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QUARTERLY

Matrix-assisted laser desorption ionization time-of-flight mass spectrometric analysis of glycosphingolipids including gangliosides*

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Long chain base compositions of gangliosides containing mainly stearic acid could be determined without any chemical modification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry with delayed ion extraction (DE MALDI-TOF MS). The analytical results for the long chain base compositions of various samples of G_{M1} from the brain tissues of patients with different diseases at different ages confirmed that the proportion of d20:1 (icosasphingosine) and d20 (icosa-sphinganine) of the total sphingosine bases increased quickly until adolescent or adult age and then remained constant slightly exceeding 50%; this value was evidently higher than the proportion of d20:1 and d20 of G_{M1} in various adult mammalian brains. A long chain base composition of G_{M1} from the brain tissue of a patient with infantile type of G_{M1}-gangliosidosis at 4y2m was abnormal and so was in two sibling patients with Spielmeyer-Vogt type of juvenile amaurotic idiocy at 19y and 21y in spite of that in the latter there was no accumulation of G_{M1} in the brain tissue. On the other hand, a patient with adult type of G_{M1} gangliosidosis at 66y showed a local accumulation of G_{M1} in the putamen and caudate nucleus, but its long chain base composition was found to be normal.

It was of interest that the white matter of Eker rat with hereditary renal carcinoma contained a large amount of plasmalocerebroside as compared with the amount of cerebroside and sphingomyelin. The individual molecular species of plasmalocerebroside were identified by DE MALDI-TOF MS.

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Abbreviations: α-CHCA, α-cyano-4-hydroxycinnamic acid; CMH, cerebroside; d18:1, sphingosine or sphingenine; d18, sphinganine; d20:1, icosasphingosine; d20, icosasphinganine; DE MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry with delayed ion extraction; 2,5-DHB, 2,5-dihydroxybenzoic acid; G_{M1}, ganglioside; G_{M1} follows L. Svennerholm's nomenclature; h, hydroxy acid; LCB, long chain base; *m/z*, mass/charge; SM, sphingomyelin.

The authors have recently been interested in the application of DE MALDI-TOF MS to micro-analysis of glycosphingolipids, including gangliosides as well as sphingomyelin.

The sensitivity, resolution and accuracy of the new instrument of DE MALDI-TOF MS has been improved enough to detect isotopically resolved peaks of 1 pmol to about 100 pmol of glycosphingolipids. Thus, the glycosphingolipids responsible for biological function, even if present in a small amount, might be identified by DE MALDI-TOF MS which shows only their molecule-related ion peaks. Actually, we have been able to detect different molecule-related peaks of various kinds of lysoglycosphingolipids and gangliosides [1–4]. Further analyses of individual molecular species of glycosphingolipids differing in their ceramide moieties consisting of various long chain bases and fatty acids, have been attempted for the first time by DE MALDI-TOF MS. We have first analysed various gangliosides which, as compared with cerebroside, sulfatide and sphingomyelin, have a relatively simple ceramide moieties and which may be involved in structural functions and modulation in the central nervous system [4]. In the present study the long chain base composition of gangliosides determined by the DE MALDI-TOF MS analysis reconfirmed that the changes in the long chain base composition of gangliosides in mammalian and human brains do occur during the development and ageing as it has been found by other workers for sphingosine base [5–9] using a completely different analytical method.

Also, the authors have intended to reconfirm the abnormality in the sphingosine base composition of gangliosides in the brains of patients with juvenile amaurotic idiocy (Spielmeyer-Vogt's syndrome) because it was found that G_{M1} locally accumulated in the brain putamen of a patient with adult G_{M1} gangliosidosis had a normal sphingosine base composition. The DE MALDI-TOF MS has been also applied to detect individual molecular species of each kind of sphingolipid of the sphingolipid fraction of the brain total lipids of Eker rat with hereditary renal carcinoma. It has been found, in addition to identification of the individual molecular species of cerebroside, sulfatide and sphingomyelin, that a rather large amount of individual molecular species of plasmalocerebrosides, which were recently found in the white matter of human [10] and equine [11] brains, were present also in the white matter of Eker rat brain.

Thus, these findings obtained due to the application of DE MALDI-TOF MS, are presented in more detail in the present paper.

MATERIALS AND METHODS

Materials. Various lysoglycosphingolipids and glycosphingolipids, G_{M1} and other gangliosides obtained earlier from human brains at autopsy of patients with different diseases and of different age [1–4, 9, 12–14] were available for this experiment. A tissue sample of the cerebral putamen of a patient with adult G_{M1} gangliosidosis at 66y of age was kindly provided by Dr. S. Ikeda of the Department of Medicine (Neurology), Shinshu University School of Medicine [15]. Brain tissues of Eker rat with and without hereditary renal carcinoma were kindly provided by Dr. O. Hino of the Department of Experimental Pathology, Cancer Institute in Tokyo. α-Cyano-4-hydroxycinnamic acid (α-CHCA) and 2,5-dihydroxybenzoic acid (2,5-DHB), used as the matrices for DE MALDI-TOF mass spectrometry, were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Acetonitrile and trifluoroacetic acid for the solvent of the matrices were from Nacalai Tesque (Tokyo).

