Isolation and characterization of ganglioside G_{M1b} from normal human brain

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Abstract A sialidase-susceptible monosialoganglioside was isolated from normal human brain by DEAE-Sephadex A-25 and Iatrobeads column chromatography. The yield of this ganglioside was about 6 mg per whole brain. Its structure was elucidated by sugar analysis, sialidase digestion, permethylation, and proton NMR studies. This ganglioside had carbohydrate, fatty acid, and long-chain base compositions identical to those of brain G_{M1a}. However, the sialosyl residue was found to be linked (α2-3) to the terminal galactosyl residue of the asialo-G_{M1a} backbone. The complete structure of this ganglioside was therefore identified as G_{M1b} or $IV^3NeuAcGgOse_4Cer$. —**Ariga, T., and R. K. Yu.** Isolation and characterization of ganglioside G_{M1b} from normal human brain. *J. Lipid Res.* 1987. **28:** 285–291.

Supplementary key words G_{M1b} ganglioside • human brain • NMR • FAB-MS

Yip (1) first demonstrated the enzymatic synthesis of a sialidase-susceptible monosialoganglioside from ganglio-N-tetraosyl ceramide ($GgOse_4Cer$) and CMP-sialic acid using rat brain homogenate as the source of the sialosyltransferase. Subsequently the structure of this ganglioside was characterized as $IV^3NeuAcGgOse_4Cer$, and was named G_{M1b} (1, 2). G_{M1b} ganglioside has since been recognized in various cell lines such as rat ascites hepatoma AH-7974 F (3, 4), bone marrow (5), mouse myeloid leukemia (6), and lymphocytes of C57 BL/6 mouse (7). Watanabe, Powell, and Hakomori (8) first reported its natural occurrence in the human erythrocyte membrane. Nakamura et al. (9) recently characterized G_{M1b} containing N-glycolyl neuraminic acid as a major component from ICR mouse spleen and also tentatively identified G_{M1b} containing N-acetylneuraminic acid as a minor component. Therefore, G_{M1b} ganglioside was considered to be a natural ganglioside species present in various mammalian tissues.

In this report, we describe the isolation and characterization of G_{M1b} from normal adult human brain. Although Chou, Nolan, and Jungalwala (10) provided preliminary data for the presence of this ganglioside in peripheral nerves, our work represents the first conclusive report for its presence in mammalian central nervous system tissues.

MATERIALS AND METHODS

Gangliosides were isolated by the procedure of Ando and Yu (11) with minor modifications. The whole brain (about 2.3 kg) from an autopsied normal adult subject was homogenized with 10 volumes each of chloroform–methanol 2:1, 1:1, and 1:2 (v/v). The lipid extracts were combined and the solvent was evaporated. The residue was dissolved in 12 liters of chloroform–methanol–water 30:60:8 (v/v). The lipid solution was divided into two equal portions and each was applied to a DEAE-Sephadex A-25 (acetate form, 5 cm i.d. × 150 cm, bed volume 1750 ml, 310 g) column. The neutral lipids were eluted with 15 liters of chloroform–methanol–water 30:60:8 (v/v) and 2 liters of methanol. The acidic lipids were recovered with 13 liters of 0.2 M sodium acetate in methanol. After the solvent was removed by evaporation, the residue was dissolved in 1 liter of water, dialyzed against distilled water for 3 days and the retentate was lyophilized. The residue was dissolved in 500 ml of chloroform–methanol–water 30:60:8 (v/v) and applied again

Abbreviations: NMR, nuclear magnetic resonance; FAB, fast atom bombardment; GLC, gas-liquid chromatography; HPTLC, high performance thin-layer chromatography. The ganglioside nomenclature follows the system of Svennerholm (1964). G_{M1} or G_{M1a}: $II^3NeuAcGgOse_4Cer$; G_{M1b}: $IV^3NeuAcGgOse_4Cer$; G_{M1b}-GalNAc:IV^3NeuAc, $IV^4GalNAcGgOse_4Cer$; G_{M3}: $II^3NeuAcLacCer$; G_{D3}: $II^3NeuAc_2LacCer$; G_{D1a}: $II^3NeuAc,IV^3NeuAcGgOse_4Cer$; G_{D1b}: $II^3NeuAc_2GgOse_4Cer$; G_{T1b}: $II^3NeuAc_2,IV^3NeuAcGgOse_4Cer$.

