Characterization of a Major Neutral Glycolipid in PC12 Cells as III⁺Galα-globotriaosylceramide by the Method for Determining Glycosphingolipid Saccharide Sequence with Endoglycoceramidase*

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Neutral glycolipids in PC12 cells were examined. A major neutral glycosphingolipid, isolated from a chloroform/methanol extract of the cells, was found to contain only galactose and glucose at a ratio of 3:1 and identified as ceramide tetrahexoside by fast atom bombardment (FAB) mass spectrometry. Its saccharide sequence was determined by a new method developed here using endoglycoceramidase (Ito, M., and Yamagata, T. (1988) J. Biol. Chem. 261, 14278–14282). The glycosphingolipid was digested with endoglycoceramidase to produce oligosaccharide which was subsequently pyridylaminated. The fluorescence-labeled oligosaccharide was digested with a series of specific exoglycosidases and fractionated by high performance liquid chromatography. The 2-aminoxyldipyrildoligosaccharide was hydrolyzed by α-galactosidase to give a 2-aminoxyldipyrildoligosaccharide which was identified as 2-aminoxyldipyril lactose by high performance liquid chromatography, indicating the glycolipid structure to be Galα1-3Galα1-4Galβ1-4Glcβ1-1Cer. Ceramide trihexoside obtained by limited digestion of the intact glycolipid was clearly identical with ceramide trihexoside obtained from human erythrocytes, according to NMR spectroscopy and methylation analysis. From these and other data on the intact glycolipid, obtained by methylation analysis and NMR spectroscopy, its structure was confirmed as Galα1-3Galα1-4Galβ1-4Glcβ1-1Cer, III⁺-Galα-globotriaosylceramide. This is the first report indicating the presence of this glycosphingolipid in PC12 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells (kindly provided by Dr. T. Amano of this Institute) were cultured as described (1) in RPMI-1640 medium supplemented with 5% fetal calf serum and 5% heat-inactivated horse serum.

Extraction and Fractionation of Glycolipids from PC12 Cells—The cells were extracted with chloroform/methanol (2:1 and 1:2) sequentially. The extracts were combined and then chromatographed on a DEAE-Sephadex A-25 column equilibrated in chloroform/methanol/water (30:60:5) as previously described (8) so as to separate neutral glycolipids from gangliosides. This neutral glycolipid fraction was purified by HPLC (Gilson, France) with an Inertsil column (Iatron Laboratories, Japan) column. Glycolipids were eluted in chloroform/methanol gradients ranging from 90:10 to 65:35. Individual glycolipids were further purified on high performance liquid chromatography (HPTLC) plates (E. Merck AG, Federal Republic of Germany) developed in chloroform/methanol/0.02% aqueous CaCl₂ (5:2:4).

Labeling Cells—In some experiments, cells were labeled with [1-¹³C]Gal (6.9 Ci/mmol, Amersham) at a concentration of 10 μCi/ml for 24 h. Neutral glycolipids were prepared from the chloroform/methanol extract of the cells as described above and analyzed by HPTLC. Glycolipid bands were detected by fluorography for 40 days at -80 °C using Kodak X-Omat film after soaking the plate in diethyl ether solution of 2,5-diphenyloxazole.

Saccharide Composition Analysis—Saccharide compositions of glycolipids were analyzed by the modified method of Honda et al. (9). Briefly, a glycolipid sample was hydrolyzed in 2.5 N trifluoroacetic acid for 5 h at 100 °C. The solvent was evaporated, and the residue was redissolved with 50 μl of water. An aliquot of the solution was injected into an HPLC system (Gilson, France) equipped with a CDR-10 column (Mitsubishi Kasei Corp., Japan). The separated monosaccharides were postlabeled with 1% 2-cyanoacetamide and detected by a voltmeter detector (VMD-901, Yanako, Japan).

Fatty Acid Analysis—Glycolipids were treated with 5% HCl in methanol at 80 °C for 11 h, and the fatty acid methyl esters thus produced were analyzed by gas-liquid chromatography (GLC). GLC was carried out with a GC-5A chromatograph and CBF-10 capillary column (Shimadzu, Japan). The fatty acid methyl ester mixture used

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1 The abbreviations used are: Gal-Gb, III⁺Galα-globotriaosylceramide; HPTLC, high performance thin layer chromatography; HPLC, high performance liquid chromatography; CTH, ceramide trihexoside; Gb, globotetraosylceramide; GA1, asialoligomannosylhexaosylceramide.