Preparation of sphingolipid fraction of Eker rat brain for DE MALDI-TOF mass spectrometric analysis. The brain tissues of Eker rat with and without hereditary renal

carcinoma were carefully separated into the white and grey matters. About 100 mg of the wet tissue was used for extraction of total lipid with chloroform/methanol (2:1 and 1:1, v/v). The lipid extraction was followed by the Folch's partition and the upper and the lower phases were separated. The lower phase was evaporated under N_2 gas and the lipid residue was weighed. A sample (a few milligrams) was treated with 1 ml of 0.1 M NaOH in methanol at 50°C for 30 min. After cooling, the reaction mixture was acidified with a few drops of 1 M HCl and shaken vigorously with 1 ml of hexane. After the hexane solution forming the upper phase was removed, the obtained lower phase was treated again with 1 ml of hexane. The lower phase obtained by this treatment was evaporated under N_2 gas and the residue was treated by Folch's partition to remove salt present in the aqueous upper phase. The lower phase was used as the sphingolipid fraction for DE MALDI-TOF mass spectrometric analysis.

DE MALDI-TOF mass spectrometry of sphingolipid fraction. The sphingolipid fraction, 1 μl of its solution and 1 μl of the matrix solution (10 mg of 2,5-DHB in 1 ml of 10% ethanol in water, or 10 mg of α-CHCA in 1 ml of 1:1 mixture of acetonitrile/water containing 0.1% trifluoroacetic acid) in a 1.5 ml Eppendorff tube were shaken vigorously on a vortex mixer, then spinned down on a microcentrifuge (Chibitan, Japan Millipore, Tokyo). One microliter (1–100 pmol of sphingolipids) of the mixed solution was loaded onto sample plate with 100 sample positions, then allow to dry and crystallize for about 20 min at room temperature. Then, the sample plate was loaded into the load position of Voyager Elite XL (6.6 m flight length in the reflector mode) Biospectrometry Workstation (PerSeptive Biosystem, Framingham, MA, U.S.A.). Nitrogen laser at wavelength 337 nm was used for ionization of the sample. A two-point external calibration was performed as described elsewhere [4]. The resolution of the ion peak was determined by the resolution calculator as described elsewhere [1, 4]. Five-point Savitsky-Golay smoothing was applied to each spectrum.

New determination of long chain base composition of gangliosides. In general, ceramide moieties of glycosphingolipids differ due to various combinations of long chain base and fatty acids. Thus, it is very difficult to determine the long chain base compositions from the mass spectra of the glycosphingolipids, but it is easier to determine them from the spectra of the lysoglycosphingolipids because the latter show only a few peaks corresponding to different kinds of the long chain bases [1, 2]. However, the gangliosides in general have relatively simple ceramide moieties consisting of a few long chain bases and mainly stearic acid. Thus, for the determination of the long chain bases composition of the gangliosides there is no need to prepare in advance the lysoganglioside and molecule-related and isotopically resolved peaks of ganglioside can be identified. Molecule-related and isotopically resolved peaks of d18:1 or d20:1 sphingosine containing G_{M1} were in sequence identified by monoisotopic molecular mass and molecular mass containing one, two and three of ^{13}C atoms (natural abundance of ^{13}C, 1.108%). The percentage of each peak count was calculated and recognized to be fairly identical with the theoretical value of its probability. If G_{M1} contains d18 or d20 sphinganine, the difference between the found value and theoretical one of the peak containing two ^{13}C atoms is indicative of d18 or d20 sphinganine. Thus the long chain base composition of G_{M1} is determined by the percentage of the found values (counts) of G_{M1} peaks containing d18:1, d20:1 and d20. The percentage of d18 or d20 was obtained from the difference between the probabilities (counts) of the peaks and those of the proposed only d18:1 or d20:1 containing ganglioside.