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to a DEAE-Sephadex A-25 column (2.5 cm i.d. × 200 cm). At this stage, there were still small amounts of residual neutral lipids, which were eluted with 1 liter of chloroform-methanol-water 30:60:8 (v/v) and 500 ml of methanol. The acidic glycolipids were then eluted with 4 liters of a linear gradient system prepared from sodium acetate in methanol (0.05 M and 0.3 M). The monosialoganglioside fractions were combined and the solvent was evaporated. The residue was dissolved in 200 ml of distilled water, dialyzed against distilled water and the retentate was lyophilized. The residue was dissolved in 20 ml of chloroform-methanol-water 70:30:1 (v/v) and applied to an Iatrobeads column (2.2 cm i.s. × 100 cm). The column was eluted with 2 liters of a linear gradient system of chloroform-methanol-water 65:35:4 and 45:55:5 (v/v). G_{M1b} ganglioside chromatographed with the tailing part of G_{M1a} ganglioside. Fractions containing this ganglioside were combined, evaporated, and then applied again to another Iatrobeads column (1.5 cm i.d. × 100 cm) which was eluted with 1 liter of a linear gradient system of chloroform-methanol-water 65:35:4 and 35:65:5 (v/v). The final residual amounts of G_{M1a} ganglioside were removed by another Iatrobeads column (15 g, 1.2 cm i.d. × 56 cm), eluting with 500 ml of n-propanol-water 85:15 (v/v). The purity of the isolated ganglioside was examined by high performance thin-layer chromatography (HPTLC) with the following solvent system: (A) chloroform-methanol-water 50:45:10 (containing 0.02% $CaCl_2 \cdot 2H_2O$) and (B) chloroform-methanol-5 M ammonia-0.4% aq. $CaCl_2 \cdot 2H_2O$ 60:45:4:5 (v/v).

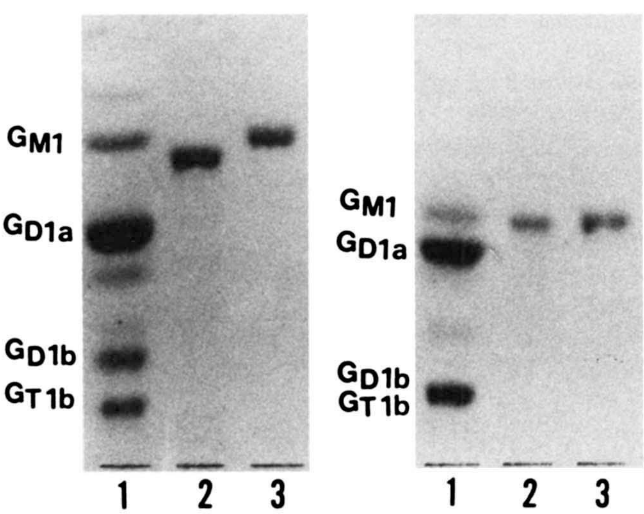

Fig. 1. Thin-layer chromatogram of the isolated ganglioside. Lane 1, ganglioside mixture from human grey matter; lane 2, isolated unknown ganglioside from normal human brain; and lane 3, GM_{1a} ganglioside standard from human brain. The left plate was developed with the solvent system chloroform-methanol-water 50:45:10 containing 0.2% $CaCl_2 \cdot 2H_2O$ (v/v) and the right plate was developed with chloroform-methanol-5 M ammonia-0.2 M $CaCl_2 \cdot 2H_2O$ 60:45:4:5 (v/v). The bands were visualized with the resorcinol-hydrochloric acid reagent.

TABLE 1. Chemical composition of the G_{M1b} ganglioside from normal human brain

	Ratio	
	GLC[a]	NMR[b]
Glucose	1.00	1.00
Galactose	2.15	2.09
Galactosamine	1.01	0.93
Sialic acid	1.05	0.90
Long-chain base	1.07	

[a]As N, O-trifluoroacetyl derivatives.
[b]Four hundred MHz proton nuclear magnetic resonance.

