Isolation and Characterization of a Novel Monosialosylpentahexosyl Ceramide from Tay-Sachs Brain*

Toshihiro Itoh†§, Yu-Teh Li‡, Su-Chen Li‡, and Robert K. Yu¶

From the †Department of Neurology, Yale University School of Medicine, New Haven, Connecticut 06510 and the ‡Department of Biochemistry, Tulane University, School of Medicine, New Orleans, Louisiana 70112

A novel monosialosylganglioside was isolated from Tay-Sachs brains. It represented about 0.1% of the total ganglioside mixture. Compositional analysis by gas-liquid chromatography indicated that it contained glucose, galactose, N-acetylgalactosamine, N-acetyl-neuraminic acid, and long chain base in the molar ratio of 1:2:2:1:1. The ganglioside was found to be resistant to neuraminidase (Clostridium perfringens), β-hexosaminidase (jack bean), and β-galactosidase. However, it could be degraded by a human liver β-hexosaminidase preparation in the presence of an activator to produce a glycolipid chromatographically identical with authentic IV\NeuAc-GgOse4-ceramide. This glycolipid product was resistant to β-galactosidase (jack bean), but could be readily degraded to GgOse4-ceramide by neuraminidase. Mild formic acid hydrolysis degraded the intact ganglioside to an asialo derivative chromatographically identical with the pentahexosyl ceramide (GalNAc-Gal-GalNAc-Gal-Glc-ceramide) derived from Gd\GalNAc. The asialo derivative could then be degraded to GgOse4-ceramide and GgOse3-ceramide by sequential treatment with jack bean β-hexosaminidase and β-galactosidase. These data suggest that the novel ganglioside is a monosialosylpentahexosyl ceramide with the sialosyl group attached to the penultimate galactose moiety of the pentahexosyl ceramide backbone, and it has the following structure:

\[
\text{GalNAc}(\beta 1-4)\text{Gal}(\beta 1-3)\text{GalNAc}(\beta 1-4)\text{Gal}(\beta 1-4)\text{Glc-ceramide} \\
3 \text{ NeuAc}
\]

 Tay-Sachs disease (variant B of infantile GM2 gangliosidosis) is a well known inborn disorder affecting the nervous system. Biochemically, it is characterized by the accumulation of GM2 ganglioside in the nerve tissue as the result of a deficiency of the isoenzyme hexosaminidase A (4). Although the brain ganglioside composition of Tay-Sachs disease has been the subject of extensive studies in the past (2-8), relatively little attention has been directed toward minor gangliosides other than GM2. In the course of isolating large quantities of GM2, we examined in detail other gangliosides in brains of Tay-Sachs patients and found the accumulation of several other minor gangliosides. The accumulated minor gangliosides include GM\~, GD\~, GD\~, and GD\~\~. In addition, we found that the concentrations of GM\~, GM\~, GD\~, GD\~, GD\~, and GD\~ were reduced compared with normal human brain. These results were nearly identical to those reported independently by Iwamori and Nagai (10) for another case of Tay-Sachs disease. In addition to the above abnormalities in ganglioside composition, we have isolated a novel monosialosylpentahexosyl ceramide from the brains of three cases of Tay-Sachs disease. This paper describes the isolation and structural analysis of this novel ganglioside.

EXPERIMENTAL PROCEDURES

Materials—Tay-Sachs brains were obtained from Dr. Edwin Koldony (E. K. Shriver Center, Walhama, MA), Dr. Robert Ledeen (Albert Einstein College of Medicine, Bronx, NY), and Dr. E. E. Manuelidis (School of Medicine, Yale University, New Haven, CT). The brains were stored at −70°C for 5 to 6 months to 2 years before use. The organic solvents were of reagent grade and were freshly distilled before use. Other organic and inorganic reagents, of analytical grade or of the highest quality commercially available, were used without further purification. All authentic gangliosides were prepared in our laboratories as described previously (11). GM\~, isolated from rat ascites hepatoma (12) was a gift from Dr. Y. Hirabayashi.

