Accumulation of a Globo-Series Glycolipid Having Galα1–3Gal in PC12h Pheochromocytoma Cells†

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ABSTRACT: In a previous paper, we reported the presence of globoside as a major neutral glycolipid in PC12 pheochromocytoma cells [Ariga, T., Macala, L. J., Saito, M., Margolis, R. K., Greene, L. A., Margolis, R. U., & Yu, R. K. (1988) *Biochemistry 27*, 52–58]. Recently, we found that subcloned PC12h cells accumulated another unusual neutral glycolipid. In order to characterize this glycolipid, PC12h cells were subcutaneously transplanted into rats. The induced tumor tissue accumulated two major neutral glycolipids, which were purified by Iatrobeads column and preparative thin-layer chromatographies. One of the glycolipids was found to be globoside, and the other had a globotriaosyl structure with an additional terminal Galα1–3 residue. Its structure was determined by fast atom bombardment mass spectrometry, two-dimensional proton nuclear magnetic resonance spectrometry (2D NMR), permethylation study, sequential degradation with exoglycosidase, and mild acid hydrolysis to be Gal(α1–3)Gal(α1–4)Gal(β1–4)Glc(β1–1′)Cer.

Glycolipids are important constituents of living cells and are generally located on plasma membranes (Fishman & Brady, 1976; Hakomori, 1981). The carbohydrate chains in glycolipids are exposed at the cell surface and can play several roles, e.g., as receptors for ligands and in cell–cell interaction and cellular adhesion (Lee et al., 1982; Hakomori & Kannagi, 1983; Hakomori et al., 1984; Westrick et al., 1983). The glycolipid composition changes dramatically during differentiation and oncogenic transformation (Hakomori & Kannagi, 1983; Hakomori et al., 1984). PC12 pheochromocytoma cells, a clonal cell line derived from rat adrenal medullary tumor tissue, display many properties associated with normal adrenal chromaffin cells (Greene & Tischler, 1976; Burnstein & Greene, 1978). We previously reported the structures of several complex glycolipids that are accumulated in PC12 cells (Yu et al., 1985; Ariga et al., 1987; Ariga et al., 1988). In particular, we found the presence of several fucogangliosides and blood group B active gangliosides, and these gangliosides might be expressed by the activation of α-fucosyltransferase and/or α-galactosyltransferase (Ariga et al., 1987). The increased levels of fucogangliosides in response to nerve growth factor (NGF) treatment may reflect a molecular event underlying neural differentiation (Margolis et al., 1983, 1984). In addition, we (Ariga et al., 1988) and Schwarting et al. (1986) also found globotetraosylceramide (globoside) as the major neutral glycolipid in PC12 cells maintained in culture. In the present paper, we describe the characterization of the major neutral glycolipids of subcloned PC12h cells and changes in the glycolipid composition during subcloning.

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Experimental Procedures

Cell Culture and Transplanted Tumor Tissues. PC12 cells were kindly provided by Drs. L. Greene and R. U. Margolis, New York University, New York. PC12h cells were subcloned by Dr. H. Hatanaka, Mitsubishi Kasei Institute of Life Science, Machida, Japan (Hatanaka, 1981). This cell line was maintained in The Tokyo Metropolitan Institute for Neuroscience, Fuchu, Japan. Cells were grown in polystyrene culture dishes in RPMI 1640 medium (Gibco) containing 10% heat-inactivated horse serum and 5% fetal calf serum (Margolis et al., 1983). Batches of cells were rinsed free of medium with phosphate-buffered saline and then harvested by scraping with a rubber policeman. PC12h cells, ca. 1×10^7, were injected subcutaneously into New England Deaconess Hospital rats, which were kindly provided by Dr. Kazuo Honda, Yamanouchi Pharmaceutical Co. When the tumor had grown to ca. 2.5 cm in diameter, the animals were killed. The tumors were removed and then stored at −20 °C until used.

Isolation of Neutral Glycolipids. The isolation procedures for the neutral glycolipids in the cells were described previously (Macala et al., 1983; Ariga et al., 1988). Briefly, total lipids were extracted from the cells, ca. 1×10^8, with 5 mL of chloroform/methanol (2:1 and 1:1 v/v) and 5 mL of chloroform/methanol/water (30:60:8 v/v) and filtered. The combined filtrates were adjusted to a final concentration of chloroform/methanol/water (30:60:8 v/v) by adding appropriate amounts of methanol and water. The extracts were then applied to a DEAE-Sephadex A-25 column (acetate form, 3-mL bed volume), which was further eluted with 30 mL of chloroform/methanol/water (30:60:8 v/v). The neutral glycolipids were eluted in this fraction.

