Identification of Novel Gangliosides Containing Lactosaminyl-\(\text{GM}_1\) Structure from Rat Spleen*

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Two novel monosialogangliosides were isolated from rat spleen. The structures of the gangliosides (shown below, where NeuNgc is \(N\)-glycolylneuraminic acid) were characterized by compositional analysis, methylation analysis, hydrolyses with \(\alpha\)-galactosidases, direct probe fast atom bombardment mass spectrometry, and proton nuclear magnetic resonance spectrometry. A novel structure common to both gangliosides was \(N\)-acetyllactosaminyl-\(\text{GM}_1\), \(\text{LaG-N-GM}_1\); where \(\text{G}_1\) is \(\text{II}^{2}\text{NeuAc-GgOse.Cer}\). and this is the first paper to report the occurrence of a new group of gangliosides.

\[
\begin{align*}
\text{Gal}\text{3} & -1\text{4GlcNAc}\text{2} - 1\text{3Gal}\text{1} - 1\text{3GlcNAc}\text{1} - 4\text{Gal}\text{1} - 4\text{Glc}\text{2} - 1\text{1Cer} \\
\text{NeuNgcGc} & - 3 \\
\text{LaG-N-GM}_1 \\
\text{Gal}\text{3} & -1\text{4GlcNAc}\text{2} - 1\text{3Gal}\text{1} - 1\text{3GlcNAc}\text{1} - 4\text{Gal}\text{1} - 4\text{Glc}\text{2} - 1\text{1Cer} \\
\text{NeuNgcGc} & - 3 \\
\text{GgOse.Cer} & - \text{GgOse.Cer} \\
\end{align*}
\]

Furthermore, in a monosialoganglioside fraction of rat spleen, the occurrence of a ganglioside having two lactosamine units ( \(\text{Gal}\text{3} -1\text{4GlcNAc}\text{2} - 1\text{3Gal}\text{1} - 1\text{3GlcNAc}\text{1} - 4\text{Gal}\text{1} - 4\text{Glc}\text{2} - 1\text{1Cer} \text{NeuNgcGc} - 3\text{GgOse.Cer} \text{GgOse.Cer}\) ) was suggested. These gangliosides have a unique structure, which includes the ganglio series ganglioside core and the extended modification characteristic of the lacto series.

Gangliosides are characteristic cell membrane components and are involved in a variety of cellular events, including differentiation, maturation, and transformation (1, 2). Important functions of these molecules as antigens, and immunomodulators have also been recognized (3).

Recently, gangliosides of murine spleen have been the subject of several investigations (4-6). In rat spleen, two major gangliosides were shown to correspond to \(\text{GM}_1\) and \(\text{G}_{0,1}\) on TLC. The occurrence of minor gangliosides, which increased with the progress of development, was also demonstrated (4). However, further characterization of these gangliosides has not been carried out. We have been trying to establish the chemical structures of gangliosides present in rat spleen. During this investigation, we found a series of novel gangliosides containing the \(\text{GM}_1\) core which are modified with \(N\)-acetyllactosamine, and we have named this series the lactosaminyl-\(\text{GM}_1\) group. In this paper, we describe the isolation and characterization of the lactosaminyl-\(\text{GM}_1\) group gangliosides from rat spleen.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wistar rats (male and female) were supplied by Clea Japan (Tokyo). The spleens were pooled and stored at \(-20^\circ\text{C}\) before use. The reference ganglioside, \(\text{GM}_1\) (NeuNgc), was prepared from pig brain in our laboratory. DEAE-Sephadex A-25 was purchased from Pharmacia LKB Biotechnology Inc., Iatronbeads 6RS-8060 were from Iatron Laboratories, Inc. (Tokyo), and precoated thin-layer plates (HPTLC Silica Gel 60) were from Merck. Green coffee bean \(\alpha\)-galactosidase and jack bean \(\beta\)-N-acetylhexosaminidase were obtained from Sigma. Jack bean \(\beta\)-galactosidase was from Seikagaku Kogyo Co. (Tokyo).