Extraction and Purification of Gangliosides—The brain ganglioside mixture was obtained by a modification of the procedure of Ledeen et al. (13). A Tay-Sachs brain sample (about 50 g wet weight) was homogenized with 15 volumes of chloroform/methanol (1:1). After filtration to remove the insoluble residue, the solvent of the lipid extract was dried using a rotary evaporator. The total lipid was redisolved in 200 ml of chloroform/methanol/water (30:60:8) and applied to a column (50 x 300 mm) of DEAE-Sephadex (A-25, acetate form). After the neutral lipids were eluted with 500 ml of chloroform/methanol/water (30:60:8), the acidic lipids were eluted with 1000 ml of chloroform/methanol/0.8 M sodium acetate (30:60:8). After the solvent was removed by evaporation, the acidic lipid fraction was treated with 0.5 M NaOH in methanol for 60 min at 37°C. The solution was neutralized with acetic acid, evaporated to near dryness, and then dissolved in 100 ml of water. The solution was dialyzed against 1000 volumes of water for 24 h.

*For publication, August 8, 1980
against distilled water and lyophilized to dryness. The residue was dissolved in 20 ml of chloroform/methanol (95:5) and the solution was applied to a column (15 x 300 mm) packed with Iatrobeads (Iatron, Tokyo).

After free fatty acids, fatty acid methylesters, and sulfatides were eluted with 290 ml of chloroform/methanol (4:1), the ganglioside mixture in 300 ml of chloroform/methanol (1:1) was added (15). After the solvent was evaporated, the ganglioside mixture was redissolved in 300 ml of methanol and applied to a DEAE-Sephacel (acacetate form) column (16 x 1050 mm; bed volume, 260 ml). The column was washed with 200 ml of methanol. Gangliosides were sequentially eluted with a linear gradient with increasing concentration of sodium acetate (0 to 0.5 M) in methanol (total volume, 2100 ml). Fractions of 15 ml each were collected (Fig. 1). Monosialogangliosides were recovered in the first peak. Disialogangliosides were separated into three peaks. Triasialogangliosides and tetrasialogangliosides were recovered in the fifth peak and sixth peak fractions, respectively. The monosialoganglioside fraction was then reapplied to an Iatrobeads column (19 x 1114 mm; bed volume, 350 ml), and eluted with a linear gradient system prepared from a first solvent mixture of chloroform/methanol/water (70:30:2) and a second solvent mixture of chloroform/methanol/water (30:60:4). The total volume of the eluting solvent was 1500 ml. Fractions of 15 ml each were collected. The elution pattern was monitored by thin layer chromatography. Fractions numbered 44 to 68 contained GM₃ gangliosides. These fractions were combined to yield about 370 mg of pure GM₃ ganglioside. Fractions 74 to 76, which contained the novel ganglioside, were combined. It was further purified by preparative thin layer chromatography. The final GM₁D₃-GNA₄ fraction, recovered from the thin layer plates, still contained small amounts of contaminants coming from TLC plates. It was redissolved in a small volume of 0.1 M acetic acid and was applied to a small column (10 x 70 mm) of Sephadex G-50 (medium). The elution was carried out with 0.1 M acetic acid. The purified GM₁D₃-GNA₄ was collected in the fraction of void volume of the column (14).

Thin Layer Chromatography—High performance thin layer chromatographic plates of Silica Gel 60 were purchased from E. Merck, Darmstadt, West Germany. The following solvent systems were used in this study, A) chloroform/methanol/water (55:45:10, containing 0.2% CaCl₂; 2H₂O), B) chloroform/methanol/2.5 M ammonium hydroxide (60:35:8), C) 1-propanol/water (75:25), D) 1-propanol/2.5 M ammonium hydroxide (75:25), E) 1-propanol/pyridine/water (70:10:20), and F) 1-butanol/pyridine/water (9:6:4, containing 0.1% KCl). Gangliosides were visualized by spraying with resorcinol/HCl reagent (356), followed by heating the covered plate at 95°C on an aluminum block heater (16). Anthrone reagent and α-naphthol reagent were used for detecting neutral glycolipids.

