Presence of an Unusual GM2 Derivative, Taurine-conjugated GM2, in Tay-Sachs Brain*

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Tay-Sachs disease (TSD) is a classical glycosphingolipid (GSL) storage disease. Although the genetic and biochemical bases for a massive cerebral accumulation of ganglioside GM2 in TSD have been well established, the mechanism for the neural dysfunction in TSD remains elusive. Upon analysis of GSLs from a variant B TS brain, we have detected a novel GSL that has not been previously revealed. We have isolated this GSL in pure form. Using NMR spectroscopy, mass spectrometry, and chemical synthesis, the structure of this unusual GSL was established to be a taurine-conjugated GM2 (tauro-GM2) in which the carboxyl group of N-acetylenuraminic acid was amidated by taurine. Using a rabbit anti-tauro-GM2 serum, we also detected the presence of tauro-GM2 in three other small brain samples from one variant B and two variant O TSD patients. On the other hand, tauro-GM2 was not found in three normal human brain samples. The presence of tauro-GM2 in TS brains, but not in normal brains, indicates the possible association of this unusual GM2 derivative with the pathogenesis of TSD. Our findings point to taurine conjugation as a heretofore unelucidated mechanism for TS brain to cope with water-insoluble GM2.

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§The abbreviations used are: TSD, Tay-Sachs disease; GSL, glycosphingolipid; UC, unknown compound; HPAC, high performance anion exchange chromatography; HMBCC, heteronuclear multiple bond connectivity; HSQC, 1H-detected hetero-nuclear single quantum coherence; ROESY, rotating frame nuclear Overhauser effect spectroscopy; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; DPA, diphenylamine-aniline-phosphoric acid; GM1, Galβ1→3GalNAcβ1→4Neu5Acα2→3Galβ1→4Glc1→1′-Cer; GM2, GalNAcβ1→4Neu5Acα2→3Galβ1→4Glc1→1′-Cer; GM3, Neu5Acα2→3Galβ1→4Glc1β1→1′-Cer; GM4, Neu5Acα2→3Galβ1→4Glc1β1→1′-Cer; GM5, Neu5Acα2→3Galβ1→4Glc1β1→1′-Cer; tauro-GM2, 2-aminoethanesulfonic acid.

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Preparation of Rabbit Antiserum against Tauro-GM2—Production of rabbit antiserum against tauro-GM2 was carried out by the Core Facility of Louisiana State University Health Sciences Center, New Orleans, LA, according to the following protocol. Chemically synthesized tauro-GM2 (0.5 mg) and methylalbumin (0.5 mg) in 0.6 ml of water were emulsified with an equal volume of complete Freund’s adjuvant. One-half of this emulsion was injected into the front pads of an adult male New Zealand White rabbit (2.5 kg), and the remainder was injected into the back of the rabbit at 3 sites. The same procedure was repeated at 10, 25, and 39 days after the initial injection. The antiserum was harvested 2 weeks after the last injection. In parallel, the same protocol was used to immunize a rabbit with GM2. The Ouchterlony double-diffusion method (23) was used to detect the production of anti-tauro-GM2 antibody. Under these conditions, tauro-GM2, but not GM2, elicited the antibody production in the rabbit, indicating that tauro-GM2 is more immunogenic to rabbit than GM2.

By the Ouchterlony double-diffusion method (23), the rabbit anti-tauro-GM2 serum did not cross-react with GM1, GM2, GM3, GD1a, and sulfatide (see Fig. 6).

TLC Immunostaining—The specific binding of rabbit anti-tauro-GM2 antibody to different gangliosides was carried out by overlaying the thin layer chromatograms with the rabbit anti-tauro-GM2 serum (1:20 dilution), followed by reacting with peroxidase-conjugated goat anti-rabbit IgG (ICN Biochemicals, Irvine, CA). The antibody binding was revealed with a substrate solution containing 3 mg of 4-chloro-1-naphthol dissolved in 1 ml of methanol, 4 ml of 25 mM Tris-HCl buffer, pH 7.4, 75 mM NaCl, and 2 μl of 30% H2O2 as described by Magnani et al. (24), except that the peroxidase-conjugated second antibody was used instead of radiodinated second antibody and autoradiography.

Partial Purification of Selgenic GSLs from Small Pathological Brain Samples—For the detection of the presence of tauro-GM2 in small pathological brain samples by TLC immunostaining, monosialogangliosides from 1 to 2 g of brain samples were prepared separately as described (3). Tauro-GM2 in the ganglioside mixture was subsequently enriched by HPAC (7).