The tumor tissues, 100 g, were homogenized in 1 L of chloroform/methanol (2:1 v/v), followed by successive extraction with 1 L each of chloroform/methanol (1:1 and 1:2 v/v) and methanol. The combined extracts were evaporated, dissolved in 2 L of chloroform/methanol/water (8:4:3 v/v), and then partitioned. The lower chloroform layer was washed twice with Folch's theoretical upper phase (Folch et al., 1957).

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In order to remove the gangliosides from the neutral lipid fraction, we used a DEAE-Sephadex A-25 column as follows: The lower chloroform layer was evaporated to dryness. The residue was dissolved in 300 mL of chloroform/methanol/water (30:60:4.5 v/v) and then applied to a DEAE-Sephadex A-25 column (acetate form, 200-mL bed volume), which was further eluted with 1 L of methanol. The neutral glycolipids were eluted in this fraction. They were evaporated to dryness. The dried material was dissolved in 200 mL of 0.2 M NaOH in methanol and incubated for 2 h at 40 °C (Ando & Yu, 1977). After removal of the released free fatty acids by the addition of *n*-hexane, the methanolic solution was evaporated, and the residue was suspended in distilled water and then dialyzed against water. The retentate was lyophilized, and the dried material was dissolved in a small amount of chloroform/methanol (4:1 v/v) and then divided into three portions. One portion was applied to an Iatrobeads column (42 g, 1.2 cm × 120 cm) and eluted with 800 mL of a linear gradient elution system prepared from chloroform/methanol/2.5 N ammonium hydroxide (70:30:1 and 45:55:4 v/v). The effluent was collected in fractions of 7 mL. Final purification of the neutral glycolipids was achieved by preparative thin-layer chromatography (TLC).[1] Briefly, the TLC plates were developed with chloroform/methanol/2.5 N ammonium hydroxide (55:50:10 v/v). The bands were scraped separately from the TLC plates, and each glycolipid was extracted successively with 5 mL each of chloroform/methanol (1:2 and 1:4 v/v) and methanol. The extracts were combined and then evaporated to dryness. The dried residue containing crude glycolipids was dissolved in a small amount of chloroform/methanol (4:1 v/v) and then applied to an Iatrobeads column (0.5 cm × 15 cm). The column was eluted stepwise with 15 mL each of chloroform/methanol (4:1, 2:1, 1:1, and 1:2 v/v). The effluent was collected in fractions of 2 mL. The pure glycolipid was recovered in the chloroform/methanol (1:1 v/v) fraction.

Analytical Procedures. Compositional analysis was carried out by gas–liquid chromatography (GLC) and nuclear magnetic resonance spectrometry (NMR). Neutral sugars, amino sugars, fatty acids, and long-chain bases were analyzed as described previously (Ariga et al., 1982). After the neutral glycolipids had been subjected to methanolysis, the sugars and fatty acid methyl esters were trimethylsilylated and then analyzed by GLC on a fused silica capillary column (0.32 mm × 25 m) of methylsilicone, with the temperature programmed at a rate of 2 °C/min from 180 to 250 °C.

Exoglycosidase Digestion and Mild Acid Hydrolysis. A neutral glycolipid, ~0.5 mg, was dissolved in 200 μL of 0.1 M sodium citrate buffer (pH 5.0) containing 10% sodium taurocholate and 30 μL of α-galactosidase (green coffee beans, EC 3.2.1.22; 50 units/1.2 mL of 3.2 M ammonium sulfate; Sigma Chemical Co., St. Louis, MO). The reaction mixture was incubated for 5 h at 37 °C, and then the reaction was terminated by the addition of 1.2 mL of chloroform/methanol (2:1 v/v). After the mixture was partitioned, the lower chloroform layer was washed twice with 0.5 mL of Folch's theoretical upper phase and then evaporated to dryness under nitrogen. The residue was dissolved in a small volume of chloroform/methanol (2:1 v/v) and then applied to a preparative TLC plate. After the plate had been developed with chloroform/methanol/2.5 N ammonium hydroxide (55:50:10 v/v), the glycolipid bands were visualized with iodine vapor and then scraped off. The glycolipids were purified as described above. In a separate experiment, a neutral glycolipid, ~20 μg, was subjected to mild acid hydrolysis in 0.3 mL of 0.1 N hydrochloric acid at 100 °C for 1.5 h. In addition, a neutral glycolipid, ~20 μg, was dissolved in 200 μL of 0.1 M citrate buffer (pH 5.0) containing 10% sodium taurocholate and 100 μL of β-hexosaminidase (green coffee beans, EC 3.2.1.30; 5 units/mL of 3.2 M ammonium sulfate; Sigma Chemical Co., St. Louis, MO). The reaction mixture was incubated at 37 °C for 18 h, and then the reaction was terminated by the addition of 1.5 mL of chloroform/methanol (2:1 v/v). After the mixture was partitioned, the lower chloroform layer was evaporated, and the glycolipid products obtained on mild acid treatment and exoglycosidase digestion were examined by TLC.