Compositional Analysis—The molar ratios of carbohydrate and long chain base constituents were determined as their N-trifluoroacetoamido derivatives by gas-liquid chromatography (17). A sample containing 5 μg of sialic acid was methanolyzed for 3 h at 100°C with 50 μl of anhydrous methanolic HCl (35%, w/v) in a 0.3-ml Reacti-vial capped with Tefbond (Applied Science Laboratories). After fatty acid methylesters were removed by extraction with n-hexane, the solvent and HCl were evaporated under a stream of nitrogen. The residue was then dissolved in 10 μl of trifluoroacetic anhydride/ethylacetate mixture (1:1) and heated for 10 min at 60°C. The trifluoroacetic anhydride and ethylacetate were evaporated under a stream of nitrogen in an ice bath. The residue was dissolved in 10 μl of ethylacetate. Aliquots of the solution were injected into a glass column (6 feet x 2 mm) packed with Gas-chrom Q coated with a mixture of 8% (w/w) SP-2401 and 0.5% (w/w) OV-225 (11). The fatty acid methylesters were analyzed on a 10% SP-222 PS column by gas-liquid chromatography. Long chain base composition was determined by a modified procedure of Carter and Glover (18).

Mild Acid Hydrolysis—A ganglioside sample, containing 20 μg of sialic acid, was dissolved in 100 μl of 1 M formic acid and heated at 100°C, according to Svensonholm et al. (19). The solution was then cooled in an ice bath, and formic acid and water were evaporated with a stream of nitrogen. The residue was dissolved in 50 μl of methanol and then analyzed by thin layer chromatography.

Neuraminidase Treatment—Treatment of gangliosides with neuraminidase was performed using the procedure of Ando and Yu (11). A ganglioside sample, containing 20 μg of sialic acid, was dissolved in 50 μl of 0.1 M sodium acetate buffer. The sample was then mixed with 10 μl of enzyme solution with 300 ml of 102.18, Clostridium perfringens (EC 3.2.1.18), type VI, Sigma, 1 unit in 1 ml of the same buffer. The solution was incubated for 30 min at 37°C. The enzyme reaction was stopped by the addition of 100 μl of methanol. The solution was then taken to dryness under a stream of nitrogen at room temperature. The residue was dissolved with 20 μl of methanol and analyzed by thin layer chromatography.

Glycosidase Treatment—β-Hexosaminidase and β-galactosidase from jack bean (20), and a β-hexosaminidase preparation from human liver (21) were used for the structural analysis of the novel ganglioside employing previously described procedures (22, 23).

Smith Degradation—Periodate oxidation-borohydride reduction of gangliosides was carried out according to the procedure reported previously (11). After methanolation, the carbohydrate components were analyzed by gas-liquid chromatography (11).

RESULTS AND DISCUSSION

Although GM₃ has long been recognized as the most characteristic and conspicuous stored lipid in Tay-Sachs brains, recent studies have indicated that the distributions of other gangliosides (1, 2, 9, 10) and neutral glycolipids (23–25) are also significantly altered. Among the gangliosides, the most notable alterations are the accumulation of G₀₂ and G₁D₃-GNA₄ (9, 10). The latter ganglioside, which was first isolated and characterized by Svennerholm et al. (19) as a minor ganglioside of normal human brain, was particularly abundant. Its concentration was found to be 10 to 20 times higher than normal (2, 9). Evidence has been provided by Iwamori and Nagai (10) that the accretion of G₁D₃ and G₁D₄-GNA₄, both of which contain a terminal β-N-acetylgalactosaminyl residue, is also correlated with the inherent deficiency in β-hexosaminidase activities in Tay-Sachs patients. Conceivably, other glycoconjugates which possess the same terminal carbohydrate structure would be similarly stored in Tay-Sachs tissues (4).