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Fig. 1. TLC patterns of glycolipid composition of chloroform/methanol extracts of PC12 cells. A, high performance thin layer chromatogram developed in chloroform/methanol/0.02% aqueous CaCl₂ (65:25:4) and visualized by orcinol/H₂SO₄ reagent. B, fluorograph of high performance thin layer chromatogram of the extract from metabolically labeled cells with [³H]Gal developed in chloroform/methanol/0.02% aqueous CaCl₂ (50:50:6). Abbreviations: Chol, cholesterol; PC, phosphatidylcholine; PS, phosphatidylserine; CM₁:₂, chloroform/methanol (1:2) extract of PC12 cells; CM₂:₁, subsequent chloroform/methanol (2:1) extract of PC12 cells; Gal Cer, galactosylceramide; Glc Cer, glucosylceramide; Lac Cer, lactosylceramide; CTH, ceramide trihexoside obtained from porcine erythrocytes; GA₁, asialogangliotetraosylceramide obtained from bovine brain; Gb₄, globotetraosylceramide obtained from porcine erythrocyte; Sul, sulfatide. * indicates the position of cold Gal-Gb₄.

Fig. 2. Negative ion FAB-mass spectrum of Gal-Gb₄. A JEOL JMS HX-100 mass spectrometer (JEOL Ltd., Japan) was used. A glycolipid sample dissolved in chloroform/methanol (2:1) was mixed with triethanolamine and 1,1,3,3-tetramethylurea as sample holder, and the analysis was performed by detecting negative ions according to Arita et al. (12).

Methylation Analysis—Methylation of glycolipids was performed by the method of Hakomori (10), and glycosyl linkages were analyzed according to Waeghe et al. (11). A mixture of partially methylated alditol acetates was analyzed by GLC-MS. For GLC-MS analysis, a JEOL JMS HX-100 mass spectrometer (JEOL Ltd., Japan) and MS- GC06 gas chromatograph (JEOL Ltd., Japan) with fused silica capillary column (DB-5; J & W Scientific Co.) were used.

Partial Degradation of Gal-Gb₄—Gal-Gb₄ (about 1 mg) was incubated with α-galactosidase (from green coffee, Sigma, 250 milliunits) in 0.1 M acetate buffer (300 µl, pH 6.0) containing sodium taurodeoxycholate (1 mg/ml) at 37 °C for 10 h. The digest was extracted with chloroform, and the components thus obtained were separated by preparative TLC using HPTLC plates. Glycolipids were located by I₂ vapor. The remaining Gal-Gb₄ was recovered and digested with α-galactosidase again. The digest was fractionated in the same manner as above. The resulting CTH was collected and subjected to 1H NMR spectroscopic analysis and then to methylation analysis.

Determination of the Saccharide Sequence of Gal-Gb₃—Gal-Gb₃ (50 nmol) was digested with endoglycoceramidase (total, 12 milliunits) prepared as described below in 0.1 M acetate buffer (pH 6.0), containing 1 mg/ml sodium taurodeoxycholate, 50 µl) for 3 days at 37 °C. Chloroform/methanol (2:1, 1 ml) was added to the digest. The solution was shaken and centrifuged at 2000 rpm for 10 min. The upper phase containing the resulting oligosaccharide was removed, lyophilized, and pyridylaminated according to the method of Hase et al. (13). The 2-aminopyridyl oligosaccharide was purified by Sephadex G-15 column chromatography (13) and further purified by HPLC in the same manner for analysis of 2-aminopyridyl oligosaccharides as described below. Two-fifths of the 2-aminopyridyl oligosaccharide were used to determine the saccharide sequence of Gal-Gb₃. The 2-aminopyridyl oligosaccharide was dissolved in 10 µl of 0.05 M acetate buffer (pH 5.5) and digested with α-galactosidase (0.5 unit, from green coffee, Sigma) for 1 day at 37 °C. It was further digested with β-galactosidase (0.5 unit, from Escherichia coli, Sigma) for 1 day at 37 °C. These reactions were monitored by HPLC analysis, using a Trisrotar VI HPLC system (JASCO, Japan), TSK gel NH₂60 column (4.6 × 260 mm, Tosoh, Japan), and FP-210 fluorescence spectrometer (JASCO, Japan), under the following conditions: elution, CH₃CN/H₂O linear gradient ranging from 75:25 to 20:80; flow rate, 0.5 ml/min; column temperature, 43 °C. Detection was conducted by a fluorescence spectrometer with excitation at 310 nm and emission at 400 nm.