Compositional analysis was carried out by gas-liquid chromatography (GLC). Neutral sugars, sialic acids, and long-chain bases were analyzed as their N,O-trifluoroacetyl derivatives according to the procedure of Ando and Yu (12). The sample, 100 μg, was methanolyzed for 16 hr at 75°C with 0.3 ml of 3% hydrochloric acid in methanol. After fatty acid methyl esters were removed by extraction with n-hexane, the methanolic solution was evaporated under N_2 gas and dried under vacuum. The residue was derivatized with 20 μl of 33% N-methyl-bis trifluoracetamide in pyridine at room temperature for 10 min and a portion of this solution was injected into a GLC column (6 feet) packed with Gas-Chrom Q, which was coated with a mixture of 6% SP-2401 and 0.5% OV-225 (Applied Science Labs). Fatty acid methyl esters were analyzed using a 3% OV-1 column at 200°C isothermally. The long-chain base composition was also determined by GLC of their fatty aldehydes after periodate oxidation according to Sweeley and Moscatteli (13). The sialic acid species of the ganglioside was determined by the method of Yu and Ledeen (14).

Enzymatic treatment with sialidase from *Arthrobacter ureafaciens* (EC 3.2.1.18, Nakarai Chemical Company, Kyoto) was carried out by the method of Sugano, Saito, and Nagai (15). The sample, 250 μg, was dissolved in 70

TABLE 2. Fatty acid and long-chain base compositions of G_{M1b} from normal human brain (%)

Fatty Acid		Long-Chain Base	
	% of total		*% of total*
C16:0	4.5	C16:0	
C18:1	6.5	C18:1	47.6
C18:0	81.4	C18:0	4.8
C20:0	4.7	C20:1	40.0
C20:1		C20:0	7.6
C22:0	1.4		
C23:0			
C24:1	0.8		
C24:0	0.7		