Permethylation Study. Permethylation of the neutral glycolipids was carried out by a modification of the procedure of Ciucanu and Kerek (1984). Briefly, a glycolipid sample, ~100 μg, was dissolved in 100 μL of dry dimethyl sulfoxide in a culture tube (1 × 10 cm) fitted with a Teflon-lined screw cap, and then 5 mg of powdered sodium hydroxide and 25 μL of dry methyl iodide were added under a nitrogen atmosphere in an ice bath. The reaction mixture was sonicated at 25 °C for 20 min, and the reaction was terminated by the addition of 3 mL each of chloroform and distilled water. After the upper aqueous layer had been removed, the chloroform layer was washed twice with 3 mL of distilled water and then evaporated to dryness under a nitrogen atmosphere. The permethylated glycolipid was purified on a minicolumn of Iatrobeads (0.2 g) with stepwise elution with 3 mL each of chloroform/*n*-hexane (1:1 v/v) and chloroform/methanol (4:1 v/v). The permethylated glycolipid was recovered in the chloroform/methanol fraction. Then, the permethylated glycolipid was hydrolyzed in 90% acetic acid containing 0.7 N hydrochloric acid at 80 °C for 18 h. The methylated sugars were reduced with sodium borohydride and then acetylated (Yang & Hakomori, 1971). The alditol acetate derivatives of sugars were analyzed by GLC–EI mass spectrometry (Shimadzu QP-1000) on a fused silica capillary column (0.32 mm × 25 m) of 5% (phenylmethyl)silicone, with the temperature programmed at a rate of 5 °C/min from 180 to 240 °C.

Negative Ion Fast Atom Bombardment (FAB) Mass Spectrometry. FAB mass spectra were obtained by a JEOL-HX 100 high-resolution mass spectrometer equipped with an FAB ion source and a JMA-3500 computer system (JEOL, Tokyo, Japan). Xenon gas was used at 6 kV as the ionization beam. A sample, 100 μg, was dissolved in 30 μL of chloroform/methanol (1:1 v/v). About 1 μL of the solution was placed on a stainless steel holder (1 × 4 mm). Triethanolamine/tetramethylurea (1:1 v/v), ~2 μL, was added, and then the sample was analyzed as described previously (Ariga & Yu, 1987).

Nuclear Magnetic Resonance (^1H NMR) Spectrometry. A sample, 1.5 mg, was dissolved in 0.5 mL of dimethyl-d_6 sulfoxide/D_2O (98:2 v/v). All spectra were obtained on a Bruker WH-500 spectrometer equipped with an Aspect 2000 computer, operating in the Fourier transform mode with quadrature detection. One-dimensional spectra were obtained at 313 K with 256 transients, 4K complex points, a sweep width of 3205 Hz, and a 1.2-s repetition rate. Two-dimensional *J*-correlated (COSY) spectra were obtained at 313 K with 96

[1] Abbreviations: FAB, fast atom bombardment; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; HPTLC, high-performance TLC; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; EI, electron impact ionization. The neutral glycolipid nomenclature used follows the system of the IUPAC–IUB Commission on Biochemical Nomenclature (1978).

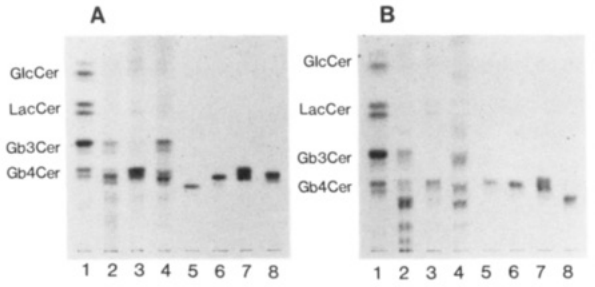

FIGURE 1: Thin-layer chromatograms of neutral glycolipids from PC12 cells, subcloned PC12h cells, and rat tumor tissue induced on PC12h cell transplantation. Lane 1, neutral glycolipid mixtures from pig erythrocyte membranes; lanes 2–4, neutral glycolipid fractions from subcloned PC12h cells, PC12 cells, and tumor tissue, respectively; lane 5, Forssman glycolipid from sheep erythrocyte membranes; lane 6, paragloboside from human erythrocyte membranes; lanes 7 and 8, isolated glycolipids G1 and G2 from tumor tissue induced on PC12h cell transplantation. The plates were stained with the orcinol/sulfuric acid reagent. Plate A was developed with chloroform/methanol/water (60:40:10 v/v) and plate B with chloroform/methanol/2.5 N ammonium hydroxide (55:50:10 v/v).