RESULTS

Changes with age in long chain base composition of gangliosides in human and mammalian brains

Various samples of G_{M1} prepared from the brain tissues of human patients with different diseases and of different age were dissolved in chloroform/methanol (1:1, v/v) at the concentration indicated and routinely subjected to DE MALDI-TOF mass spectrometric analysis. As shown in Fig. 1, an $[M-H]^-$ peak at *m/z* 1545.32 corresponded to the monoisotopic molecular mass of G_{M1} containing a ceramide moiety of d18:1 and stearic acid (C18). Another $[M-H]^-$ peak at *m/z* 1573.36 corresponded to the monoisotopic molecular mass of G_{M1} containing a ceramide moiety of d20:1 and C18. Each peak region was expanded according to the high resolution of DE MALDI-TOF MS, to detect isotopically resolved peaks as shown in Figs. 2 and 3. The counts of the isotopically resolved peaks at *m/z* 1545.32, 1546.30, 1547.32 and 1548.28 accounted for 37.7%, 30.2%, 20.6% and 11.6%, respectively, while the probabilities of the corresponding peaks were calculated as 39.9%, 32.6%, 15.7% and 5.5%. From these results, the percentages of d18:1 and d18 were assumed to be 95.1% and 4.9%, respectively. Similarly, the counts of the isotopically resolved peaks at *m/z* 1573.37, 1574.35, 1575.40 and 1576.39 accounted for 33.2%, 31.9%, 22.7% and 12.2%, while the probabilities of the corresponding peaks were calculated as 39.0%, 32.8%, 16.1% and 5.8%. Thus, the percentages of d20:1 and d20 were assumed to be 93.4% and 6.6%, respectively. As a result, the long chain base composition of G_{M1} obtained from the brain of a 48 year old patient with Sudanophilic leukodystrophy was determined to be d18:1 (44.2%), d18 (2.3%), d20:1 (49.9%) and d20 (3.6%). In the same way, other samples of G_{M1} obtained from the brain tissues of patients with different diseases at different age were analysed by DE MALDI-TOF MS and the analytical results are shown in Table 1. The new method for the determination of the long chain base composition of G_{M1} reconfirmed with certainty that the proportion of d20:1 and d20 in G_{M1} increased with age [5, 6, 9]. A long chain base composition of G_{M1} from a patient with infantile type of G_{M1}-gangliosidosis seemed to be abnormal as compared with those of other neonatal and infantile human brains. Also, the long chain base compositions of G_{M1} from

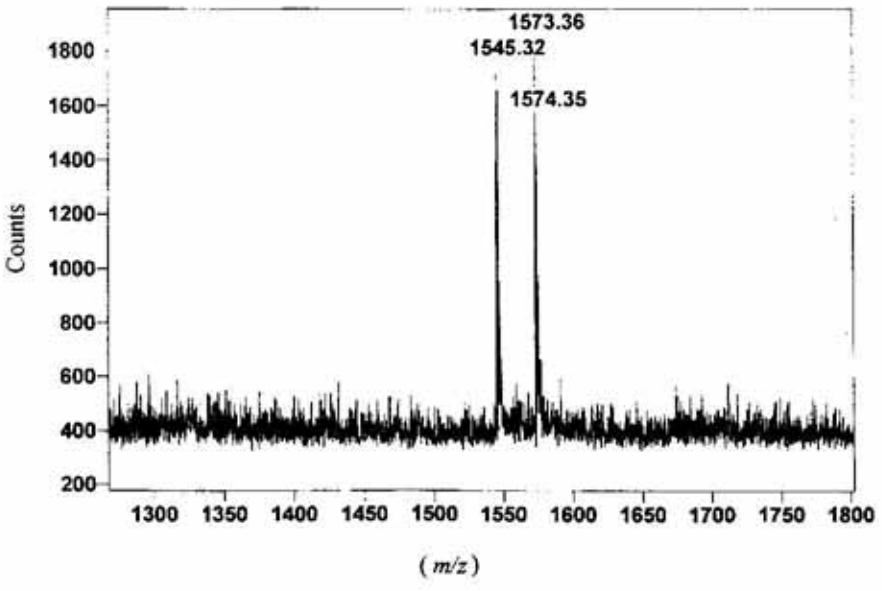

Figure 1. DE MALDI-TOF MS spectrum of G_{M1} obtained from the brain of a patient with Sudanophilic leukodystrophy at 48y of age in the reflector; negative ion mode with 2,5-DHB used as the matrix.