**Preparation of Ganglioside Fraction**—The total gangliosides were prepared from rat spleens (2.2 kg) according to the procedures described previously (7). Briefly, glycolipids were extracted three times with chloroform/methanol/water (4:8:3, v/v) by the method of Svennerholm and Fredman (8). The combined extracts were partitioned with adding water to adjust the solvent composition to chloroform/methanol/water (4.8:5:0). The total gangliosides (257 mg as the sialic acid content) were obtained from the upper phase after mild alkali treatment, dialysis, and evaporation. The gangliosides were applied subsequently to a DEAE-Sephadex column (acetate form) equilibrated with chloroform/methanol/water (5:7:1) (7). After neutral lipids had been eluted with 5 volumes of the same solvent, gangliosides were separated by stepwise elution with 5 volumes of a series of chloroform/methanol (3:7) mixtures containing one-tenth volume of 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, or 2.0 \(\text{M}\) aqueous ammonium acetate. The three novel monosialogangliosides were eluted with the 0.1 \(\text{M}\) aqueous ammonium acetate-containing chloroform/methanol/methanol mixture. These were tentatively named M15, M14, and M15 in order of decreasing mobility on TLC.2

**Isolation of Gangliosides M13-M15**—The fraction eluted with the 0.1 \(\text{M}\) aqueous ammonium acetate-containing chloroform/methanol/methanol mixture from the DEAE-Sephadex column was applied to a large Iatronbeads 6RS-8060 column (5.0 cm x 100 cm) and gangliosides were separated successively by stepwise elution with chloroform/methanol/water (70:30:1.5, 4 liters; 60:40:2, 4 liters; 50:50:2.5, 4 liters; and 40:60:3, 2 liters). Each 25-ml eluate was collected in a test tube (total of 560 tubes). Gangliosides obtained in each tube were analyzed by TLC, and the eluates were grouped into six fractions. Gangliosides M13 and M14 were eluted in the fourth fraction (tubes 361-419) with another ganglioside. The fifth fraction (tubes 420-520) contained M15 as the major ganglioside. The fraction containing M13 and M14 was then subjected to HPLC on an Iatronbeads 6RS-8010 column (1 x 25 cm) with a linear gradient of chloroform (A) and methanol, 7.5 \(\text{N}\) \(\text{NH}_2\text{OH}\) (9:1) (B) (solvent

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† The abbreviations for gangliosides follow the nomenclature of Svennerholm (23). Other abbreviations used are: HPLC, high-performance liquid chromatography; NeuNgc, \(N\)-glycolylneuraminic acid; GLC, gas-liquid chromatography; Me-, methyl.
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Three novel monosialogangliosides were isolated from rat spleen and tentatively named M13, M14, and M15 in order of decreasing mobility on TLC. TLC profiles obtained with two different solvent systems are shown in Fig. 1. M13 migrated slightly faster than GD1b, M14 between GD1b and GT1b, and M15 near GT1b with the neutral solvent system A (Fig. 1a). These gangliosides migrated slower with the ammonia-

![Fig. 1. TLC profiles of gangliosides M13–M15. RB, total gangliosides from rat brain.](image-url)

**TABLE I**

| Alditol acetate | Position of O-Me | Ganglioside |
|----------------|------------------|-------------|
| Gal            | 2,3,4,6          | M13         |
| Glc            | 1                | M13         |
| Gal            | 1.4              | M14         |
| Gal            | 0.9              | M14         |
| GlcNAc         | 1.1              | M15         |
| GlcNAc         | 0.1              | M15         |
| GalNAc         | 0.4              | M15         |

* Product after sequential treatment of M14 with α- and β-galactosidases.

† Mixed fraction of M15 and unknown ganglioside.

**RESULTS**

The accelerating voltage was 8 kV, and the primary beam for the bombardment was 3.0 kV of Xe.

*NMR Spectroscopy—* The gangliosides were analyzed in 0.4 ml of dimethyl sulfoxide-d$_6$ containing 2% D$_2$O and 10 μl of 0.1 N NaOD with a JEOL JNM-GX 500 500-MHz NMR spectrometer at 60 °C. Tetramethylsilane was used as an internal standard for chemical shifts.

