Structural Characterization of a Novel Cholinergic Neuron-specific Ganglioside in Bovine Brain*

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A new ganglioside antigen, termed Chol-1α-b, recognized by cholinergic neuron-specific antibody, Chol-1α, was isolated from bovine brain ganglioside mixture using Q-Sepharose. The yield was approximately 1.3 mg from 5 g of the total ganglioside. The chemical structure was characterized as a novel ganglioside by means of gas-liquid chromatography, a permethylation study, mild acid hydrolysis, thin layer chromatography-enzyme immunostaining, fast atom bombardment mass spectrometry, and proton nuclear resonance spectroscopy. The ganglioside has the following unique structure.

\[
\text{NeuAc}_2 \\
\text{Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide} \\
\text{NeuAc}_2 \\
\text{NeuAc}_2 \\
\text{NeuAc}_2
\]

When examined by thin layer chromatography immunostaining and enzyme-linked immunosorbant assays, this ganglioside has the most intense immunoreactivity with Chol-1α antibody among bovine brain gangliosides. As combined with our previous results (Hirabayashi, Y., Hyogo, A., Nakao, T., Tsuchiya, K., Suzuki, Y., Matsumoto, M., Kon, K., and Ando, S. (1990) \textit{J. Biol. Chem.} 265, 8144–8151; Ando, S., Hirabayashi, Y., Kon, K., Inagaki, F., Tate, S., and Whitaker, X. (1992) \textit{J. Biochem. (Tokyo)}, 111, 287–290), the present study indicates the occurrence of a new series of gangliosides containing N-acetylneuraminic acid residue attaching to N-acetylgalactosamine as cholinergic specific antigens.

By immunizing sheep with synaptosomal membranes prepared from the electric organ of \textit{Torpedo marmorata}, Whitaker and his colleagues (1–4) have raised an antiserum which reacts with at least three gangliosides in mammalian brains, designated as Chol-1α, Chol-1β, and Chol-1γ. Since the antiserum can recognize only cholinergic neurons in central and peripheral neurons of the rat, the antigenic gangliosides are thought to be selectively localized on cholinergic neurons (1, 2, 5). Despite their significance, elucidation of their chemical structures has not been successful due to their extremely low concentration in mammalian brain tissues. Giuliani et al. (6) isolated 100 μg (as sialic acid) of the ganglioside Chol-1β from 100 kg of pig brain, and they characterized in part the chemical structure of the novel antigen.

We have recently developed a new method using Q-Sepharose to efficiently purify extremely minor gangliosides from complex mixtures of bovine brain gangliosides (7). This method was applied for isolation of G\textsubscript{M\textsubscript{5}n}, G\textsubscript{D\textsubscript{2}n} and G\textsubscript{T\textsubscript{1}n}(NeuGc) from bovine brain in pure form (8, 9). More recently, we successfully have isolated and characterized one of the Chol-1α gangliosides, termed Chol-1α-a from bovine brain (10). During the work, we detected a ganglioside antigen more strongly immunoreactive with anti-Chol-1α antibody than Chol-1α-a. This paper describes the isolation and immunochemical characterization of this novel tetrasialoganglioside antigen, Chol-1α-b, from bovine brain.

**EXPERIMENTAL PROCEDURES**

Materials—Gangliosides from bovine brain were prepared as described previously (7). G\textsubscript{M\textsubscript{5}n} and G\textsubscript{D\textsubscript{2}n}, were purified from bovine brain ganglioside mixture (8). A novel trisialoganglioside GT\textsubscript{4}n, or IV\textsubscript{NeuAc},II\textsubscript{NeuAc},II\textsubscript{NeuAc-GD\textsubscript{1}n}Gc was a gift from Drs. K. Nakamura and Y. Tamai (Department of Biochemistry, Kitazato University School of Medicine) (11). The same ganglioside identified as Chol-1α-a antigen was also isolated from bovine brain (10). Sheep anti-Chol-1 was raised as described by Jones et al. (1). Affinity purified anti-Chol-1α (4) was kindly provided by Drs. E. Derrington and E. Borroni (Max-Planck-Institute). Peroxidase-labeled rabbit anti-sheep IgG was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). \textit{Vibrio cholerae} toxin B subunit conjugated to horseradish peroxidase was purchased from List Biological Laboratories (Campbell, CA).