Preparation of Endoglycoceramidase—Endoglycoceramidase was isolated from the culture filtrate of Rhodococcus sp. G-74-2 as described previously (6). Endoglycoceramidase was further purified by
DEAE- and octyl-Sepharose column chromatography in order to remove contaminating oligosaccharides at the final stage of enzyme preparation.

RESULTS

Glycolipid Composition of PC12 Cells—PC12 cells were extracted with chloroform/methanol (2:1 and 1:2), and the components obtained were developed on HPTLC. Fig. 1A shows clearly the appearance of phospholipids and cholesterol. In addition, a major band of a glycolipid, judging from its characteristic color, is present nearly at the position of the Gal, isolated from porcine erythrocytes. Although this glycolipid (tentatively named Gal-Gb) was predominant on the TLC plate (Fig. 1A), another faint spot representing another glycolipid was also noted at the position of the marker, asialo-gangliotetraosylceramide (GA1). Because of its scanty amount, this glycolipid is not dealt with in this paper. The occurrence of the glycolipid, Gal-Gb, was also noted at the position of the marker, asialo-gangliotetraosylceramide (GA1). Because of its scanty amount, this glycolipid is not dealt with in this paper. The occurrence of the glycolipid, Gal-Gb, was confirmed by the labeling experiment of PC12 cells with 1-[^3H]Gal. PC12 cells metabolically labeled with 1-[^3H]Gal were extracted with chloroform/methanol and processed in the same manner as the unlabeled cells to prepare the neutral glycolipid fraction. It was developed on HPTLC, and glycolipids were detected by fluorography (Fig. 1B). A pair of bands (due to the heterogeneity of the ceramide portion), one clear and the other weak, was observed, its position coinciding with that of the unlabeled Gal-Gb. To characterize the main glycolipid of PC12 cells, Gal-Gb was isolated from unlabeled PC12 cells and analyzed for detailed structure.

Isolation and Characterization of Gal-Gb—Chloroform/methanol extracts of PC12 cells were fractionated by HPLC equipped with an Iatrobeads column. The eluates were monitored by TLC, and the fractions containing Gal-Gb were combined. Gal-Gb was further purified by preparative HPTLC. From about 1 g (wet weight) of cells, 0.1 mg of Gal-Gb was obtained. When analyzed for saccharide composition, only galactose and glucose in the ratio of 3:1 were found present. Considering this and its mobility on HPTLC, the new glycolipid (Gal-Gb) may be reasonably concluded to be ceramide tetrahexoside. This was confirmed by FAB-mass spectrometry (Fig. 2) and $^1$H NMR spectroscopy (Fig. 3). The molecular ion (M – H)$^-$ was observed at m/z 1184, representing ceramide tetrahexoside with fatty acid composition (16:0) and sphingosine base composition (18:1). Also, molecular ions were observed at m/z 1212, 1240, at intervals of 28 due to heterogeneity in fatty acid and sphingosine base lengths with (CH$_2$)$_n$ units. Analysis by GLC indicated C16 and C18 fatty acids to be the main components. The $^1$H NMR spectrum of Gal-Gb showed four signals for anomeric protons. Signals at 4.903 ppm ($J = 3.64$ Hz) and 4.886 ppm ($J = 3.85$ Hz) were assigned to $\alpha$-anomeric protons. Signals at 4.303 ppm ($J = 7.49$ Hz) and at 4.194 ppm ($J = 7.69$ Hz) were assigned to $\beta$-anomeric protons. Olefinic protons peculiar to ceramide were observed at 5.3–5.6 ppm. From these results, it is clearly evident that Gal-Gb is ceramide tetrahexoside.