transients. Seven hundred t_1 points were obtained. Each t_1 point consisted of 1K complex points, a sweep width of 3205 Hz, and a 1-s repetition rate. Data were processed as magnitude spectra with an FTNMR program, written by Dr. D. R. Hare, running on a Vax 11-750 computer. Data were processed with sine-bell weighting functions and zero filling in both dimensions, respectively, to yield a 2K × 2K matrix. Two-dimensional pure absorption NOE (NOESY) spectra were obtained at 303 K with 96 transients. Quadrature was achieved in the t_1 dimension by means of time proportional phase incrementation (TPPI). Three hundred t_1 points were obtained. Each t_1 point consisted of 2K complex points, a sweep width of 3205 Hz, and a 3-s repetition rate. Data were processed with cosine-bell weighting functions in both dimensions and zero filling in the t_1 dimension to yield a 2K × 2K data matrix (Koerner et al., 1983, 1984; Scarsdale et al., 1986).

Results and Discussion

The PC12h cells were subcloned by Dr. H. Hatanaka, in which the tyrosine hydroxylase activity was significantly increased in the presence of the physiological concentration of nerve growth factor (Hatanaka, 1981). The neutral glycolipid patterns of parent PC12 cells, subcloned PC12h cells, and tumor tissue induced on PC12h cell transplantation are shown in Figure 1. The PC12 cells contained one major glycolipid, which cochromatographed with authentic globoside obtained from pig erythrocyte membranes on HPTLC with two different solvent systems (Ariga et al., 1988). However, the subcloned PC12h cells contained two glycolipids that cochromatographed with authentic globotriaosylceramide and globoside and three unknown glycolipids that accounted for approxiamtely 68.5% of the total neutral glycolipids, on the basis of the TLC pattern obtained on development with the basic solvent system (Figure 1B, lane 2). The major unknown glycolipid, which migrated to the ceramide tetrasaccharide region, accounted for 45% of the total neutral glycolipids. In order to characterize this glycolipid, we transplanted PC12h cells into rats. In the induced tumor tissue, three major glycolipids and two minor glycolipids were observed with the basic solvent system (Figure 1B, lane 4). The glycolipid patterns in the tumor tissue were quite similar to these in parent PC12h cells (Figure 1B, lane 2). The fast-migrating glycolipid cochromatographed with authentic globotriaosylceramide and the second fastest migrating glycolipid (G1) with globoside.

Table I: Compositional Analysis of the Isolated Neutral Glycolipids G1 and G2[a]

	molar ratio	
	G1	G2
GlC	1.00	1.00 (1.00)
Gal	1.79	2.77 (3.07)
GalNAc	0.85	
LCB	0.92	0.87
	%	
	G1	G2
fatty acid		
C16:0	15.8	14.0
C18:0	10.4	4.7
C20:0	17.6	14.7
C22:0	19.4	22.5
C23:0	2.2	
C24:1	8.0	13.0
C24:0	25.0	31.2

[a] LCB, long-chain base. The numbers in parentheses are based on NMR.

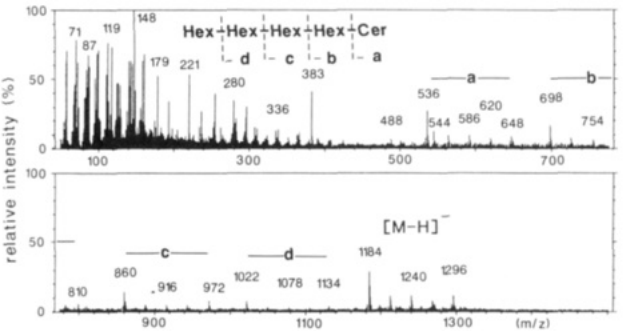

FIGURE 2: Negative ion FAB mass spectrum and fragmentation diagrams for glycolipid G2.