In the present study, we employed three cases of Tay-Sachs brains. All of them showed nearly identical ganglioside composition, including the presence of the novel monosialogangliosyl-2-ethoxyl-2-hexosylceramide, GM₁D₃-GNA₄. The isolation of this ganglioside was achieved by utilizing a linear gradient elution system of DEAE-Sephacel column chromatography for the subfractionation of the complex ganglioside mixture. This was a modification of the procedure originally developed by Momoi et al. (26) who used DEAE-Sephadex as the ion exchange resin. However, DEAE-Sephadex, which had been used extensively in our laboratory (11, 27–29), showed remarkable shrinkage with the change of salt concentrations in the eluting solvent. DEAE-Sephadex alleviated this problem and further

![Fig. 1. Ganglioside elution profile from DEAE-Sephacel column. Gangliosides were eluted from the column with a linear gradient of increasing concentrations of sodium acetate in methanol. Peaks I, II, III, and IV corresponded to mono-, tri-, and tetrasialoganglioside fractions, respectively. Peaks II, III, and IV were ascribed to disialoganglioside fractions.](image-url)
improved the resolution. A typical elution profile of a TaySachs brain ganglioside mixture is shown in Fig. 1. The bulk of the total gangliosides was recovered in the first peak fraction which contained primarily monosialogangliosides. Fractions II, III, and IV consisted of disialogangliosides with \( \text{G}_{\text{Dla-GalNAc}} \) heavily concentrated in Fraction IV. Fractions V and VI were tri- and tetrasialogangliosides, respectively. After removing the excess salts by washing with 5 column volumes of methanol, the DEAE-Sephacel column could be reused without any noticeable loss of resolving power.

The monosialoganglioside fraction (Fraction I) which contained a large amount of \( \text{G}_\text{M3} \), together with small amounts of \( \text{G}_\text{M2} \), \( \text{G}_\text{M1} \), and \( \text{G}_\text{M4} \), was rechromatographed on an Iatrobeads column employing a linear gradient elution system. The fractionation was monitored by thin layer chromatography and a chromatogram is shown in Fig. 2. \( \text{G}_\text{M4} \) ganglioside, which is one of the major gangliosides in human brain white matter and myelin (12, 30), was not detectable in any of these fractions. Instead, we detected two resorcinol positive spots (GX and GY) on the thin layer chromatogram (Fig. 2). GX migrated faster than \( \text{G}_\text{M4} \), whereas GY had a nearly identical migratory rate as \( \text{G}_\text{M4} \). However, after mild base treatment, GX and GY were converted into \( \text{G}_\text{M2} \) and \( \text{G}_\text{Dib} \), respectively. Iwamori and Nagai (31) mentioned that the lactonization of \( \text{G}_\text{Dib} \) was easily achieved by mild acid treatment. Presumably, small amounts of \( \text{G}_\text{M2} \) and \( \text{G}_\text{Dib} \) were converted to lactones during chromatography.

Fractions 74, 75, and 76 (Fig. 2) contained \( \text{G}_\text{M2} \), \( \text{G}_\text{M1} \), and the novel ganglioside \( \text{G}_\text{M3-GalNAc} \). The latter was finally isolated from these fractions by preparative thin layer chromatography with a final yield of about 0.1% of the total brain ganglioside mixture. The isolated \( \text{G}_\text{M3-GalNAc} \) was homogenous as judged by chromatography on thin layer plates employing six different solvent systems (see above). Typical thin layer chromatograms are shown in Figs. 3 and 4. \( \text{G}_\text{M3-GalNAc} \) migrated between \( \text{G}_\text{M1} \) and \( \text{G}_\text{Dib} \) on silica gel plates developed with solvent system A or B. Its chromatographic behavior is therefore different from that of \( \text{G}_\text{M1-GalNAc} \) which migrates below \( \text{G}_\text{M1} \) with solvent system B (32). The structure of \( \text{G}_\text{M3-GalNAc} \), isolated from normal human brain has recently been characterized by Iwamori and Nagai (32) as a derivative of \( \text{G}_\text{M1} \), with an additional GalNAc attached to the terminal galactose residue through a \( \beta \rightarrow 4 \) linkage.