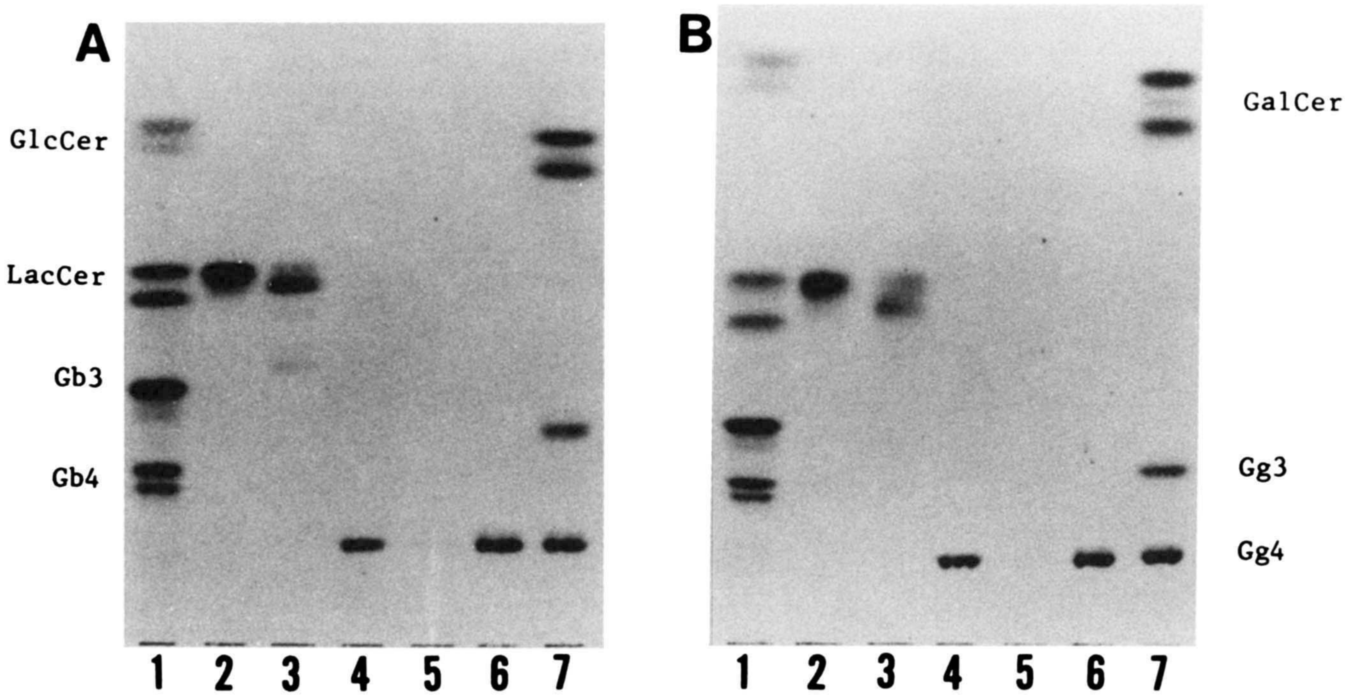

Fig. 2. Thin-layer chromatogram of the glycolipid products after sialidase digestion. Lane 1, standard glycolipid mixture (from top to bottom, glucosyl ceramide (GlcCer) from Gaucher's spleen, lactosyl ceramide (LacCer), galactosyl-lactosyl ceramide (Gb3), and globoside (Gb4) from pig erythyrocyte membrane); 2, lactosyl ceramide derived from G_{M3} ganglioside of bovine adrenal medulla following sialidase treatment; 3, lactosyl ceramide derived from G_{D3} of human brain after sialidase treatment; 4, glycolipid product derived from the isolated unknown ganglioside following sialidase treatment; 5, G_{M1a} ganglioside after sialidase treatment; 6, authentic ganglio-N-tetraosylceramide; 7, standard glycolipid mixture (from top to bottom, galactosyl ceramide (GalCer), ganglio-N-triaosyl ceramide (Gg3), and ganglio-N-tetraosylceramide (Gg4). Plate A was developed with chloroform- methanol-water 65:35:8 (v/v) and plate B with chloroform-methanol-2.5 N ammonia 65:35:8 (v/v). The bands were visualized with the oricinol-sulfuric acid reagent (33). Note the absence of any glycolipid product in lane 5.

μl of distilled water and 100 μl of 0.1 M sodium acetate buffer (pH 5.0). Then 20 μl of neuraminidase solution (1 unit in 1 ml of the same buffer) without detergent was added and the reaction mixture was incubated for 16 hr at 37°C. The reaction was terminated by the addition of 1 ml of chloroform-methanol 2:1 (v/v). The lower phase was evaporated under nitrogen gas. The glycolipid products after the enzymatic degradation were examined by HPTLC with the following solvent systems: (A) chloroform-methanol-water 65:35:8 (v/v) and (B) chloroform-methanol-2.5 M ammonia 65:35:8 (v/v). For comparison, an authentic sample of G_{M1a} was treated and analyzed similarly.

Permethylation was carried out by the method of Tanaka et al. (16) and Ariga et al. (17). The sample, 100 μg, was peracetylated with 20 μl of acetic anhydride-pyridine 2:3 (v/v) at room temperature for 1 hr. The reaction mixture was evaporated under nitrogen and desiccated with P_2O_5. The residue was dissolved in 20 μl of anhydrous dimethyl foramide, followed by the addition of 40 μl of sodium hydride in dimethyl formamide (20 mg per ml) and 15 μl of methyl iodide (freshly prepared, stored under molecular sieves 3 Å) at 0°C. After 30 min, 10 μl of methyl iodide was added at room temperature. This reaction mixture was incubated for 3 hr at room temperature, evaporated to dryness under nitrogen, and dried under vacuum. The residue was subjected to reacetylation and remethylation as described above in order to assure complete methylation. The permethylated glycolipid was then purified by HPTLC with a developing solvent system of chloroform-methanol-n-hexane 4:1:2 (v/v) (17).

The partially methylated alditol acetates were prepared from methylated glycolipid by the method of Yang and Hakomori (18) and analyzed by GLC-mass spectrometry (Model QP-1000 Shimadzu Co., Ltd.) using a perfused silica capillary column. The glycolipid product after the sialidase treatment was also subjected to permethylation and the aldohexitol acetates were analyzed by GLC-mass spectrometry.

Proton nuclear magnetic resonance (NMR) spectra were obtained by a JEOL GX-400 spectrometer. The sample, 2 mg, was dissolved in 0.5 ml of dimethyl sulfoxide-d_5-D_2O 98:2 (v/v) containing tetramethylsilane (19).

TABLE 3. Chemical shifts of the anomeric protons and their coupling constants

	-3GalNAc-	-3Gal-	-4Gal-	-4Glc-
Anomeric proton (ppm) (H-1)	4.52	4.25	4.19	4.16
$J_{1,2}$ (Hz)	7.93	7.63	7.23	7.33

NMR spectra were recorded by 400 MHz NMR spectrometry at 27°C.