They accounted for 15% and 35.5% of the total neutral glycolipids, respectively. The tumor tissue also contained an unknown glycolipid (G2) associated with PC12h cells. It accounted for approximately 40% of the total neutral glycolipids. Attempts to isolate the two major glycolipids, G1 and G2, from the transplanted tumor tissue by Iatrobeads column chromatography with the basic solvent system or by TLC with the neutral solvent system were not successful. Final separation of these two glycolipids, G1 and G2, was achieved by preparative high-performance TLC with the basic solvent system. Figure 1 shows that these two glycolipids are homogeneous and each migrates as two bands. The yields of glycolipids G1 and G2, starting from 30 g of tumor tissue, were 2.7 and 3.2 mg, respectively. Table I shows the compositional analysis of the carbohydrates and fatty acids. Glycolipid G1 was found to contain galactose, glucose, *N*-acetylgalactosamine, and long-chain bases in a molar ratio of 2:1:1:1. Glycolipid G2 contained galactose, glucose, and long-chain bases in a molar ratio of 3:1:1, but it did not contain any hexosamine. These two glycolipids contained mainly palmitic, stearic, behenic, lignoceric, and nervonic acids but did not contain any α-hydroxy fatty acids (Table I). The long-chain bases consisted of mainly C18 sphingenine (over 93%) and small amounts of the C16 and C20 homologues.

Negative ion FAB mass spectrometry of the glycolipids can provide information on their molecular weights and sugar sequences. In addition, information with respect to their fatty acid and long chain base compositions can also be obtained (Ariga & Yu, 1987). The glycolipid G1 gave a mass spectrum identical with that of a major glycolipid (globoside) in the PC12 cells (Ariga et al., 1988). Figure 2 shows the negative

Table II: Chemical Shifts (ppm) and $J_{1,2}$ Coupling Constants (Hz) of Glycolipid G2

Galα1-3Galα1-4Galβ1-4Glcβ1-1′Cer
IV III II I R

	proton						
	1	2	3	4	5	6a	6b
residue							
I	4.16[a,b] ($J_{1,2}$ = 7.87)	3.03[a-c]	3.34[a,b]	3.29[b]	3.28[b]	3.60[a]	3.74[a]
II	4.26[a,b] ($J_{1,2}$ = 7.68)	3.31[a,b]	3.39[a,d]	3.82[a,b]	3.57[a,b]	3.58[b]	3.67[b]
III	4.84[a,b] ($J_{1,2}$ = 3.88)	3.75[a,b]	3.68[a,b]	3.97[a,b]	4.09[a,b]	3.49[a]	
IV	4.87[a,b] ($J_{1,2}$ = 3.65)	3.61[a,b]	3.64[a,b]	3.75[a,b]	4.01[a]	3.46[a]	3.52[a]

	proton						
	1a	1b	2	3	4	5	6
R	3.44[a,b]	3.95[a,b]	3.77[a,b]	3.87	5.35[a]	5.53[a]	1.92[a]

[a] Connectivity observed to resonance in COSY experiment. [b] Connectivity observed to resonance in pure absorption NOESY experiment. [c] ppm scale referred to I-2 resonance as 3.03 ppm. [d] Connectivity obscured by HDO resonance in NOESY experiment.

ion FAB mass spectrum of glycolipid G2. The mass spectrum showed prominent dehydrogenated molecular ions $(M - H)^-$, which corresponded to glycolipid molecular species containing fatty acids with chain lengths ranging from C16:0 to C24:0 and C18 sphingenine. Glycolipids G1 and G2 showed the same fragment ions corresponding to ceramide (a) and ceramide mono- (b), ceramide di- (c), and ceramide trisaccharides (d). However, in glycolipids G1 and G2, the terminal sugar was found to be hexose (m/z 162) and *N*-acetylhexosamine (m/z 203), respectively. The FAB mass spectral data suggested that glycolipids G1 and G2 were ceramide tetrasaccharides having different terminal sugars, as follows: G1, ceramide-Hex-Hex-Hex-HexNAc; G2, ceramide-Hex-Hex-Hex-Hex.

The results of mild acid hydrolysis and α-galactosidase digestion of the unknown glycolipid G2 showed the presence of two major glycolipid products, which cochromatographed with authentic lactosylceramide and globotriaosylceramide samples as shown in Figure 3, parts A and B. These glycolipid products were further purified by preparative TLC (see Experimental Procedures; Figure 3C). The results of α-galactosidase digestion suggested that the unknown glycolipid G2 contained 2 mol of α-D-galactopyranose in the molecule and had a lactosylceramide core structure. Glycolipid G2 did not hydrolyze with the β-galactosidase (EC 3.2.1.22) from green coffee beans (data not shown). Glycolipid G1 produced globotriaosylceramide after treatment with β-hexosaminidase (Figure 3D).