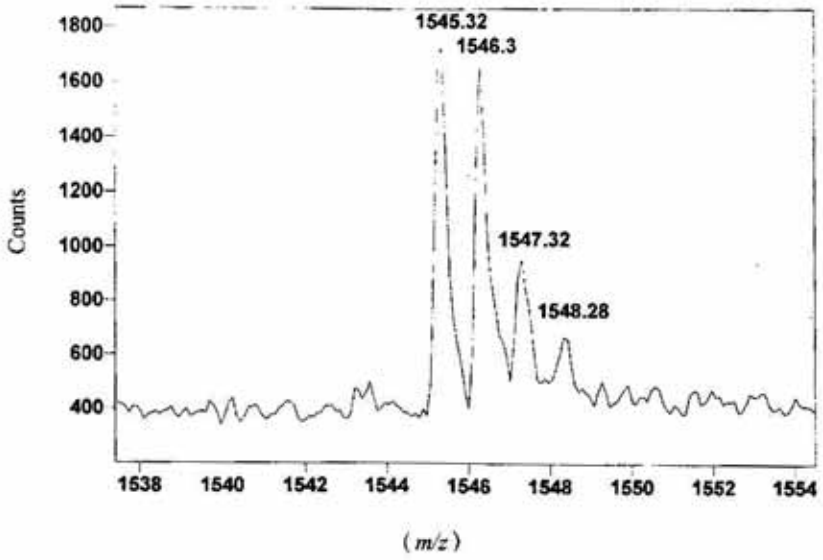

Figure 2. An expansion of the spectrum in the region of the peak at *m/z* 1545.32 in Fig. 1.

two patients as sisters with Spielmeyer-Vogt type of juvenile amaurotic idiocy seemed to be abnormal as compared with those of other adult human brains. However, G_{M1} from a patient with adult type of G_{M1} gangliosidosis at 66y [15] showed rather a normal long chain base composition as compared with those of other adult human brains. It was also noted that long chain base composition of G_{M1} from two patients (sister) with Spielmeyer-Vogt's disease at 19y and 21y of age [12] was very similar to that of G_{M1} from a patient with Gaucher's disease at 1y7m of age, whereas G_{M1} from a patient with adult G_{M1} gangliosidosis at 66y of age [15] showed an almost normal long chain base composition as found in adult human brains. On the other hand, each G_{M1} from the brain tissues of various adult mammals showed a relatively similar long chain base composition, which was significantly different from those of neonatal, infantile and adult human brains, but seemed to be rather similar to that of Spielmeyer-Vogt type of juvenile amaurotic idiocy.

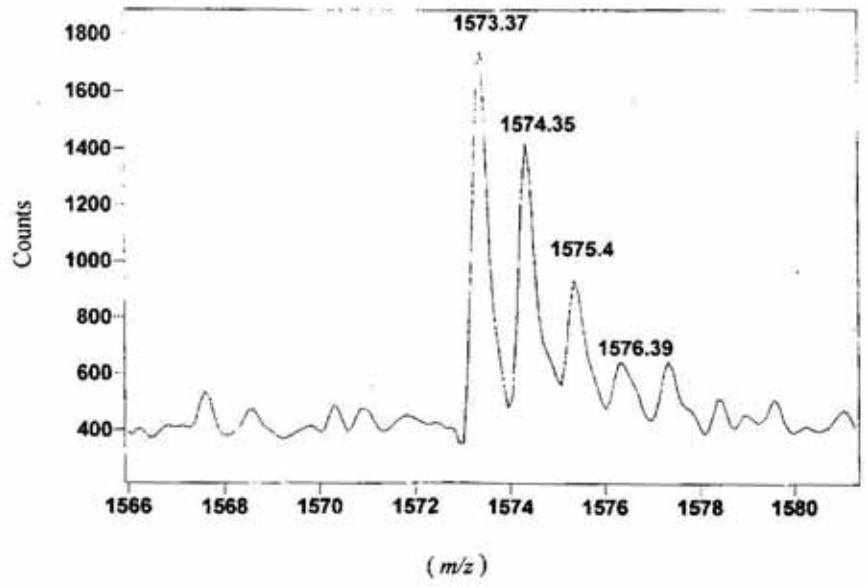

Figure 3. An expansion of the spectrum in the region of the peak at *m/z* 1573.37 in Fig. 1.

Table 1. Long chain base (LCB) composition (%) of brain G_{M1} from patients with different diseases at different age, and from adult mammals

	Patients									
Age/ LCB	2m*	5m	1y7m	4y2m	19y	21y	45y	48y	53y	66y
d18:1	79.3	68.6	56.8	76.3	57.8	54.9	47.9	44.2	43.9	42.1
d18	2.7	3.6	3.9	3.0	3.1	2.5	3.1	2.3	2.1	4.2
d20:1	16.4	26.3	36.2	20.4	37.3	40.3	45.1	49.9	51.0	49.0
d20	1.6	1.5	3.1	0.3	1.8	2.3	3.9	3.6	3.0	4.7

*2m: edema; 5m: nuclear jaundice; 1y7m: Gaucher's disease; 4y2m: infantile type of G_{M1}-gangliosidosis; 19y and 21y: Spielmeyer-Vogt's syndrome; 45y: Schilder's disease; 48y: Sudanophilic leukodystrophy; 53y: schizophrenia; 66y: adult G_{M1} gangliosidosis.