Negative ion fast atom bombardment (FAB) mass spectra were obtained by a JEOL HX-100 high resolution mass spectrometer equipped with a FAB ion source and JMA-3500 computer system (JEOL, Tokyo). The sample, 100 μg, was dissolved in 30 μl of chloroform-methanol 3:1 (v/v) and then 2 μl of triethanolamine-tetramethylurea 1:1 (v/w) was added (20). The solvent mixture, about 1 μl, was applied onto a stainless-steel holder (1 × 4 mm) and analyzed.

RESULTS AND DISCUSSION

The yield of the isolated unknown ganglioside was about 6 mg from one adult human brain, which was about 2.6 μg/g wet tissue and 0.25% of the monosialoganglioside fraction. Hence this represents a minor ganglioside previously undetected in brain tissue. On thin-layer chromatography, it migrated slightly slower than brain G_{M1a} ganglioside using the neutral solvent system (**Fig. 1**). The ganglioside was found to contain glucose, galactose, N-acetylgalactosamine, sialic acid, and long-chain base in the molar ratio of 1:2:1:1:1 by GLC and proton NMR data (**Table 1**). The sialic acid was of the N-acetyl type only. The predominant fatty acid was stearic acid and the long-chain bases were C18 and C20 sphingenine (**Table 2**). These structural features are therefore, very similar to those of brain G_{M1a} ganglioside (21, 22). However, this ganglioside could be digested by sialidase from *A. ureafaciens,* without the detergent sodium cholate, to yield a glycolipid product that co-chromatographed with authentic asialo-G_{M1} using both the neutral and basic solvent systems. Under this condition, brain G_{M1a} ganglioside was not hydrolyzed (**Fig. 2**). This is consistent with the results of Sugano et al. (15) who reported that G_{M1a} ganglioside can be hydrolyzed to yield asialo-G_{M1a} only in the presence of sodium cholate, whereas G_{M1a} ganglioside is known to be susceptible to sialidase digestion (1, 2). The degraded glycolipid product obtained from the sialidase treatment was found to contain glucose, galactose, N-acetylgalactosamine, and long-chain base in the molar ratio of 1:2.12:0.90:1.10, based on GLC analysis, which is also compatible with an asialo-G_{M1} structure.

Analyses of the permethylated ganglioside and the resulting partially methylated aldohexitol acetates derived from it by capillary GLC and GLC-electron impact mass spectrometry revealed that this ganglioside produced

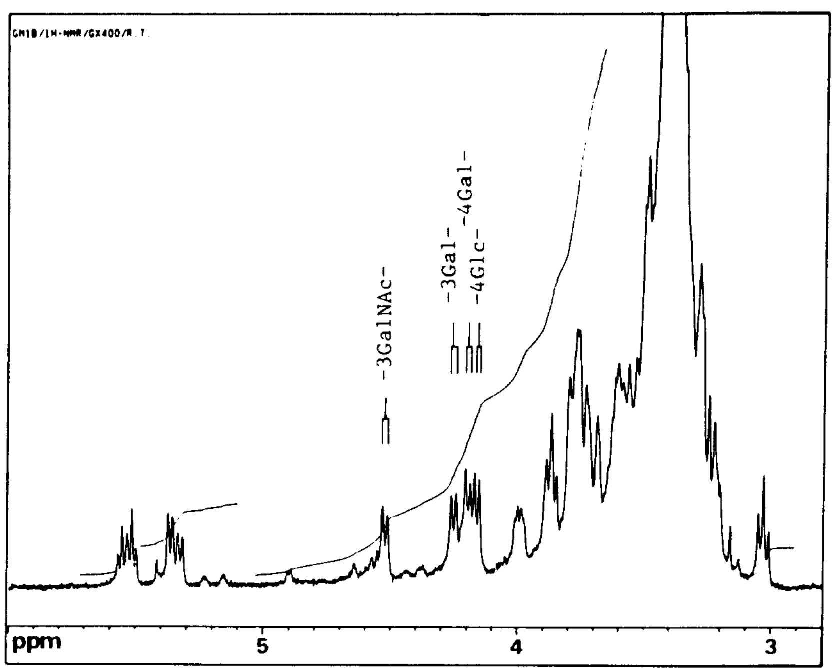

Fig. 3. Four hundred MHz proton NMR spectrum of the isolated unknown ganglioside.