RESULTS AND DISCUSSION

Detection and Isolation of a UC from a Variant B TS Brain Sample—Fig. 1A shows the fractionation of the crude GSL extract prepared from a variant B TS brain sample by Q-Sepha-rose chromatography (4). TLC analysis of the column fractions revealed that GM2, the major ganglioside, had eluted over a wide range of fractions (Fig. 1A, fractions 80–180). Although the fractions two-thirds of these fractions (Fig. 1A, fractions 80–147) contained mainly GM2, the latter fractions (Fig. 1A, fractions 148–180) contained 1–2 GSLs with TLC mobilities slightly faster than that of GM2. Because the TLC mobilities of these two fast moving GSLs were very close to those of GM3 (see Fig. 1A), and the level of GM3 had been reported to be also elevated in TS brains (25–29), we initially regarded them as a doublet of GM3. However, clostridial sialidase (Sigma) converted only about 30% of these fast moving bands to lactosylceramide, indicating that these bands contained a UC other than GM3. Furthermore, the fast moving bands gave a greenish-gray color in response to the DPA spray (see Fig. 2, A and B), which was distinct from the purple color typically given by sialoglycoconjugates such as gangliosides (5). Fractions 148–180 (containing UC, shown in Fig. 1A) were pooled and subsequently fractionated by high resolution silicic acid (Iatrobeads, Iatron Laboratory) chromatography (6). As shown in Fig. 1B, UC was well separated from GM2 by this procedure. The UC-containing fractions (Fig. 1B, fractions 50–64) were pooled and further purified by HPAC (7) to obtain the pure UC. Through these purification steps, we obtained about 0.8 mg of pure UC from 75 g of wet brain sample. Using the same procedure, we did not detect the presence of UC in a normal brain sample (100 g) from a 4-year-old boy. The pure UC migrated as a single band by TLC (see Fig. 2B, lane 3).

Structural Characterization of UC by NMR Spectroscopy—We have assigned the NMR chemical shifts of 1H, 13C, and 15N for the sugar chain of UC and GM2 isolated from TS brain (Table I). The 1H–1H double-quantum filtered correlation spectroscopy and total correlation spectroscopy spectra of UC revealed the proton relay signals corresponding to seven spin systems. The HMBC and ROESY two-dimensional NMR experiments supplied definitive structural information regarding the interconnection of the seven spin systems and provided an overall view of the structure. Analysis of HSQC and HMBC spectra showed that, as in the case of GM2, the ceramide (Cer) of UC contained two spin systems: the sphingosine and the fatty acyl amide. In addition to the four spin systems found in GM2 (Neu5Ac, Gal, GalNAc, and Glic), the sugar chain of UC contained a fifth spin system, 2-aminoethanesulfonic acid (taurine). The assignment of the sugar sequence was based on the analysis of ROESY and HMBC. In the ROESY spectrum, the anomeric proton of β-Glc (4.156 ppm; 1J_HH = 7.8 Hz; 1J_CH =
Several lines of evidence indicate the presence of an amide bond between the carboxyl group of Neu5Ac and a taurine residue in UC. In the HMBC spectrum (Fig. 3), we detected the two-bond correlation between the taurine amide proton (3.459 ppm) and the H3 proton (3.898 ppm) of Neu5Ac and also the three-bond correlation between the more upfield aliphatic proton on C1 of taurine (3.342 ppm) and the C1 carbon (168.55 ppm) of Neu5Ac. In the ROESY spectrum, we see the following inter-residue interactions: taurine-NH/Neu5Ac-NH, taurine-NH/Neu5Ac-OH8, taurine-NH/Neu5Ac-H4, and taurine-H2/Neu5Ac-OH8. Diffusion coefficient measurements (22) provide additional evidence showing that taurine is a part of UC. The value of the translational diffusion coefficient of UC, calculated from the well separated signal (2.700 ppm of C2 methylene protons of taurine), is identical to the value obtained for any other proton on the sugar chain (D = 1.05e-10 m2/s). These results show that taurine is an integral part of the molecule and establish the structure of UC as tauro-GM2 (see Fig. 5A for the structure).

Analysis of UC by Electrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS)—Subsequently, we used mass spectrometry to ascertain the presence of a conjugated taurine in UC. By FT-ICR-MS, the protonated GM2 isolated from the variant B TS brain sample was detected at m/z 1384.801 (Fig. 4A, peak a), which corresponds to the
calculated monoisotopic m/z 1384.832 for the GM2 containing octadecasphingenine and stearic acid. In addition, several peaks separated by 26 Da (representing an additional \(-CH=CH–\) moiety) or 28 Da (representing an additional \(-CH_2–\) moiety) appeared in the mass spectrum, indicating the homogeneity and the complexity of the ceramide moiety in GM2. The assignments for the major monoisotopic mass peaks shown in Fig. 4A (peaks b–f) are presented in the legend to this figure. On the other hand, the protonated molecule of UC was detected at m/z 1491.855 (Fig. 4B, peak a). This value corresponds to the calculated monoisotopic m/z 1491.836 for the protonated GM2 (containing octadecasphingenine and stearic acid) conjugated with one residue of taurine. The FT-ICR mass spectrum of UC also contained sodium adducts, sodium/proton substitutions, dimers, and peaks corresponding to heterogeneous varieties with different chain lengths of the long chain base and fatty acid (peaks b–h), analogous to the variation found in Fig. 4A for GM2. The assignments of these major monoisotopic mass peaks are presented in the legend to Fig. 4B. Thus, the FT-ICR-MS confirmed the conjugation of taurine to GM2 in UC.