Analysis by capillary GLC–EI mass spectrometry of glycolipid G1 revealed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol (-3Gal1-), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol (-4Gal1-), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol (-4Glc1-), and 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxygalactitol (-0GalNAc1-) (Figure 4A). On the other hand, glycolipid G2 yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (-0Gal1-), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol (-3Gal1-), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol (-4Gal1-), and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol (-4Glc1-) (Figure 4B). For the glycolipid product, which migrated to the ceramide trisaccharide region after the α-galactosidase treatment, a peak corresponding to -3Gal1- was not observed (Figure 4C). In addition, the peak corresponding to -4Gal1- was lost in the case of the glycolipid product that cochromatographed with authentic lactosylceramide (Figure 4D).

The one-dimensional NMR spectrum of glycolipid G2 showed the presence of four protons in the anomeric region (4–5 ppm) (Figure 5 and Table II). From the chemical shifts and coupling constants, they were assigned as the anomeric

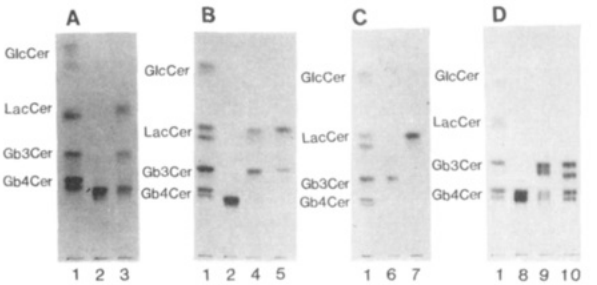

FIGURE 3: Thin-layer chromatograms of glycolipid products from glycolipids G1 and G2 after mild acid hydrolysis and exoglycosidase (green coffee beans) treatment. Lane 1, neutral glycolipid mixture from pig erythrocyte membranes; lane 2, isolated glycolipid G2; lanes 3 and 4, glycolipid products from glycolipid G2 after mild acid hydrolysis and α-galactosidase digestion, respectively; lane 5, glycolipid products from authentic globotriaosylceramide (Gb3) sample after α-galactosidase digestion; lanes 6 and 7, isolated glycolipid products from glycolipid G2 after α-galactosidase treatment; lane 8, isolated glycolipid G1; lanes 9 and 10, glycolipid products from glycolipid G1 and globoside after β-hexosaminidase treatment, respectively. The plates were developed with chloroform/methanol/2.5 N ammonium hydroxide (55:50:10 v/v), and the bands were visualized with the orcinol/sulfuric acid reagent.

Table III: NOESY Connectivities for Glycolipid G2

H-1	intra-ring	inter-ring	structure
I	I-3, I-5	R1a, R1b, R2	Glc(β1-1′)Cer
II	II-5, II-3[a]	I-4[b]	Gal(β1-4)Glc
III	III-2	II-4,[b] II-6a, II-6b	Gal(α1-4)Gal
IV	IV-2	III-3,[b] III-4[c]	Gal(α1-3)Gal

[a] Obscured by HDO resonance in NOESY experiment. [b] Trans glycosidic connectivity. [c] Linkage between IV-1 and II-4 does not allow simultaneous NOESY connectivities for IV-1 and the III-3,4 pair.

protons of α-Gal, α-Gal, β-Gal, and β-Glc, respectively, and the ratio of their relative intensities was approximately 1:1:1:1 (Table I and Figure 5). The ^{1}H NMR spectrum of glycolipid G2 was completely assigned on the basis of the results of a COSY (Figure 6 and Table II), which revealed scalar coupling (J) connectivities between pairs of protons. The resonance assignments of the residue types were confirmed on the basis of chemical shifts of anomeric protons and their coupling constants. The chemical shifts for residues I, II, and III were very similar to those for residues I, II, and III in globoside (Koerner et al., 1984).

The two-dimensional NOE (NOESY) experiment revealed connectivities between pairs of protons that were spatially proximate (Figure 7). This method is useful for determining the linkage sites and linkage stereochemistries of the various carbohydrate residues (Table III). The connectivities for residues I, II, and III were nearly identical with those for