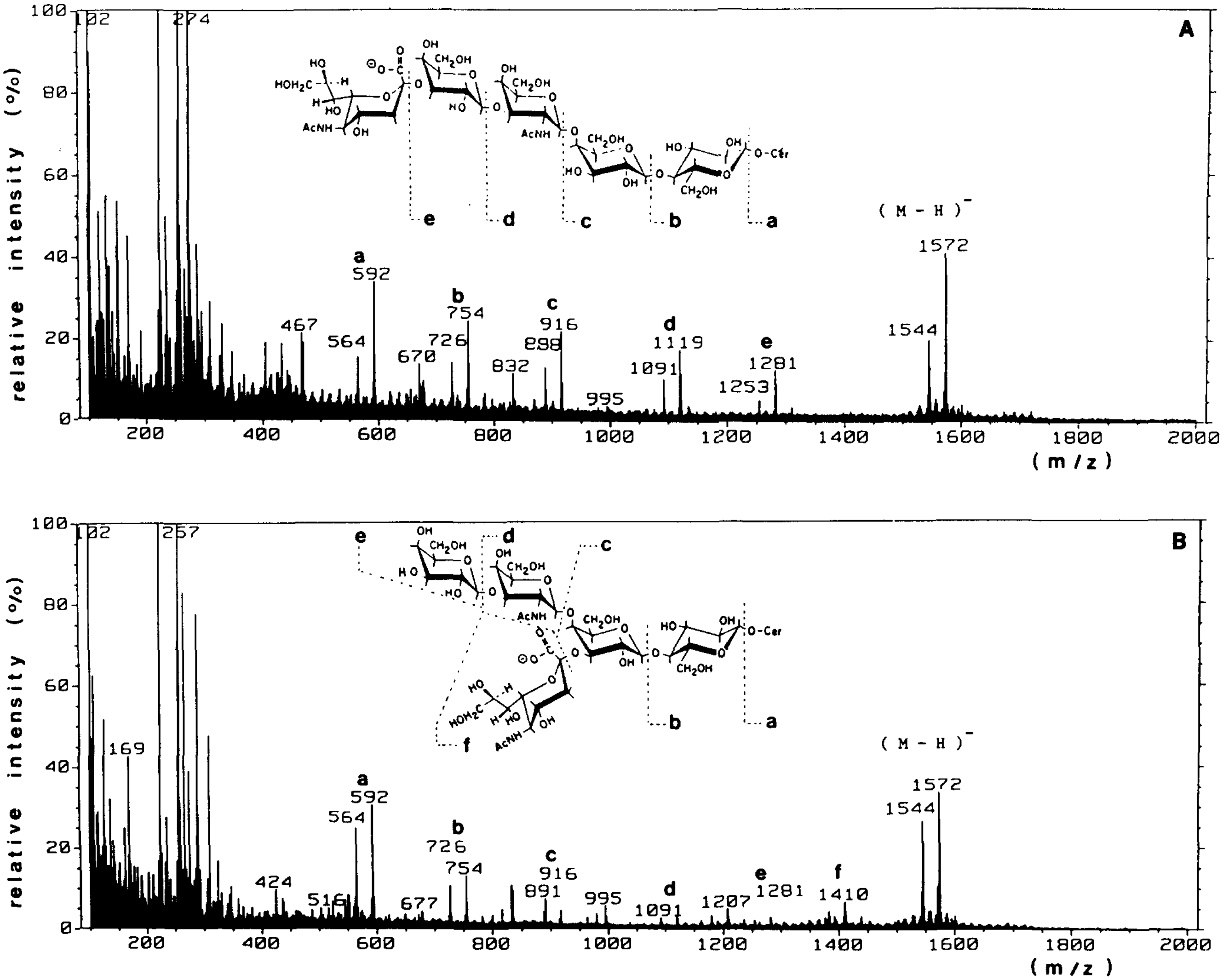

Fig. 4. Negative FAB mass spectra of isolated unknown ganglioside (A) and G_{M1a} ganglioside (B).