**TABLE I**

| Position of O-Me | Ganglioside |
|------------------|-------------|
| 2,3,4,6          | M13         |
| 1                | M13         |
| 1.4              | M14         |
| 0.9              | M14         |
| 1.1              | M15         |
| 0.1              | M15         |
| 0.4              | M15         |

* Product after sequential treatment of M14 with α- and β-galactosidases.

† Mixed fraction of M15 and unknown ganglioside.
containing solvent system B than with solvent system A, in contrast to the reference di- and trisialogangliosides (Fig. 1b). M13 and M14 showed a single band on TLC obtained with solvent systems A–C. Although M15 showed a single band on TLC with the three solvent systems, the results of exoglycosidase treatment revealed that the fraction contained two components as mentioned below. Yields of M13–M15 were 120, 810, and 920 µg from 2.2 kg of wet tissue, respectively.

The carbohydrate constituents of M13 and M14 were: Gal, Glc, GalNAc, GlcNAc, and NeuNGc at molar ratios of 2.9:1:1.1:0.9:1.1 and 4.1:1:1.2:1.0:1.1, respectively. These gangliosides were characterized by the presence of both N-acetylgalactosamine and N-acetylglucosamine. M15 also contained two types of hexosamines, and the carbohydrate constituents were Gal, Glc, GalNAc, GlcNAc, and NeuAc at a molar ratio of 5.3:1:1.0:2.1:1.0. The sialic acid in M13 and M14 was identified as N-glycolyneuraminic acid, and that of gangliosides in M15 was found only in the N-acetyl form.

Characterization of M13 and M14—Methylation analysis of M13 (Table I) showed the presence of terminal galactose with the detection of 2,3,4,6-tetri-O-Me Gal. The rest of the alditol acetates included those derived from GM1 substituted at C-3 of the terminal galactose (2,3,6-tetri-O-Me-Glc, 2,4,6-tetri-O-Me-Gal, and 2,6- and 4,6-di-O-Me-Gal) and that derived from C-4-substituted GlcNAc (3,6-di-O-Me-GlcNAc). Although the yield of 4,6-di-O-Me-GalNAc was 40% of the yield of 2,3,6-tetri-O-Me-Glc, no other derivatives of GalNAc were detected. Thus, N-acetylgalactosamine was assumed to be substituted at C-3. Methylation analysis of M14 gave identical derivatives to those of M13, except for producing 2 mol rather than 1 mol of 2,4,6-tetri-O-Me-Gal. The sequential treatment of M14 with α- and β-galactosidases (M14b) produced 2,4,6-tetri-O-Me-GlcNAc instead of 3,6-di-O-Me-GlcNAc, indicating the presence of N-acetylgalactosamine residues at the nonreducing terminus. The disappearance of 2,3,4,6-tetri-O-Me-Gal and the reduction of 1 mol of 2,4,6-tetri-O-Me-Gal were also shown.

When M14 was subjected to exoglycosidase treatment (Fig. 2), the terminal galactose was liberated by α-galactosidase. The product showed exactly the same Rf value as that of M13 and was further sequencially hydrolyzed with β-galactosidase and β-N-acetylgalactosaminidase, being converted to a ganglioside with the same TLC mobility as GM1(NeuNGc).2 The terminal galactose of M13 was shown to possess the β-anomeric configuration by β-galactosidase treatment. The product also gave the same TLC mobility as the product after sequential treatment of M14 with α- and β-galactosidases. From the results of methylation analysis and exoglycosidase treatment, M13 and M14 were suggested to contain the terminal sequences Galαl-4GlcNAcαl- and GalLul-3GalBl-4GlcNAcβ1- attaching to the external galactose of the GM1(NeuNGc) core through 1–3 linkages, respectively.