	Monkey	Dog	Bear	Pig	Rabbit
d18:1	52.9	54.6	61.4	58.4	51.4
d18	3.9	4.4	4.8	4.1	5.1
d20:1	39.1	38.0	31.5	35.6	40.5
d20	4.1	3.0	2.2	1.9	3.0

DE MALDI-TOF mass spectrometric analysis of sphingolipid fraction of Eker rat brain

Alkali-stable sphingolipid fractions from total lipids of the white matter and the grey matter of the brain tissues of Eker rat with or without hereditary renal carcinoma were tentatively subjected to DE MALDI-TOF mass spectrometric analysis because individual molecular species of each different glycosphingolipid and sphingomyelin could be identified by DE MALDI-TOF MS (not shown) and because the mixed sample of different gangliosides was amenable to their identification by DE MALDI-TOF MS [4]. As shown in Fig. 4, the patterns of mass spectra of the sphingolipid fractions were found to be different depending on the sources of the brain tissues. Then, four different regions of the spectrum were expanded to detect the individual molecular species of various sphingolipids as shown in Figs. 5 and 6. Each ion peak corresponding to monoisotopic molecular mass of cerebrosides and sphingomyelins containing different ceramide moieties by combination of different long chain bases and fatty acids could be identified in the brain tissues of Eker rats (Fig. 5). While, as shown in Fig. 6, all ion peaks corresponding to monoisotopic molecular mass of plasmalocerebroside containing both different ceramide moieties and different fatty aldehydes linked to galactose moiety by a cyclic acetal linkage proposed by Levery *et al.* [10] and Yachida *et al.* [11], were detected only in the white matter of the brain tissue of Eker rat with hereditary renal carcinoma. As shown in Fig. 4, the white matter of the brain tissue of Eker rat without hereditary renal carcinoma analysed, as a control, seemed to contain much less of the plasmalocerebroside than the Eker rat with hereditary renal carcinoma, whereas the grey matter of the Eker rat with hereditary renal carcinoma had almost no plasmalocerebroside. Although the white matter of Eker rat with hereditary

renal carcinoma contained a fairly larger amount of plasmalocerebroside as compared to cerebroside, sphingomyelin and sulfatide (not shown), it had no plasmalopsychosine which was found in the human brain by Nudelman *et al.* [16].

DISCUSSION

The determination of long chain base composition of glycosphingolipids including gangliosides has been attempted by DE MALDI-TOF MS analysis. The counts of molecule-