The chemical composition of \( \text{G}_\text{M3-GalNAc} \) is shown in Table I. The ganglioside contained glucose, galactose, N-acetylgalactosamine, N-acetylneuraminic acid, and long chain base in the molar ratios of 1:2:2:1:1. Its composition is therefore identical with that of \( \text{G}_\text{M1-GalNAc} \). Stearic acid was the predominant fatty acid (93%) and the major long chain bases were sphingosine (58.8%), icosasphingenine (15.8%), and hexadecasphingenine (14%).

![Fig. 2. Thin layer chromatograms of the monosialoganglioside fractions eluted from the Iatrobeads columns. A 20-μl aliquot from each fraction was spotted on a silica gel plate which was then developed with solvent system A. Gangliosides were revealed by resorcinol/HCl reagent.](image)

![Fig. 3. Thin layer chromatogram of \( \text{G}_\text{M1-GalNAc} \). The plate was developed with solvent system A. Lane 1, \( \text{G}_\text{M1} \); Lane 2, \( \text{G}_\text{M2} \); Lane 3, \( \text{G}_\text{M3} \); Lane 4, \( \text{G}_\text{M4} \); Lane 5, \( \text{G}_\text{M5} \); Lane 6, \( \text{G}_\text{M6} \); Lane 7, \( \text{G}_\text{M7} \); Lane 8, \( \text{G}_\text{Tib} \). The bands were revealed with resorcinol/HCl reagent.](image)

![Fig. 4. Thin layer chromatogram of \( \text{G}_\text{M4-GalNAc} \). The plate was developed with solvent system B. Lane 1, total gangliosides from human brain white matter; Lane 2, \( \text{G}_\text{M4-GalNAc} \); Lane 3, \( \text{G}_\text{Dib} \); Lane 4, \( \text{G}_\text{Dla} \); Lane 5, \( \text{G}_\text{M4} \); Lane 6, \( \text{G}_\text{M5} \); Lane 7, \( \text{G}_\text{M6} \); Lane 8, \( \text{G}_\text{Tib} \). The bands were revealed with resorcinol/HCl reagent.](image)

\( \text{G}_\text{M3-GalNAc} \) was treated with neuraminidase (\textit{C. perfringens}) in the absence of detergent. In analogy to \( \text{G}_\text{M2} \), this ganglioside was completely resistant to the action of the enzyme. Even in the presence of a detergent (bile acid), the ganglioside still was not degraded by neuraminidase. The results suggested that the sialic acid must be attached to an inner position of the neutral sugar chain.

The ganglioside was also completely resistant to \( \beta \)-hexosaminidase (jack bean) and \( \beta \)-galactosidase (jack bean). However, mild acid treatment (1 M formic acid, 100°C, 30 min) could convert this ganglioside to a pentahexosyl ceramide which was chromatographically identical to that derived from \( \text{G}_\text{Dla-GalNAc} \) (18) (Fig. 5, Lanes 4 and 7). The pentahexosyl ceramide, \( \text{GalNAc(β1-4)Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)} \) ceramide, could then be degraded to asialo-\( \text{G}_\text{M1} \) (\( \text{G}_\text{A1} \)) by the action of \( \beta \)-hexosaminidase (jack bean) (Fig. 5, Lane 5). The resulting asialo-\( \text{G}_\text{M1} \) could be converted to asialo-\( \text{G}_\text{M2} \) (\( \text{G}_\text{A2} \)) by hydrolysis with \( \beta \)-galactosidase (jack bean) (Fig. 5, Lane 6). These results indicated that the novel ganglioside indeed possessed a pentahexosyl ceramide backbone as shown above.
TABLE I