To confirm the proposed structures of M13 and M14, the gangliosides were examined by negative-ion fast atom bombardment mass spectrometry and 1H NMR spectrometry. As shown in Fig. 3B), the main molecular ion ([M – H]+) of M14 was demonstrated at m/z 2170. The mass number is 1 unit higher than that calculated for the proposed structure with C24:1 fatty acid and C18 sphingenine, which is due to the isotope effect. This assignment was supported by the fatty
TABLE II
Chemical shifts and J, L coupling constants of the anomeric protons of gangliosides M13 and M14
Values in parentheses are coupling constants (in hertz).

| VII | VI | V | IV | III | II | I |
|-----|----|---|----|-----|----|---|
| Galna1-3Galβ1-4GlcNacβ1-3Galα1-3GalNAcβ1-4Galα1-4Glcβ1-1-NeuNGcν2-3 |

| ppm | ppm |
|-----|-----|
| 4.24 | 4.83 | 4.29 | 4.15 |
| (6.6) | (8.1) | (7.7) | (7.3) |

| VII | VI | V | IV | III | II | I |
|-----|----|---|----|-----|----|---|
| Galna1-3Galβ1-4GlcNacβ1-3Galα1-3GalNAcβ1-4Galα1-4Glcβ1-1-NeuNGcν2-3 |

| ppm | ppm |
|-----|-----|
| 4.23 | 4.06 | 4.30 | 4.85 | 4.29 | 4.16 |
| (6.6) | (8.1) | (7.3) | (8.1) | (8.1) | (8.1) |

| VII | VI | V | IV | III | II | I |
|-----|----|---|----|-----|----|---|
| Galna1-3Galβ1-4GlcNacβ1-3Galα1-3GalNAcβ1-4Galα1-4Glcβ1-1-NeuNGcν2-3 |

| ppm | ppm |
|-----|-----|
| 4.86 | 4.31* | 4.66 | 4.31* | 4.85 | 4.29 | 4.16 |
| (3.7) | (8.1) | (8.8) | (8.1) | (8.1) | (8.1) | (8.1) |

TABLE III
Fatty acid composition of M13 and M14

| Ganglioside | Fatty acid |
|------------|------------|
|            | 16:0 | 18:0 | 18:1 | 20:0 | 22:0 | 24:0 | 24:1 | 24:2 |
| % of total fatty acids |
| M13 | 8    | 7    | 2    | 11   | 12   | 4    | 14   | 36   | 6    |
| M14 | 7    | 6    | 7    | 9    | 3    | 13   | 47   | 8    | 8    |

**Fig. 5.** Sequential degradation study of M15 fraction with exoglycosidases. Lane a, M15 treated with α-galactosidase; lane b, product from the lipids in lane a with β-N-acetyllactosaminidase; lane c, product from the lipids in lane b with β-N-acetyllactosaminidase; lane d, product from the lipids in lane c with β-N-acetyllactosaminidase; lane e, product from the lipids in lane d with β-N-acetyllactosaminidase; lane RB, total gangliosides from rat brain. The chromatogram was obtained in chloroform, methanol, 0.2% CaCl₂ (60:40:9).

The fatty acid composition (see Table III), which indicated that C24:1 is the major fatty acid in M14. Fragment ions due to the successive elimination of carbohydrates were also detected at m/z 2007, 1845, 1642, 1480, 1277, 970, 808, and 646 and were assigned as noted in Fig. 3B. The presence of the fragment ion at m/z 1277 clearly demonstrated that the sialic acid is linked to the innermost galactose and that the core structure of the ganglioside is GM1, not GM1b. In the mass spectrum of M13 (Fig. 3A), the main pseudo molecular ion was detected at m/z 2007, which is consistent with the value calculated for the proposed structure of M13 with C24:1 fatty acid and C18 sphingosine. Characteristic fragment ions were also detected and were assigned as shown (Fig. 3).