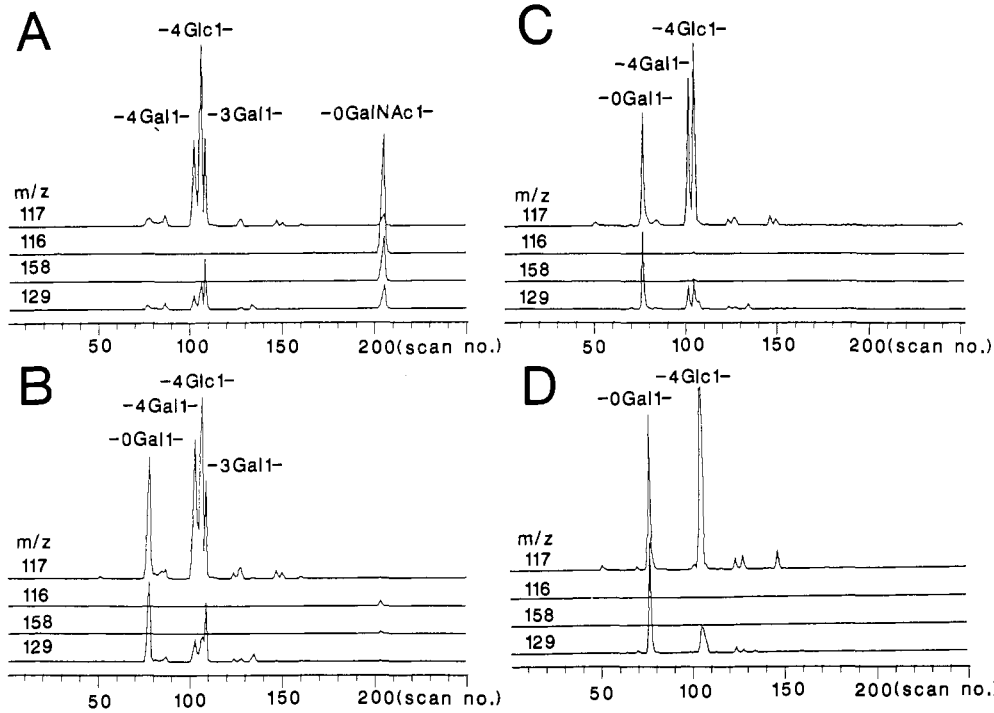

FIGURE 4: GLC–EI mass chromatograms of sugar alditol acetates from isolated glycolipids G1 and G2. The vertical axis shows relative intensity. (A) glycolipid G1; (B) glycolipid G2; (C) glycolipid product, which cochromatographed with globotriaosylceramide from glycolipid G2 by α-galactosidase treatment; (D) glycolipid product, which cochromatographed with lactosylceramide from glycolipid G2 obtained on α-galactosidase treatment.

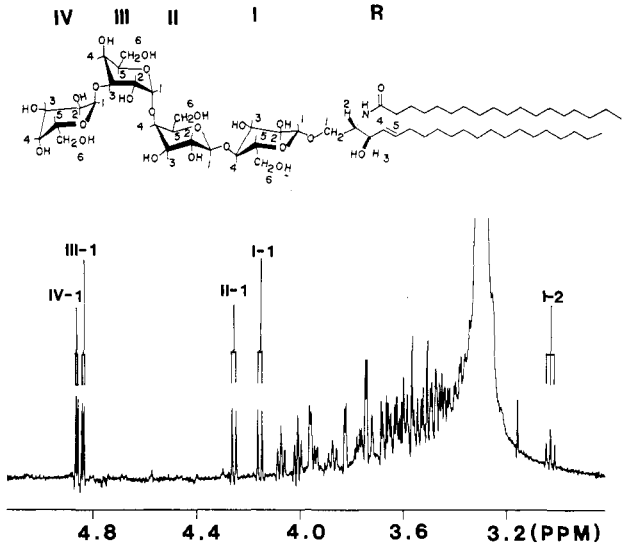

FIGURE 5: 490-MHz one-dimensional proton nuclear magnetic resonance spectrum of glycolipid G2.

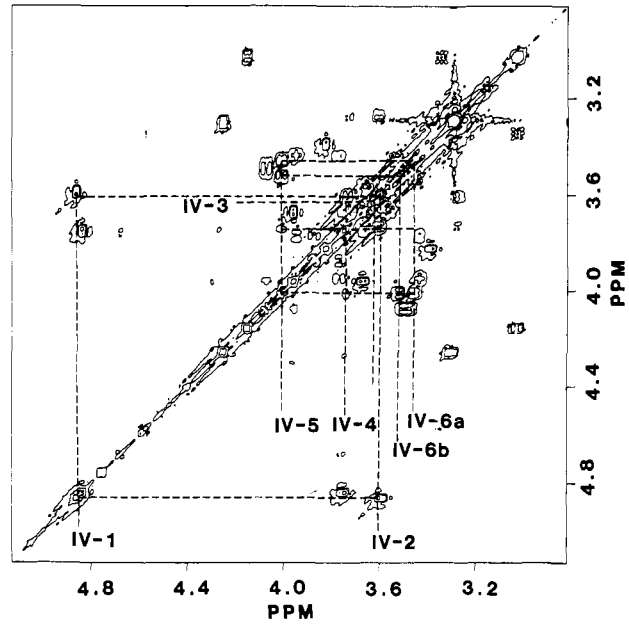

FIGURE 6: Contour plot of the two-dimensional proton nuclear magnetic resonance COSY spectrum showing the connectivities for residue IV of glycolipid G2.

