Glycosphingolipid Composition of Murine Neuroblastoma Cells: *O*-Acetylesterase Gene Downregulates the Expression of O-Acetylated GD3[†]

Toshio Ariga,[‡] Germaine M. Blaine,[‡] Hiide Yoshino,[‡] Glyn Dawson,[§] Takashi Kanda,[‡] Guichao C. Zeng,[‡] Takeshi Kasama,[∥] Yasunori Kushi,[∥] and Robert K. Yu*,[‡]

Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298, Department of Pediatrics, University of Chicago, Chicago, Illinois 60637, and Department of Biochemistry, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT: We have studied the glycosphingolipid composition in an F-11 neuroblastoma cell line originated from hybridization of a mouse neuroblastoma cell line (N18TG-2) with rat dorsal root ganglion cells. The total lipid-bound glucose of F-11 cells was estimated to be 0.28 μg/mg of protein and the total lipid-bound sialic acid was 0.82 μg/mg of protein. The major neutral glycosphingolipids were Gb4 (37% of the total neutral glycosphingolipids), Gb3 (15%), LacCer (21%), and GlcCer (15%). The major gangliosides were found to be GM3 (37% of the total gangliosides), GD3 (27%), O-acetylated GD3 (18%), and GD1a (4%), with trace amounts of GD2. The unusually high concentration of O-acetylated GD3 is consistent with its putative role as a tumor marker. Immunocytochemical localization studies of GD3 and O-acetylated GD3, examined by mouse monoclonal antibodies R24 and D1.1, respectively, revealed that the cell bodies and processes were all positively stained. To elucidate the role of O-acetylated GD3 in tumorigenesis, we transfected F-11 cells with the *O*-acetylesterase gene from influenza C virus. Compared with the original cell line, the transfected cells showed a dramatic increase in the level of GD3 (150% of that in the control cells) and a significant decrease of the concentration of O-acetylated GD3 (27% of control cells). In addition, the transfected F-11 cells exhibited a morphology different from the parental cells with enlarged cell bodies and elongated neurites. We conclude that alteration of ganglioside composition, particularly the expression of GD3 and O-acetylated GD3, may be associated with the morphological changes observed in this cell line. Furthermore, the study has provided direct evidence that cellular ganglioside expression can be manipulated by transfection of cells with a foreign gene and this approach may represent a powerful means of elucidating the biological and physiological functions of gangliosides.

Glycosphingolipids (GSLs) constitute part of the glycocalyx network of the cellular surface (Hakomori, 1994; Yu & Saito, 1989). Gangliosides, GSLs with one or more sialic acid residues, are important constituents of the plasma membrane and are particularly abundant in the nervous system (Yu & Saito, 1989; Yu, 1994). These cell surface glycoconjugates have been implicated in cell−cell recognition, neurite outgrowth, synaptogenesis, transmembrane signaling, and cellular growth and differentiation (Hakomori & Kannagi, 1983; Hakomori, 1994; Ledeen & Wu, 1993). Studies from many laboratories have indicated that the expression of the various GSLs is cell specific and developmentally regulated (Yu, 1994). Thus, certain stage-specific GSLs are expressed only transiently during specific proliferative phases of cellular maturation (Hakomori, 1994; Yu, 1994). For example, O-acetylated gangliosides are known to be abundant in proliferating cells of neuroectodermal origin and play a critical role in cellular proliferation and migration (Cheresh et al., 1984; Levine et al., 1984; Schlosshauer et al., 1988; Varki et al., 1991; Chou et al., 1990). It has also been shown that some of these early embryonic GSLs are reexpressed in proliferating tumor cells, and these "oncofetal antigens" have been considered as useful markers for these tumor cells (Hakomori 1994; Ariga et al., 1987). Alterations of the GSL content and pattern have been reported in transformed cells in culture and have been correlated with changes in cellular properties such as decreased contact inhibition and accelerated growth due to the expression of tumor-specific GSL antigens (Hakomori & Kannagi, 1983; Ariga et al., 1991). These changes, which may play an important role in tumor metastasis or cellular differentiation, are frequently accompanied by alterations in the metabolism of these tumor-specific GSL antigens (Hakomori & Kannagi, 1983).