<table>
<thead>
<tr>
<th>Ratios of carbohydrate and long chain base</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.1</td>
</tr>
<tr>
<td>N-acetylgalactosamine</td>
<td>2.1</td>
</tr>
<tr>
<td>N-acetylneuraminic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Long chain base</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Fatty acid composition

| C16:0 | 0.1 |
| C18:1 | 1.6 |
| C18:0 | 92.3 |
| C18:1 | 5.0 |
| C20:0 | 1.0 |

Long chain base composition

| d16:0 | 1.0 |
| d16:1 | 14.1 |
| d18:0 | 7.3 |
| d18:1 | 58.8 |
| d20:0 | 2.8 |
| d20:1 | 15.8 |

* Hexadecasphinganine.
* (4E)-Hexadecasphingenine.
* Sphinganine.
* (4E)-Sphingenine.
* Icosasphinganine.
* (4E)-Icosasphingenine.

The remaining question then was the point of attachment for the sialic acid residue. Since the intact ganglioside was completely resistant to jack bean β-hexosaminidase, it suggested that the sialic acid must be linked to the penultimate galactose moiety. Conclusive evidence came from a hydrolysis experiment employing a β-hexosaminidase preparation of human liver (21). Previously, Li et al. (21) showed that this enzyme could degrade GM₂ to GM₁, and asialo-GM₁, respectively. Lane 4, pentahexosyl ceramide obtained from G₂M₁-G₂N₂Ac. Lane 5, β-hexosaminidase hydrolysis products of Lane 4. Lane 6, β-galactosidase hydrolysis product of Lane 5. Lane 7, pentahexosyl ceramide obtained from G₂M₁-G₂N₂Ac. Lane 8, asialo-GM₁. Lane 9, asialo-GM₁. Bands were revealed by spraying with resorcinol/HCl reagent followed by spraying with an anthrone reagent.

Finally, Smith degradation of the intact ganglioside reduced GM₁ to GM₂ (data not shown). This is in contrast to G₂M₁, which is resistant to hydrolysis by neuraminidase, but can be degraded to G₂M₁ by β-galactosidase.

Fig. 6. Enzymatic hydrolysis of G₂M₁-G₂N₂Ac by β-hexosaminidase (human liver). The plate was developed with solvent system A. Lane 1, mixture of asialo-GM₁, G₂M₁, and G₂M₁. Lane 2, mixture of GM₂, GM₁, and G₂M₁. Lane 3, hydrolysis products of G₂M₁-G₂N₂Ac. Lane 4, authentic GM₂. Bands were revealed by spraying with resorcinol/HCl reagent.
derivative (N-acetylheptulosaminic acid), and 1 mol each of galactose and galactosamine was destroyed (Table I). These data, together with those obtained by the hydrolysis experiment, suggest that this is a monosialosylpentahexosyl ceramide.

Because the yields of intact derivative of G\textsubscript{M\textsubscript{b}-GalNA\textsubscript{c}} isolated from adult human brain by Iwamori and Nagai (32) were not able to detect this novel ganglioside in normal human brain although it may be present in extremely low concentrations. Presumably, the deficiency in \(\beta\)-hexosaminidase, which is known to occur in Tay-Sachs brains, is the cause for the accumulation of minor gangliosides such as G\textsubscript{M\textsubscript{b}-GalNA\textsubscript{c}}, G\textsubscript{D\textsubscript{a}-GalNA\textsubscript{c}}, and G\textsubscript{D\textsubscript{c}-GalNA\textsubscript{c}}. Like G\textsubscript{M\textsubscript{c}}, all these gangliosides possess a similar terminal oligosaccharide structure.

**REFERENCES**


5. Klenk, E. (1942) Ber. 75, 1632-1636


20. Li, Y.-T., and Li, S.-C. (1972) Methods Enzymol. 28, 702-713