Determination of Saccharide Sequence of Gal-Gb—Gal-Gb was digested with endoglycoceramidase (6) followed by pyridylaminating oligosaccharide thus obtained (13). The 2-aminopyridyl oligosaccharide was digested with a series of exoglycosidases. The digests were separated by HPLC, and the results are shown in Fig. 4. The 2-aminopyridyl oligosaccha-
precisely identical with the latter except for several peaks due to nonreducing end galactose and penultimate α-galactosyl residues (Galα1-3Galα1-4Galα1-4Glcα1-1Cer) of the glycolipid were only empirically assigned by chemical shifts of the H-1 protons of permethylated glycolipid in NMR spectroscopy (17). In contrast, our conclusion is based unambiguously on linkage analyses on the degradation product (CTH) of Gal-Gb

PC12 cells used in the present study are a good source of the glycolipid with the unusual structure, Galα1-3Galα1-4Galα1-4Glcα1-1Cer, since it is the major glycolipid in PC12 cells that can be used as an immunogen to obtain new classes of monoclonal antibodies as reported recently (18).

Very slight amounts of glucosylceramide, CTH, and an unidentified glycolipid migrating nearly at Galα1 on an HPTLC plate were found in PC12 cells along with Gal-Gb. Interestingly, the glycolipid compositions of PC12 cells determined by us do not agree with the previous report of Schwarting et al. (5), who showed Glcα1(GalαGalNAcα3Galα1-4Galβ1-4Glcβ1-1Cer) to be most abundant. It is known that the nature of PC12 cells transforms in part during repeated subcultures and subclones have been established (17-22). This discrepancy may possibly arise from differences in the subclones of the PC12 cells used. Differences in glycolipid compositions may also be due to the particular experimental conditions used. Our PC12 cells were cultured as a monolayer, but cells were grown in a spinner culture by Schwarting et al. (5). It is clear that we have subclones of PC12 cells whose glycolipid compositions are mutually quite different. Taking into consideration recent information on the participation of glycolipids in cell differentiation, especially in that of PC12 cells (23-25), these subclones can be used to resolve the relationships between cell differentiation and membrane glycolipids. We recently reported the presence of a glucose polymer in the same PC12 cells used in this study (26). Mutual glycolipid compositions are quite different from each other, and the presence of the glucose polymer is so unique to PC12 cells that a search for the glucose polymer in the PC12 strain of Schwarting et al. (5) may yield significant results.

We have developed a new method in which the specificity of endoglycoceramidase (6) is used for the determination of the saccharide sequence of PC12 glycolipids. For this purpose, endoglycoceramidase was further purified from contaminating

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**DISCUSSION**

Our present data show the structure of the major glycolipid in PC12 cells to be Galα1-3Galα1-4Galα1-4Glcα1-1Cer. A glycolipid having this structure was previously reported by Ångström et al. (17), although apparently without sufficient evidence to substantiate its structure. They isolated the glycolipid from rat small intestine as a minor component and postulated its structure to be Galα1-3Galα1-4Galα1-4Glcα1-1Cer, based on NMR measurement. The saccharide linkages of the nonreducing end and penultimate α-galactosyl residues (Galα1-3Galα1-4 . . . ) of the glycolipid were only empirically assigned by chemical shifts of the H-1 protons of permethylated glycolipid in NMR spectroscopy (17). In contrast, our conclusion is based unambiguously on linkage analyses on the degradation product (CTH) of Gal-Gb.

The PC12 cells used in the present study are a good source of the glycolipid with the unusual structure, Galα1-3Galα1-4Galα1-4Glcα1-1Cer, since it is the major glycolipid in PC12 cells that can be used as an immunogen to obtain new classes of monoclonal antibodies as reported recently (18).

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We have developed a new method in which the specificity of endoglycoceramidase (6) is used for the determination of the saccharide sequence of PC12 glycolipids. For this purpose, endoglycoceramidase was further purified from contaminating
oligosaccharides in the previous preparations. When a glycolipid is digested with endoglycoceramidase, the intact oligosaccharide and ceramide are obtained at the same time (6). The resulting reducing end of oligosaccharide can be labeled with 2-aminopyridine (13). A 2-aminopyridyl oligosaccharide thus obtained can be detected by a fluorophotometer at high sensitivity. The saccharide sequence can be determined by digestion with a series of specific exoglycosidases. The saccharide sequence of the new glycolipid from PC12 cells was determined only with 20 nmol of the sample in the present study. The amount of exoglycosidases to digest the material must be made as little as possible in this highly sensitive analysis of saccharide sequence since fluorescent contaminants are present in enzyme samples. Another advantage of the present method is the increased susceptibility of the sample to digestion with glycosidases as compared with the intact glycolipid. In the future, this method should greatly facilitate the sequencing of glycolipid oligosaccharides.

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