Isolation of Tetrasialoganglioside Chol-1α-b—Total bovine brain gangliosides (5 g) were applied to a Q-Sepharose column (3×75 cm) and fractionated into 23 fractions as described previously (7). As revealed by TLC immunostaining, a major component of polysialoganglioside in fraction 23 (tubes 295–310) reacted with anti-Chol-1α

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§The abbreviations for gangliosides are according to Svennerholm nomenclature (24) and our previous publications (10, 20); TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; FAB-MS, fast atom bombardment-mass spectrometry.
and was designated as Chol-la-b. Final purification of Chol-la-b was achieved by preparative TLC using precoated silica gel HPTLC plates (E. Merck) developed with chloroform/methanol/water (5:4:1, v/v/v). The compound was located by iodine vapor and eluted with methanol/water (1:1, v/v) after scraping the silica gel powder. Approximately 1.3 mg of purified Chol-la-b was obtained.

**TLC—**Analytical HPTLC was carried out on precoated HPTLC plates using the following solvent systems: Chloroform, methanol, 12 mM MgCl₂, 15 mM NH₄OH (5:4:0:7:0:3, v/v/v, solvent A) and chloroform, methanol, 12 mM MgCl₂, 15 mM NH₄OH (5:4:0:7:0:3, v/v/v, solvent B). Gangliosides were visualized using resorcinol/HCl reagent (12).

**Immunological Methods—**TLC-immunostaining was performed by the method of Higashi et al. (13). Gangliosides were applied on a plastic TLC plate (Polygram SL G, Nagel, Germany) and developed with solvent system A. Gangliosides were visualized by immunostaining with anti-Chol-la (10) or the B subunit of cholera toxin after treatment of the plate with *Clostridium perfringens* sialidase (14). The enzyme-linked immunosorbent assay was performed by the method of Higashi et al. (15).

**Mild Acid Hydrolysis—**A ganglioside sample, containing 1 nmol of sialic acid, was dissolved in 100 μl of aqueous 5.6 mM formic acid and heated at 80 °C (16) for 30 min. The solution was neutralized by adding 25 μl of concentrated NH₄OH solution followed by incubation at 30 °C for 2 h. The solution was then lyophilized and the residue thus obtained was analyzed by HPTLC using the solvent systems A and B.

**Structural Analysis—**Neutral sugars and amino sugars of each ganglioside were analyzed by gas-liquid chromatography (8) after methanolation, N-acetylation, and trimethylsilylation as described by Hatti et al. (17). Fatty acid composition was also determined by gas-liquid chromatography. The gangliosides were permethylated by the method of Hakomori (18). The partially methylated alditol acetics were prepared as described previously (19, 20) and analyzed by gas chromatography-mass spectrometry on a Shimadzu-LKH 9000B data system equipped with a capillary column of 3% OV-101 (0.2 mm × 25 m, Shimadzu, Kyoto).

**RESULTS AND DISCUSSION**

**Chemical Structure of Chol-la-b—**We have reported the presence of a ganglioside in bovine brain reacting with affinity purified antibody against cholinergic neurons (10). The chemical structure of the antigenic ganglioside, designated Chol-la-a, was determined as follows.

![FIG. 1. HPTLC of Chol-la-b ganglioside.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Chemical composition of Chol-la-b ganglioside</th>
<th>Ratio of carbohydrate</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.05</td>
<td>0.95</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>N-Acetylneuraminic acid</td>
<td>3.75</td>
<td></td>
</tr>
<tr>
<td>Fatty acid composition</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>91.3</td>
<td></td>
</tr>
<tr>
<td>C20:0</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>C22:0</td>
<td>3.0</td>
<td></td>
</tr>
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**TABLE II**

<table>
<thead>
<tr>
<th>Partially methylated alditol acetates from Chol-la-b ganglioside</th>
<th>2,3,6-O-</th>
<th>2,3,4,6-O-</th>
<th>2,3,5-O-</th>
<th>2,4,5-O-</th>
<th>2,6-O-</th>
<th>4,6-O-</th>
<th>4-O-</th>
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<tbody>
<tr>
<td></td>
<td>O-Me</td>
<td>O-Me</td>
<td>O-Me</td>
<td>O-Me</td>
<td>O-Me</td>
<td>O-Me</td>
<td>O-Me</td>
</tr>
<tr>
<td></td>
<td>Ac-galactitol</td>
<td>Ac-galactitol</td>
<td>Ac-galactitol</td>
<td>Ac-galactitol</td>
<td>Ac-galactitol</td>
<td>Ac-galactitol</td>
<td>Ac-galactitol</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chol-la-a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chol-la-b</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sialidase-treated Chol-la-b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GM1α</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
GM1a which was proven by immunostaining on TLC with horseradish peroxidase-labeled cholera toxin B subunit (data not shown). These data indicated that the antigen is a tetrasialo-ganglioside-N-tetraosylceramide structure. The major fatty acids were C18:0 (91%) and C20:0 (4%).

