS ALDEHYDES*

	4-Sphingenine† (C_{18})	Sphinganine (C_{18} dihydro-)	4-Eicosasphingenine (C_{20})	Eicosasphinganine (C_{20} dihydro-)
Mixed gangliosides	47	2	49	2
SGC	31	4	55	10

* Expressed as percentage of known aldehydes; these include the derivatives of sphinganine and eicosasphinganine and 3-*O*-methyl-4-sphingenine.

† Figures represent the sum of peaks from 4-sphingenine and 3-*O*-methyl-4-sphingenine.

The presence of C_{18}- and C_{20}-sphingosine in SGC suggests a metabolic relationship with the major brain gangliosides rather than with the brain cerebrosides. However, the composition of the fatty acid fraction complicates this interpretation. The composition of the hydroxy fatty acid fraction resembles that reported for brain cerebroside (3), while the normal fatty acid fraction is clearly distinct from the normal fatty acids of adult brain cerebrosides. O'Brien and coworkers (3) report that 24:0 and 24:1 constitute the major fatty acids of cerebrosides in both the normal and hydroxy series. The high percentage of oleic acid also distinguishes the normal fatty acid pattern from that of the major brain gangliosides.

From the data presented we have concluded that SGC is not a direct derivative of normal adult brain cerebroside. Because adult brain cerebroside contains no C_{20}-sphingosine, the possibility of a common psychosine precursor appears to be eliminated; the fatty acid differences eliminate consideration of a common ceramide precursor. The fatty acid composition of SGC seems to eliminate the possibility of a ceramide precursor in common with the major brain gangliosides. Thus, the unique ceramide composition of the isolated SGC suggests that this compound has no direct metabolic relationship with either the major brain gangliosides or adult brain cerebrosides. However, O'Brien and Sampson (20) report that the normal fatty acids of cerebroside obtained from infant and child gray matter consist mostly of 16:0, 18:0, and 18:1. Menkes, Philippart, and Concone (21) report similar data

for normal fatty acids from cerebrosides isolated from fetal and 1 day old infant gray matter. Thus, the fatty acid composition of SGC more closely resembles that reported for infant gray matter cerebroside than adult brain cerebroside. It will be of interest to determine the sphingosine composition of infant gray matter cerebroside, which contains a large percentage of these medium-chain normal fatty acids.

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