**Chemical Synthesis of Tauro-GM2**—To obtain additional evidence that the structure of UC is tauro-GM2, we chemically conjugated the carboxyl group of Neu5Ac in GM2 with taurine using diethyl phosphorocyanidate as a coupling reagent in the presence of triethylamine as a catalyst (8, 9) (see Fig. 2A, lane 2). As shown in Table I and Fig. 5B, the NMR chemical shifts of the sugar residues in the chemically synthesized tauro-GM2 were identical to those found in UC. Fig. 2B shows that the chemically synthesized tauro-GM2 and UC have the same TLC mobility and color response to the DPA reagent (5). The TLC mobility of tauro-GM2 is faster than GM2 but only slightly slower than GM3 (Fig. 2B).

**Immunological Detection of the Presence of Tauro-GM2 in Small TS Brain Samples**—Because other TS brain samples were available to us only in small quantities (1–2 g), we used a sensitive TLC immunostaining method for the detection of the presence of tauro-GM2 in these samples. As shown in Fig. 6B, the monosialoganglioside fraction obtained from one more variant B and two variant O TS patients also contained a GSL that interacted with the rabbit anti-tauro-GM2 serum. This GSL and the chemically synthesized tauro-GM2 shared the same TLC mobility and color response to the DPA spray (5). Under the same condition, we did not detect any GSL that interacted with the rabbit anti-tauro-GM2 serum in the corresponding fraction prepared from three normal human brain samples. From Fig. 6, one can also see that the anti-tauro-GM2 serum did not cross-react with GM1, GM2, GM3, or other gangliosides.

Taken together, we have used NMR spectroscopy, FT-ICR-MS, chemical synthesis, and TLC immunostaining to conclusively show the presence of tauro-GM2 in TS brain samples. Our results indicate that previous reports of the elevation of GM3 in TS brain samples (25–29), based primarily on TLC analysis, may instead be due to the presence of tauro-GM2, because the TLC mobility of tauro-GM2 is very close to that of GM3 (see Fig. 2B).
from the body by increasing their polarity and aqueous solubility (34). GM2 is very insoluble in water (35). The pK<sub>a</sub> of the carboxylic acid of Neu5Ac is 2.6 (36), whereas that of the sulffonic acid of taurine has been reported to be between 1 and 1.5 (37, 38). Thus, tauro-GM2 would be more polar and more water-soluble than GM2. The level of GM2 in a normal infant brain has been found to be around 19 nmol/g of wet tissue, whereas that in a TS brain could exceed 1000 nmol/g of wet tissue (39). Neural tissues may regard this massively elevated GM2 as a quasi-xenobiotic and employ taurine conjugation as a vehicle for its removal. However, the low pK<sub>a</sub> of taurine makes tauro-GM2 a potential surfactant that may exert an adverse effect on neural tissues. It is well known that the toxicity of a xenobiotic can be activated upon conjugation (40). Although enzymes for detoxication in the central nervous system have been studied extensively (41), the enzyme responsible for the taurine conjugation of GM2 has not yet been identified, and the possible pathophysiological effects of tauro-GM2 on the central nervous system remain to be elucidated. It should be pointed out that the taurine-conjugated Neu5Ac is a novel sialic acid derivative that has not been previously revealed. Tauro-GM2 represents an additional pathological product of TSD and should be taken into consideration in the future development of therapies for this dreadful disease.

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FIG. 5. The chemical structure of tauro-GM2 (A) and an overlay of proton-detected 13C–1H HSQC spectra of the isolated (in gray) and the synthetic (cyan) tauro-GM2 (B). Arabic numerals refer to the protons of the residues designated by the Roman numerals, and the protons of Neu5Ac (N), taurine (T), and the sphenigine backbone in Cer (S).

FIG. 6. Immunological detection of tauro-GM2 in three TS brain samples. Monosialoligosidase fractions prepared from three normal brain samples (lane 1, 4-year-old male; lane 2, 3-year-old male; lane 3, 20-year-old female) and three TS brain samples (lane 4, variant O, 2-year-old male; lane 5, variant B, 5-year-old female; lane 6, variant O, 3-year-old male) were separated by TLC and chemically stained by DPA reagent (A) or immunostained with rabbit anti-tauro-GM2 serum (B). Approximately 3–5 µg of GSLs were spotted on the TLC plate. A and B were initially developed as a single plate. St, standard GM1, GM2, and GM3; TM2, chemically synthesized tauro-GM2. The detailed conditions are described under “Experimental Procedures.”

Taurine, one of the most abundant free amino acids found in the human central nervous system (31–33), has been shown to serve a wide variety of biological functions, including bile acid and xenobiotic conjugation, osmoregulation, and calcium modulation (34). Taurine conjugation is a well known mechanism in biological systems that facilitates the clearance of xenobiotics.
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