The anomeric proton regions of the 1H NMR spectra of M13 and M14 are shown in Fig. 4, and the assignment of each signal is given in Fig. 4 and Table II. M13 showed six distinctive signals of anomeric protons. The signals at 4.16, 4.29, and 4.85 ppm were in positions almost identical to those of GM1(αGalNAcβ1-3Galα1-3GalNAcβ1-4Galα1-4Glcβ1-1-NeuNGcν2-3) and were assigned to α-GalI, β-GalII, and β-GalNAcIII, respectively. The signal at 4.23 ppm showed a chemical shift virtually identical to the terminal galactose of GM1(αGalNAcβ1-3Galα1-3GalNAcβ1-4Galα1-4Glcβ1-1-NeuNGcν2-3) and was assigned to the terminal α-GalVI; and the signal at 4.30 ppm was assumed to be β-GalIV with a downfield shift due to glycosylation. The signal at 4.66 ppm, which is a region for β-N-acetyllactosamine (18), must belong to β-GalNAcV. In the spectrum of M14, four signals showed exactly the same chemical shifts as those of M13: 4.16, 4.29, 4.66, and 4.85 ppm, assigned to β-GlcI, β-GalII, β-GlcNAcV, and β-GalACIII, respectively. The overlapping signals around 4.30 ppm were assumed to be β-GalIV and β-GalVI. The remaining signal at 4.86 ppm showed good agreement in chemical shift and coupling constant to that of the anomeric proton of terminal α-galactose in α-galactosyllacto-N-neotetraosylceramide reported by Dabrowski et al. (18) and was assigned to α-GalVII in M14.

From the results mentioned above, the structures of M13 and M14 were concluded to be as shown below.

M13 contains the GM1 core structure and is extended by a neolacto-type disaccharide (N-acetyllactosamine). M14 is a derivative of M13 with an α-galactosyl substitution.

The fatty acid compositions of M13 and M14 are summarized in Table III. They are characterized by the predominance of saturated and unsaturated C24 fatty acids including C24:2. No hydroxy fatty acid was detected in these gangliosides.

**Characterization of M15 by Sequential Exoglycosidase Treatment and Methylation Analysis—**Ganglioside M15 was converted into two distinctive components on TLC by α-galactosidase treatment (Fig. 5). Both were hydrolyzed with β-galactosidase, and two products were obtained. The predom-
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Generally, N-acetylgalactosamine is the exclusive hexosamine constituent of ganglio- and globo series glycolipids, and N-acetylgalactosamine is in that of lacto series glycolipids. In this study, two novel monosialogangliosides containing both N-acetylgalactosamine and N-acetylgalactosamine were isolated from rat spleen. These gangliosides (LacN-GM₃ and αGal–(LacN)₂-GM₃) were clearly demonstrated to be novel ones, whose structures consist of a GM₃ core extended by Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3 sequences, respectively. Moreover, the third novel ganglioside, which was suggested to contain an α-galactosylated repeating N-acetylgalactosamine and N-acetylglucosamine was isolated. The glycolipids, named "lacto-a series gangliosides" from GM₃ remains unanswered. It has been reported (22) that mouse myelogenous leukemia cells contain lacto series glycolipids, the synthesis of which were enhanced upon differentiation of the cells into macrophage-like cells. If the enzymes involved in lacto series synthesis could synthesize lactosaminyl-GM₃ group gangliosides, the expression may be associated with cell differentiation such as that of monocytes to macrophages in the spleen. Further studies are required to clarify the biosynthetic pathways including hydroxylation of sialic acid in lactosaminyl-GM₃ group gangliosides.

DISCUSSION

In general, the lower band in Fig. 5 (lane b), was further and successively hydrolysed with β-N-acetylgalactosaminidase and β-galactosidase and was converted to a ganglioside corresponding to GM₃ (NeuAc) by β-N-acetylgalactosaminidase. The other product, the upper band in Fig. 5 (lane b) was stable to treatment with β-galactosidase and β-N-acetylgalactosaminidase. The results of the enzymatic hydrolyses together with the result of the compositional analysis suggested that the major ganglioside in M15 contains the terminal sequences Galβ1-(Galβ-GlcNAcβ)₂ substituted to GM₃ (NeuAc). Further analysis will be required to show the structure of another ganglioside in M15.

The methylation analysis of M15 gave identical derivatives to those of M14 (Table I). The higher ratios of 2,4,6-tri-O-methyl Gal and 3,6-di-O-Me-GlcNAc in M15 than in M14 were consistent with a structure containing a repeating 3Galβ1-4GlcNAcβ1-3 unit in the substitution extended on GM₃. From the results mentioned above, the structure of one of the two gangliosides in M15 was deduced to be as follows.

Galβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3 → 3(Galβ1-4GlcNAcβ1-3)z Cer

 αGal–(LacN)₂-GM₃

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