1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol; 1,3,5-tri-O-acetyl-4, 6-di-O-methyl-2-deoxy-2-N-methylacetamido-galactitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol; and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol in the ratio of 0.82:1.12:1.29:1. On the other hand, the enzyme degradation product produced 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol (instead of 1,3,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol), 1,3,5-tri-O-acetyl-4,6-di-O-methyl-2-deoxy-2-N-methylacetamidogalactitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol; and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol in the ratio of 0.67:0.78:0.62:1, respectively. These overall data are consistent with the structure of a ganglio-N-tetraosyl backbone with a sialosyl residue attached to the terminal galactosyl residue at the C-3 position through an α-D-linkage.

NMR data revealed the presence of four protons in the anomeric region (4–5 ppm) having relative areas of approximately 1:1:1:1. Each had a large coupling constant (>7 Hz), suggesting a β-D-aldopyranose. The anomeric doublets at 4.16 ppm and 4.52 ppm are characteristic of β-D-glucopyranosyl and 2-acetamido-2-deoxy-β-D-galactopyranosyl residues, respectively. The β-proton of the internal galactose can be assigned at 4.19 ppm ($J_{1,2}$ = 7.23 Hz). The β-proton of the external galactose is found at 4.25 ppm, which is slightly downfield, compared to that of asialo-G_{M1} (4.21 ppm, ref. 19), presumably because of the presence of the sialic acid residue (**Table 3** and **Fig. 3**).

In the negative ion FAB mass spectrum of G_{M1a} ganglioside, there are six major ion groups, which represent ceramide, glucosyl ceramide, lactosyl ceramide, ganglio-N-triaosylceramide, ganglio-N-tetraosylceramide, and sialosyl ganglio-N-tetraosyl ceramide. The prominent molecular ions (M-H)$^-$ were detected at m/z 1544 and 1572. Elimination of the terminal sialic acid residue yields the fragment ions (e) at m/z 1253 and 1281 (M-H-291)$^-$. Fragment ions corresponding to the successive elimination of galactose, N-acetylgalactosamine, galactose, and glucose residues were detected at m/z 1091 and 1119(d), m/z 888 and 916(c), m/z 726 and 754(b), and m/z 564 and 592(a), respectively (**Fig. 4A**). The negative FAB-mass spectrum of brain G_{M1} ganglioside, previously reported by Arita et al. (19) was also analyzed as a reference (Fig. 4B). In the case of G_{M1} ganglioside, the molecular ions were confirmed at m/z 1544 and 1572 (M-H)$^-$. However its fragmentation diagrams are somewhat different, because the elimination peaks of the sialic acid residue were extremely low while those of the terminal galactose residue were clearly detected at m/z 1382 and 1410 (M-H-163)$^-$(f). The ions representing ceramide and glucosyl ceramide were detected at m/z 564 and 592(a), and m/z 726 and 754(b), respectively, as they were in G_{M1b} ganglioside.

Taken together, these results are consistent with the following structure for this ganglioside: $IV^3NeuAcGgOse_4Cer$; NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer (G_{M1b}).

Itoh et al. (23) first characterized the structure of G_{M1b}-GalNAc in Tay-Sachs brains as a minor ganglioside and suggested that G_{M1b} ganglioside might exist in the brain to serve as the biosynthetic precursor for this ganglioside. The discovery of G_{M1b} in human brain lends strong support to this contention. The possible precursor of G_{M1b}, asialo-G_{M1}, has been detected in immature brains and the brains of patients with G_{M1}-gangliosidosis (24, 25). Recently Kusunoki, Tsuji, and Nagai (26) reported the natural occurrence of asialo-G_{M1} in adult mouse brain myelin. Our finding thus provides the intermediate step for the new biosynthetic pathway in the brain, namely, asialo-$G_{M1} \rightarrow G_{M1b} \rightarrow G_{M1b}$-GalNAc. It is also well known that G_{M1b} may be involved in anti-mouse natural killer (NK) cell activity and it is considered to be a surface marker of NK cells (27-29), T-cells (30, 31), and human acute leukemia cells (32). Moreover, G_{M1b} ganglioside has recently been reported in immune cells such as mouse spleen cells (9). In light of the growing evidence that glycolipids may serve as common antigens for the nervous and immune systems (26), the possibility exists that G_{M1b} may be involved in certain autoimmune diseases of the nervous system. Additionally, it may be involved in the loss of adhesiveness in tumor cells (4) and in cell maturation (5, 33).

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