The F-11 neuroblastoma cells are hybrid cells derived from a mouse neuroblastoma cell line (N18TG-2) and embryonic rat dorsal root ganglion (DRG) cells (Platika et al., 1985). They exhibit many properties characteristic of dorsal root ganglion neurons and show physiological responses to bradykinin, opiate agonists, and prostaglandins (Francel et al., 1987). In the present study, we analyzed the GSL composition of F-11 neuroblastoma cells and found that the ganglioside composition in F-11 cells was quite different from those of rat DRG cells (Chou et al., 1989; Ohsawa, 1990), which contain significant concentrations of b-series gangliosides such as GD1b, GT1b, and GQ1b. Most strikingly, this cell line expressed a high concentration of O-acetylated GD3 and GD3, which are known to be oncofetal

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[‡] Virginia Commonwealth University.
[§] University of Chicago.
[∥] Tokyo Medical and Dental University.
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antigens and whose expression is developmentally regulated in murine embryogenesis (Varki et al., 1991; Seyfried & Yu, 1985; Yu et al., 1988). In an attempt to correlate the unusually large amounts of O-acetylated ganglioside species with cellular differentiation, we transfected F-11 cells with the *O*-acetylesterase gene from influenza C virus. In this report we present evidence that this treatment could effectively downregulate the expression of O-acetylated GD3 and upregulate the expression of GD3 in this cell line with concomitant alterations in cellular morphology and neuritogenesis. Manipulation of glycolipid expression by molecular biological approaches should represent a powerful means for studying their biological and physiological functions.

MATERIALS AND METHODS

Materials. Neutral GSLs except nLc4 and gangliosides used as reference standards were isolated from pig erythrocyte membranes and human brains in our laboratories (Ando & Yu, 1979; Ariga et al., 1980). nLc4 was isolated from guinea pig erythrocyte membrane using similar procedures (Ando & Yu, 1979; Ariga et al., 1980). Fuc-GM1 was isolated from PC12 pheochromocytoma cells (Ariga et al., 1987) and GM3 from bovine adrenal medulla (Ariga et al., 1982). GD3 and O-acetylated GD3 were isolated from bovine butter milk and hamster melanoma (Ren et al., 1992, 1993). GlcCer was purified from the kidney tissue of a patient with Gaucher's disease. GA2 and GA1 were generated from GM2 and GM1, respectively, by the procedure of Kasai et al. (1982). The monoclonal antibody R-24 (recognizing GD3) was a generous gift from Dr. Kenneth Lloyd, Memorial Sloan-Kettering Cancer Center, New York, NY; the monoclonal antibody D1.1 (recognizing *O*-acetylated GD3) was from Dr. Joel Levine, SUNY, Stony Brook, NY; the monoclonal antibody 3F8 (recognizing GD2) from Dr. Nai-Kong V. Cheung, Memorial Sloan-Kettering Cancer Center, New York, NY. All reagents used were of analytical grade or higher. Solvents were freshly redistilled before use.

Cell Culture. The F-11 mouse neuroblastoma cells were placed on Falcon dishes (60 mm, Falcon, Franklin Lake, NJ) and grown in Dulbecco's Modified Eagle (Gibco, Gaitherburg, MD) medium supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD) and antibiotics (Gibco), as described previously (Dawson, 1979).