residues I, II, and III in globoside (Scarsdale et al., 1986), suggesting that glycolipid G2 has a globotriaosyl (Galα1–4Galβ1–4Glcβ1–) core structure. Given the facts that residue IV is an α-D-galactopyranoside and that the presence of strong connectivities for both interresidue III-3 and III-4 is indicative of a Galα1–3Gal linkage, it is not possible that an anomeric proton becomes spatially proximate to both the 3 and 4 protons of an aglyconic residue in the case of a 1–4 diaxial linkage.

The combined data suggest that the two major glycolipids, G1 and G2, have the following structures: G1, GalNAc(β1–3)Gal(α1–4)Gal(β1–4)Glc(β1–1′)Cer or globotetraosylceramide (Gb4Cer); G2, Gal(α1–3)Gal(α1–4)Gal(β1–4)-Glc(β1–1′)Cer.

We previously showed that glycolipid G1 (globoside) is a major glycolipid in PC12 cells (Yu et al., 1985; Ariga et al., 1988), a finding consistent with that reported by Schwarting et al. (1986). In a preliminary report, Shimamura et al. (1987) also suggested the presence of glycolipid G2 in PC12 cells. Breimer et al. (1982) also tentatively characterized this unique glycolipid G2 and other minor complex glycolipids carrying a Galα1–3 determinant in normal rat small intestine. Metabolic labeling of the original PC12 cells with [^{3}H]galactose revealed the incorporation of unknown neutral glycolipids (Schwarting et al., 1986), and these glycolipids were increased by the treatment of nerve growth factor (Schwarting et al., 1986). In the light of the present results it is conceivable that glycolipid G2 may also be present in PC12 cells, albeit in much low concentrations (Figure 1B, lane 3). It is possible that this glycolipid G2 may be expressed on the induction of α-galac-

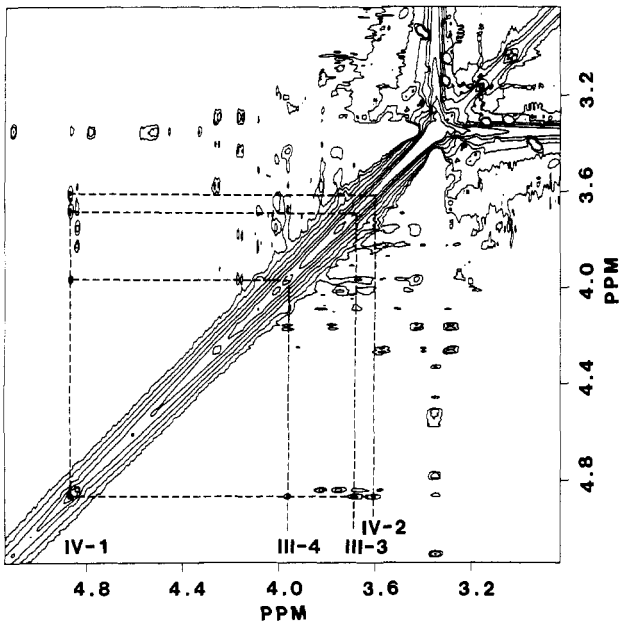

FIGURE 7: Contour plot of the two-dimensional proton nuclear magnetic resonance NOESY spectrum showing the connectivities for residue IV-1 of glycolipid G2.

tosyltransferase in PC12h cells and solid tumor cells. α-Galactosyltransferase, which catalyzes the synthesis of neutral glycolipids carrying the blood group B determinant from fucosylasialo-G_{M1} ganglioside, has been detected by Taki et al. (1985) in bone marrow cells. The accumulation of a unique glycolipid carrying a Galα1–3 determinant in subcloned PC12h cells may be involved in the activation of this transferase during cell differentiation. Since neurite outgrowth in subcloned PC12h cells is stimulated rapidly on nerve growth factor treatment (Hatanaka, 1981), the accumulation of this unique glycolipid or the activation of α-galactosyltransferase may be involved in the neurite outgrowth. Studies are in progress to explore this possibility.

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Registry No. Gal(α1–3)Gal(α1–4)Gal(β1–4)Glc(β1–1)Cer, 87501-64-2; globotetraosylceramide, 11034-93-8; globotriaosylceramide, 71965-57-6.

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