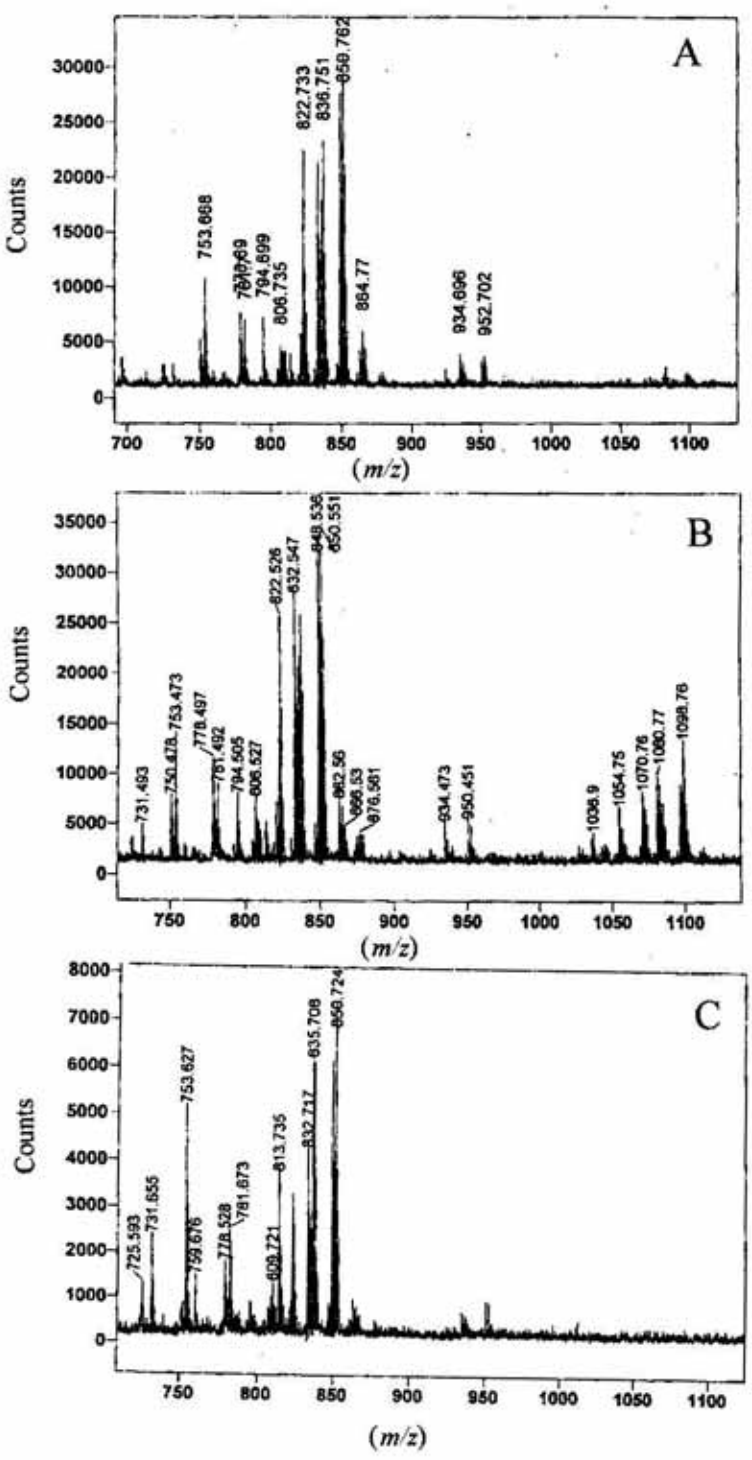

Figure 4. DE MALDI-TOF MS spectra of sphingolipid fraction of total lipids from the white matter of the brain tissue of Eker rat without hereditary renal carcinoma as a control (A), and from white matter of the brain tissue of Eker rat with hereditary renal carcinoma (B) and (C) grey matter in the reflector, positive ion mode with 2,5-DHB used as the matrix.

Accelerating voltage: 20000, grid voltage: 74% of the accelerating voltage, guide wire voltage: 0.05% of the accelerating voltage, delay: 100 ns, laser step: 2100, scans averaged: 128.

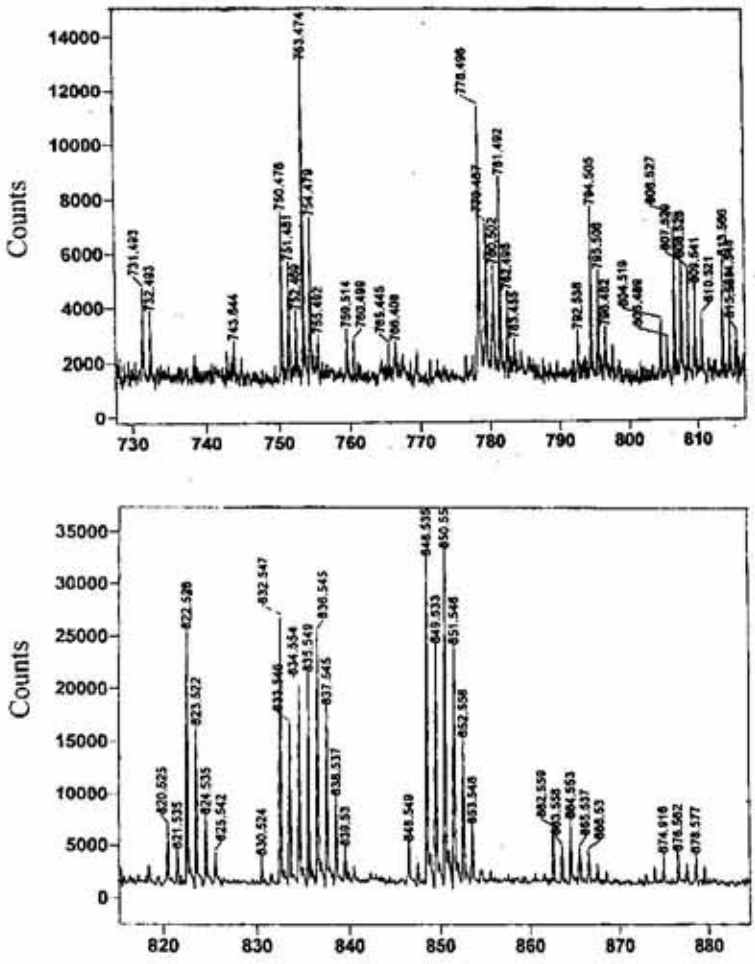

Figure 5. Legend on the next page.

related and isotopically resolved peaks of either lysoglycosphingolipids prepared from the glycosphingolipids or gangliosides themselves without any chemical modifications were found to lead to the determination of the long chain base composition. This method is completely different from the usual methods in which the sphingosine bases are produced by acidic methanolysis of a glycosphingolipid and analysed as the trimethylsilyl derivatives by GLC [5, 12], or the sphingosine bases are transformed to the fatty aldehydes by periodate oxidation or ozonolysis and analysed by GLC [6, 9]. The disadvantages of the usual methods are that they lead to the formation of by-products such as *O*-methyl-sphingosine and *threo*-sphingosine [17, 18], or do not permit to analyse sphinganine. The authors have been interested in the changes occurring with age in the long chain base composition of G_{M1} as the main ganglioside in the central nervous system. The changes of d20:1 and d20 of G_{M1} in human brains proceeded quickly to reach the proportions of about 40% which is similar to those in adult mammalian brains. However, the proportions of d20:1 and d20 of G_{M1} in the adult mammalian brains seem to be genetically constant and never exceed the proportion of 50% found in adult human brains. When changes in the enzyme 3-ketosphiganine synthase activity with palmitoyl-CoA and stearoyl-CoA in rat cerebellar granule in culture were recently studied [19], it was reported that the enzyme activity was higher