Isolation of Total Neutral Glycosphingolipids. The isolation procedures for the total lipids and neutral GSLs were described previously (Ariga et al., 1991). Briefly, the total lipids were extracted from the cells with chloroform–methanol (2:1, 1:1, v/v) and chloroform–methanol–water (solvent A; 30:60:8, v/v), successively. The combined extracts were then applied to a DEAE-Sephadex A-25 column (acetate form, 3-mL bed volume; Pharmacia, Uppsala, Sweden), which was further eluted with 15 mL of solvent A to elute neutral GSLs. The solvent was evaporated, and the dried neutral GSL fraction was redissolved in 0.5 mL of 0.3 M NaOH in methanol and incubated at 40 °C for 2 h in order to destroy alkali-labile phospholipids. The reaction mixture was desalted by passing through a Sephadex LH-20 (Pharmacia, 26-mL bed volume; 0.7-cm i.d. × 48 cm) with solvent A as the eluting solvent (Ariga et al., 1991). An aliquot of the GSL fraction was examined by high-performance thin-layer chromatography (HPTLC;[1] Merck, Darmstadt, Germany) with two different developing solvent systems: (A) chloroform–methanol–water (60:40:5, v/v), and (B) chloroform–methanol–2.5 N ammonium hydroxide (60:40:10, v/v). Neutral GSLs were visualized by spraying with the orcinol–sulfuric acid reagent and heating at 120 °C. Quantitation of neutral GSLs was carried out by gas–liquid chromatography (GLC) of the trimethylsilyl derivatives of the sugars with myoinositol as an internal standard according to the procedure of Vance and Sweeley (1967).

After recovery of the neutral GSLs from the DEAE-Sephadex A-25 column, the acidic GSL fraction was eluted with 10 mL of chloroform–methanol–0.8 M sodium acetate (30:60:8), v/v, solvent B). The acidic lipid fraction was evaporated to dryness, and the residue was redissolved in 0.5 mL of solvent A and then was desalted by Sephadex LH-20 column chromatography. A portion of this ganglioside fraction was applied to a minicolumn of DEAE-Sephadex A-25 to separate the mono-, di-, and polysialogangliosides fractions with increasing concentrations of ammonium acetate (20, 40, and 100 mM) in methanol. The recovered gangliosides were developed on a HPTLC plate with the solvent system of chloroform–methanol–water containing 0.22% $CaCl_2 \cdot 2H_2O$ (50:45:10, v/v, solvent system C) or chloroform–methanol–2.5 N ammonium hydroxide–0.4% $CaCl_2 \cdot 2H_2O$ (50:45:5:5, v/v, solvent system D). Gangliosides were visualized by spraying the plate with the resorcinol–HCl reagent followed by heating the covered plate at 100 °C for 30 min. In a separate experiment, the major gangliosides were isolated from a large-scale preparation of F-11 cells (equivalent to 200 mg of proteins) as described above. Further purification of the individual ganglioside species was achieved by preparative HPTLC using solvent system C. Each ganglioside band was scrapped from the TLC plate and extracted from the silica gel with solvent A. Final purification was performed on a small DEAE-Sephadex A-25 column as described above for gangliosides. Small aliquots of the cell homogenate were set aside for determination of the total protein.

HPTLC Overlay Method for Glycosphingolipids. HPTLC overlay for GSLs using specific anti-GSL antibodies was carried out as described previously (Saito et al., 1985). Gangliosides isolated from F-11 cells were developed on a HPTLC plate with solvent system C. After the plate was dipped in a 0.2% poly(isobutyl methacrylate) solution in *n*-hexane, the bands were overlaid with a solution of anti-GD3 (R24), anti-O-acetylated GD3 (D1.1), or anti-GD2 (3F8) monoclonal antibody at a dilution of 1:50 in 1% bovine serum albumin (BSA)–phosphate-buffered saline (PBS, pH 7.3) for 2 h at room temperature. After the plate was washed with PBS, the bands were covered with peroxidase-conjugated anti-mouse IgG or IgM (Cappel, Cooper Biomedical, Malvern, PA) at a dilution of 1:500 in 1% BSA–PBS and incubated for 2 h at room temperature. After further washing with PBS, the plate was then dipped in a solution of 4-chloro-1-naphthol (15 mg/25 mL of PBS containing 0.02% hydrogen peroxide) for a few minutes in order to