Chol-1α-b and its clostridial sialidase-treated product were permethylated and subjected to acetylation followed by reduction and acetylation. The resulting partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometry. As listed in Table II, the composition of partially methylated alditol acetates derived from Chol-1α-b was identical to that from Chol-1α-a, indicating that the antigen also has a branched structure at the N-acetylgalactosamine residue. The sialidase-treated ganglioside produced the same composition of partially methylated alditol acetates as that of Gm1a, implying that four sialic acids were one attached to the C-6 position of N-acetylgalactosamine, the second at the C-3 position of the nonreducing end of galactose, and the third at the C-3 position of the internal galactose. Thus, the last one should be attached on one of the three sialic acids.

To identify the sialyl linkage, permethylated Chol-1α-b was subjected to methanolysis, followed by acetylation and analyzed by gas chromatography-mass spectrometry (9, 23). The antigen produced 1,2,4,7,8,9-hexa-O-methyl-N-methyl-N-acetyleneuraminic glycoside and 1,2,4,7,9-penta-O-methyl-8-O-acetyl-N-methyl-N-acetyleneuraminic acid methyl ester (data not shown). This result indicates that the ganglioside has a NeuAcO2-8NeuAc side chain.

The ganglioside Chol-1α-b was subjected to mild acid hydrolysis in aqueous formic acid at 80 °C for 30 min. This treatment produced a series of partially desialylated gangliosides products, i.e. Gm1a, Gm1b, Gm2b, and Chol-1α-a (Fig. 2, lane 3). Production of Gm1b was confirmed by TLC immunostaining analysis using human serum IgM antibody against b-series gangliosides (Fig. 3). These observations indicate the presence of a NeuAcO2-8NeuAc structure at the internal galactose of gangliotetraosylceramide. In addition, a product coded as X-1 was noted. This ganglioside was located between Gm1b and Gm1b in solvent A and has an Rf value similar to that of Gm1b in solvent B. This became reactive with cholera toxin subunit B after direct treatment of clostridial sialidase on TLC (data not shown). Moreover, the product was not immunoreactive with anti-Gm1b-specific monoclonal antibody KA-17 (data not shown). These results suggest that X-1 is a disialoganglioside having one sialic acid linked at the 6 position of the N-acetylgalactosamine of ganglioside Gm1a.

When the products were developed in basic solvent system B, a faint resorcinol-positive band, termed X-2, was noticed (Fig. 2). According to our previous findings (8), this ganglioside was thought to be Gm1b. To confirm this possibility, the products were analyzed by two-dimensional TLC with anti-Gm1b monoclonal antibody (KA-17). As shown in Fig. 4, panel II, Chol-1α-b yielded Gm1b as also observed in the mild acid hydrolysis experiment with Chol-1α-a (Fig. 4, panel I). The results establish that X-2 is Gm1b. Thus, one of four sialic acid residues in Chol-1α-b is attached to the C-6 position of GalNAc.

Six-hundred MHz proton NMR spectrum of Chol-1α-b is shown in Fig. 5. The anomeric configurations of one glucose, two galactoses, and one N-acetylgalactosamine are assigned as α (Table III). The signals appearing in the range of 2.5 and 2.8 ppm were assigned as the protons with equatorial configuration at the C-3 of sialic acids. Due to the limited amount of antigen available, the precise assignment of each peak were not achieved by two-dimensional NMR analysis.

Intact Chol-1α-b antigen was analyzed by negative FAB-MS (Fig. 6). Two major molecular ion species, (M+2Na–3H)+, were seen at m/z 2489 and 2461 which corresponded to the structure composed of four N-acetyleneuraminic acid, three hexoses, one N-acetyhexosamine, and a ceramide comprised
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NeuAcα₂₆

Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Ceramide
(IV) (III) (II) (I)

NeuAcα₂

Table III
Chemical shifts of the anomeric protons and their coupling constants of Chol-1α-b antigen