Figure 5. An expansion of the spectrum in the region between the peak at *m/z* 731 and the peak at *m/z* 813 (upper) and in the region between the peak at *m/z* 820 and the peak at *m/z* 878 (lower). Peaks in the upper and lower panels were identified as follows:

Peaks (upper panel)			
Monoisotopic mass (*m/z*)	Sphingolipid	LCB	Fatty acid
731.49	SM $[M+H]^+$	d18:1	C18
743.84	CMH $[M+H]^+$	d18:1	C18h
750.48	CMH $[M+Na]^+$	d18:1	C18
753.47	SM $[M+Na]^+$	d18:1	C18
759.51	SM $[M+H]^+$	d18:1	C20
765.45	CMH $[M+Na]^+$	d18:1	C18h
778.50	CMH $[M+Na]^+$	d18:1	C20
781.49	SM $[M+Na]^+$	d18:1	C20
794.51	CMH $[M+Na]^+$	d18:1	C20h
804.52	CMH $[M+Na]^+$	d18:1	C22:1
806.53	CMH $[M+Na]^+$	d18:1	C22
808.53	CMH $[M+Na]^+$	d18	C22
813.57	SM $[M+H]^+$	d18:1	C24:1
Peaks (lower panel)			
Monoisotopic mass (*m/z*)	Sphingolipid	LCB	Fatty acid
820.53	CMH $[M+Na]^+$	d18:1	C23
822.53	CMH $[M+Na]^+$	d18:1	C22h
832.55	CMH $[M+Na]^+$	d18:1	C24:1
834.55	CMH $[M+Na]^+$	d18:1	C24
835.55	SM $[M+Na]^+$	d18:1	C24:1
836.55	CMH $[M+Na]^+$	d18:1	C23h
837.55	SM $[M+Na]^+$	d18:1	C24
846.55	CMH $[M+Na]^+$	d18:1	C25:1
848.54	CMH $[M+Na]^+$	d18:1	C25
850.55	CMH $[M+Na]^+$	d18:1	C24h
	CMH $[M+Na]^+$	d18	C25
862.56	CMH $[M+Na]^+$	d18:1	C26
	CMH $[M+Na]^+$	d18	C24h
864.55	CMH $[M+Na]^+$	d18:1	C25h
	CMH $[M+Na]^+$	d18	C26
866.53	CMH $[M+Na]^+$	d18	C25h
876.92	CMH $[M+Na]^+$	d18:1	C27
878.58	CMH $[M+Na]^+$	d18	C27

SM, sphingomyelin; CMH, cerebroside; LCB, long chain base; h, hydroxy acid.