[1] Abbreviations: HPTLC, high-performance thin-layer chromatography; LSI, liquid secondary ion; PBS, phosphate-buffered saline. The nomenclature used for glycosphingolipids is based on that recommended by the IUPAC–IUB Commission on Biochemical Nomenclature [(1976) *Lipids 12*, 455] except gangliosides, which are abbreviated according to Svennerholm [(1964) *J. Lipid Res. 5*, 145].

detect GD3, O-acetylated GD3, or GD2. In a separate experiment, the plate was developed with solvent system C and then treated with *Vibrio cholerae* neuraminidase *in situ* followed by incubation with peroxidase-conjugated cholera toxin B-subunit, which is a sensitive ligand to GM1 and fucosyl-GM1 (Fuc-GM1). The corresponding bands were scanned using a Shimadzu CS-910 scanning densitometer, and the integrated areas were computed by a Shimadzu CR1A data processor (Shimadzu, Kyoto, Japan). The integrated areas of various known amounts of standard gangliosides (10−500 ng), developed on the same plate, were used for quantification of each ganglioside.

Liquid Secondary Ion (LSI) Mass Spectrometry. LSI mass spectra were recorded in the negative ion mode on a TSQ 70 triple-stage quadruple mass spectrometer (Finnigan MAT, Inc., San Jose, CA), equipped with a cesium ion gun (Finnigan MAT). The accelerating voltage of the primary beam was maintained at 20 kV. Approximately 1 μg of sample, dissolved in 1 μL of chloroform−methanol (1:1, v/v), was loaded on the LSI mass spectrometry target with 0.5 μL of triethanolamine as the matrix (Kasama & Handa, 1991).

Immunocytochemistry of GD3 and O-Acetylated GD3 in F-11 Neuroblastoma Cells. Cultured F-11 cells on 35-mm dishes were fixed with 4% paraformaldehyde for 15 min and washed with PBS for at least three times. After incubation in PBS with 3% nonimmunized mouse serum, a mouse monoclonal anti-antibody (R24 or D1.1), diluted 1:100 in PBS, was applied at 4 °C for 72 h. The dishes were washed three times and FITC-conjugated anti-mouse immunoglobulin (Cappel, 1:200 dilution) was applied for 1 h at room temperature to visualize the labeled sites. The fluorescence was determined by a Nikon microscope, Model UFX-IIA (Nikon Inc., New York) (Kanda et al., 1995).

Plasmid Preparation and Cell Transfection. The plasmid pCHE4 was kindly provided by Dr. P. Palese of Mount Sinai School of Medicine, New York, NY, which contains the influenza C virus esterase gene (Vlasak et al., 1987). The plasmid was transformed into *Escherichia coli* DH5α and purified by a DNA purification system (Magic Minipreps, Promega). Transfection of F-11 cells was performed by the calcium phosphate precipitation method of Graham and van der Eb (1973) using Profection Mammalian Transfection System (Promega). F-11 cells, 5×10^6 cells, were placed in a 75-cm^2 flask 24 h before transfection and exposed to a precipitate of calcium phosphate formed in the presence of 5 μg of plasmid pRSV-Neo and 20 μg of pCHE4. After a 4-h exposure to the precipitate, the cells were treated with 15% glycerol in HBSP (140 mM NaCl, 10 mM KCl, 0.75 mM Na_2HPO_4, 6 mM dextran, 50 mM HEPES, pH 7.0) at 37 °C for 3 min, washed with PBS once, and cultured in the fresh medium until the cells grew to near confluency. Then, the cells were passed into Petri dishes and grown in the medium containing 600 μg/mL geneticin to select colonies resistant to the drug. Individual colonies were cultured and their gangliosides analyzed. Cell morphology was examined daily using an inverted microscope.