<table>
<thead>
<tr>
<th>Chol-1α-b</th>
<th>Chemical shifts</th>
<th>J₁,₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td>Hz</td>
<td></td>
</tr>
<tr>
<td>-3GalNAc</td>
<td>4.61</td>
<td>8.3</td>
</tr>
<tr>
<td>-3Gal</td>
<td>4.33</td>
<td>7.8</td>
</tr>
<tr>
<td>-3,4Gal</td>
<td>4.31</td>
<td>7.8</td>
</tr>
<tr>
<td>-4Glc</td>
<td>4.17</td>
<td>7.8</td>
</tr>
</tbody>
</table>

of sphingosine d20:1/fatty acid C18:0 and sphingosine d18:1/fatty acid C18:0. Fragment ions due to the successive elimination of two sialic acids from the sodium adduct ion were also detected at m/z 2198 and 1885. The presence of ions at m/z 564 and 592 indicate that the major ceramide species are sphingosine d18:1/fatty acid C18:0 and sphingosine d20:1/fatty acid C18:0, respectively. Characteristic ions, (NeuAc-Hex)⁻, (NeuAc-Hex-(NeuAc)-HexNAc + Na⁻ - H)⁻, and (NeuAc:NeuAc + Na⁻ - H)⁻, were observed at m/z 470, 986, and 621, respectively.

From all the data combined, the structure of the cholinergic ganglioside is established as follows.

NeuAcα₂

Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Ceramide
(IV) (III) (II) (I)

NeuAcα₂

NeuAcα₂

(IV⁵NeuAc,III⁵NeuAc,II⁵NeuAc2G0se4Cer).

FIG. 5. Proton NMR spectrum of Chol-1α-b ganglioside at 600 MHz.

The Chol-1α-a antigen isolated from bovine brains was characterized as a trisialoganglioside having a hybrid structure of a- and α-series gangliosides and designated G₃₁₆₈ in our previous study (9). The antigen Chol-1α-b in this paper is a tetrasialoganglioside having one additional NeuAc at the C-6 position of the GalNAc residue of ganglioside G₃₁₆₈, showing that this ganglioside has a hybrid structure of G₃₁₆₈ (b-series) and G₁₀₁₆₈ (α-series). According to our previous nomenclature system, the novel tetrasialoganglioside is termed G₃₁₆₈b.

Immunological Reactivity of Chol-1α-a and -b—Binding specificities of anti Chol-1α have been examined by enzyme-linked immunosorbant assay (Fig. 7) and TLC-enzyme immunostaining (Fig. 8). Compared to G₁₀₁₆₈, G₃₁₆₈, ganglioside reacted more strongly with affinity purified antibody under both assay systems. Interestingly, G₁₀₁₈₁, G₁₀₁₆₁, and G₁₀₁₈₁ are hardly reactive with the antibody. The results show that the 3 sialic acid residues separately linked to the gangliotetraosyl backbone structure are essential portions for the binding of the antibody to the antigenic gangliosides. In addition, the disialyl structure in the ganglioside Chol-1α-b is necessary for the maximal binding of the antibody.

Since the antiserum against Chol-1 was successfully used to detect cholinerergic neurons in the central and peripheral nervous systems of the rat (1, 2, 5), the novel ganglioside, termed Chol-1α-b, should be localized in the cell surface of cholinergetic neurons. Specific monoclonal antibody to the cholineriergic ganglioside will give us a more precise answer to the questions concerning its localization, distribution, and spatial and temporal expression of the antigen during development of mammalian brain tissues.

In addition to the finding of G₁₀₁₈₁ and G₃₁₆₈ in bovine brain tissues as minor components (8, 10), the present study indicates that in addition to a-, b-, c-, and α-series, a new series of hybrid type of gangliosides consisting of the classical (a, b, and c) and α-structures exist in bovine brain tissues. It would be of great interest to know the metabolic relationship among them and to pursue the physiological functions of Chol-1α gangliosides in cholinergetic neurons.
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Fig. 6. Negative FAB-MS analysis of the intact Chol-1α-b ganglioside.

Fig. 7. Reactivity of GT1ac and GQ1bc with anti-Chol-1α. A plastic 96-well plate was coated with the antigen, 20 pmol (●, GQ1bc; ▲, GT1bc from dogfish brain; ○, GT1bc from bovine brain; □, GQ1bc), others (GMIbc, GMIIbc, GDIIIbc, GT1bc, GT1bc, and GQ1bc).

Fig. 8. TLC immunostaining of GT1ac and GQ1bc by using anti-Chol-1α and densitometric estimation of immunostained spots. The indicated amounts of the antigens (a, GT1bc; b, GQ1bc) were applied on a plastic TLC plate and immunostained as shown in the inset. Integrated densitometric responses of GT1bc (●) and GQ1bc (○) were shown. Other gangliosides (■) such as GM1bc, GMIIbc, GDIIIbc, GT1bc, GT1bc, and GQ1bc, were also analyzed by TLC immunostaining in the same way.
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