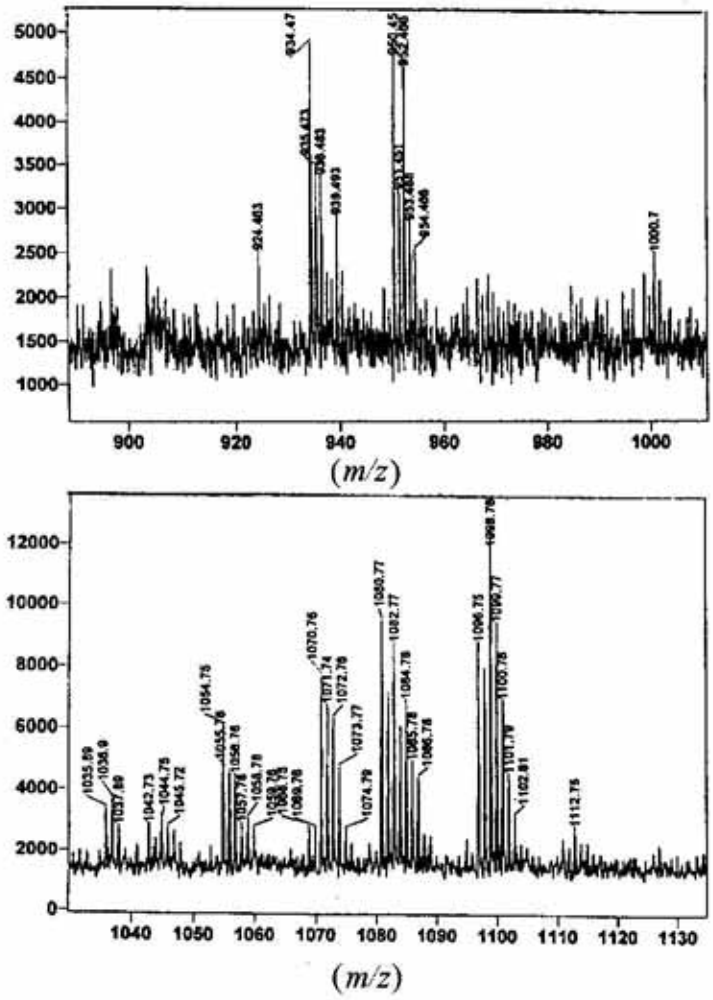

Figure 6. An expansion of the spectrum in the region between the peaks at *m/z* 900 and *m/z* 1000 (upper) and in the region between the peaks at *m/z* 1035 and 1130 (lower) in Fig. 4. Peaks in the upper and lower panels were identified as follows:

Peaks (upper panel)				
Monoisotopic mass (*m/z*)	Plasmalocerebroside	LCB	Fatty acid	Fatty aldehyde
934.47	$[M+H]^+$	d18:1	C16	C17:1
936.48	$[M+H]^+$	d18:1	C16	C17
950.45	$[M+H]^+$	d18	C16	C18:1
952.47	$[M+H]^+$	d18	C16	C18
Peaks (lower panel)				
Monoisotopic mass (*m/z*)	Plasmalocerebroside	LCB	Fatty acid	Fatty aldehyde
1054.75	$[M+Na]^+$	d18:1	C24:1	C16
1956.76	$[M+Na]^+$	d18:1	C24	C16
1058.78	$[M+Na]^+$	d18	C24	C16
1070.76	$[M+Na]^+$	d18:1	C24:1h	C16
1080.77	$[M+Na]^+$	d18:1	C24:1	C18:1
1082.77	$[M+Na]^+$	d18:1	C24:1	C18
1084.78	$[M+Na]^+$	d18:1	C24	C18
1086.78	$[M+Na]^+$	d18	C24	C18
1096.75	$[M+Na]^+$	d18:1	C24:1h	C18:1
1098.76	$[M+Na]^+$	d18:1	C24:1h	C18

For abbreviations see Fig. 5.

with palmitoyl-CoA than with stearoyl-CoA and changed during cell differentiation and the aging processes. It seems that the differences in the proportions of d20:1 and d20 of G_{M1} between human and animal brains are significant and conceivably, could be rather due to a genetic factor. The rapid increase in the proportions of d20:1 and d20 of G_{M1} until adolescent or adult age suggests that the long chain bases of gangliosides are important both because of their own roles in membrane structural functions and/or as modulators of signal transduction in adult human brains. A patient with the infantile type of G_{M1}-gangliosidosis showed abnormal accumulation of G_{M1} in the brain and other organ tissues due to the deficiency of β-galactosidase [14], and the long chain base composition of G_{M1} in the brain tissue seemed to be a little abnormal. Two patients with Spielmeyer-Vogt type of juvenile amaurotic idiocy showed no accumulation of G_{M1} in the brain tissue [12], but the long chain base compositions of G_{M1} seemed to be abnormal as already reported elsewhere [5, 12].

However, although a patient with adult type of G_{M1}-gangliosidosis showed a local accumulation of G_{M1} in the putamen and caudate nucleus [15], the long chain base compositions of G_{M1} was normal as compared with those of other human brains. As a conclusion, the abnormal long chain base composition of G_{M1} in the neonatal and infantile human brains may be influenced by the accumulation of G_{M1} as seen in the infantile type of G_{M1}-gangliosidosis, whereas the Spielmeyer-Vogt type of juvenile amaurotic idiocy may reflect an abnormal metabolism of the long chain bases of G_{M1} as judged by the change of their compositions with age [5, 6].

The authors are interested in DE MALDI-TOF MS analysis of the alkali-stable sphingolipid fraction because only the white matter of Eker rat with hereditary renal carcinoma showed a large amount of plasmalocerebroside as compared to cerebroside and sphingomyelin. Also, like the findings on the individual molecular species of cerebroside, sphingomyelin and sulfatide of the sphingolipid fraction, it is of importance that the individual molecular species of main plasmalocerebroside obtained from small amounts of brain tissue samples could be identified. However, we were unable to find plasmalopsychosine which was reported to occur in much larger amounts than the plasmalocerebrosides [15]. As it is well known that the Eker rat family shows a variety of phenotypes of tuberous sclerosis (personal communication with Dr. O. Hino), it may be suggest that the increase in plasmalocerebroside is related probably to the tuberous sclerosis in the brain tissue of Eker rat with hereditary renal carcinoma. We expect that Dr. O. Hino will publish elsewhere these results in more detail.

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