RESULTS

Neutral GSL Composition of F-11 Neuroblastoma Cells. The content of neutral GSLs in F-11 cells was estimated to be 0.28 ± 0.07 μg (mean ± SD, $n = 4$) of lipid-bound glucose (Glc) per mg of protein. F-11 cells contained globo-series GSLs, including glucosylceramide (GlcCer, 15.3 ± 2.6% of the total neutral GSLs), lactosylceramide (LacCer, 21.2 ± 0.9%), globotriaosylceramide (Gb3, 14.7 ± 2.4%), and globotetraosylceramide (Gb4, 37.3 ± 3.9%), which were characterized by comigration with authentic GSLs (Figure 1). The presence of Gb4 was confirmed by HPTLC

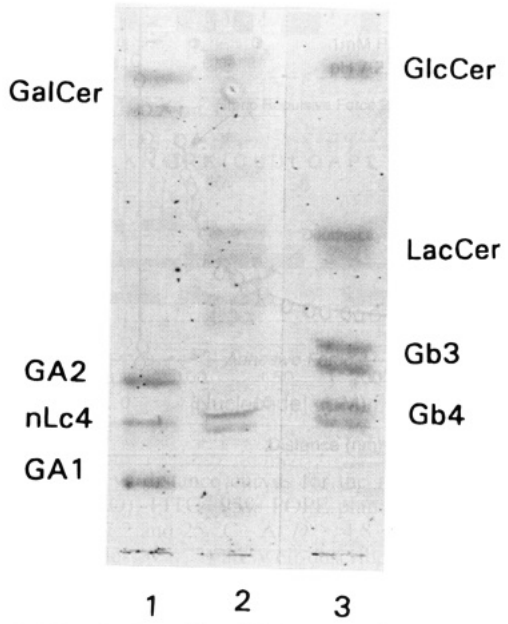


FIGURE 1: Neutral glycosphingolipid pattern of F-11 neuroblastoma cells: (lane 1) GalCer from human brain, asialo-GM2 (GA2) from human brain, nLc4 from guinea pig erythrocyte membrane, and asialo-GM1 (GA1) from human brain (from top to bottom); (lane 2) neutral GSLs isolated from F-11 cells; and (lane 3) GlcCer from the kidney of a patient with Gaucher's disease, LacCer, Gb3, and Gb4 from pig erythrocyte membrane. The developing solvent was solvent system B. The bands were visualized with the orcinol−sulfuric acid reagent.

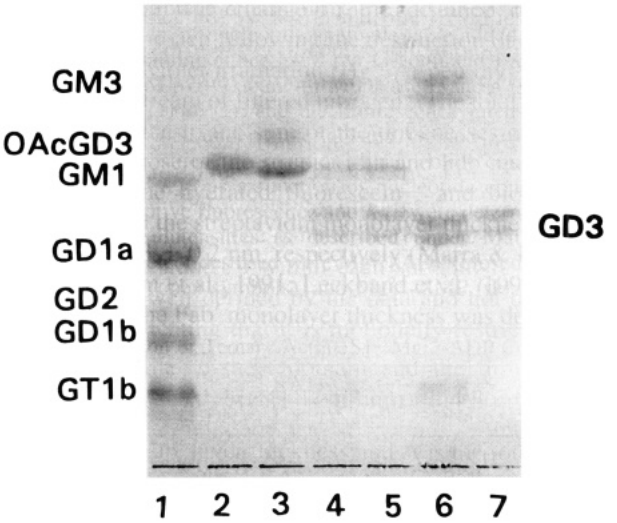


FIGURE 2: Ganglioside pattern of F-11 neuroblastoma cells. (lane 1) human brain ganglioside mixture; (lanes 2 and 3) O-acetylated GD3 from hamster melanoma cells and bovine butter milk (Ren et al., 1992, 1993), respectively; (lanes 4 and 5) total gangliosides and the disialoganglioside fraction of F-11 cells, respectively, based on 1.65 mg of protein of the original cell homogenate; (lanes 6 and 7) total gangliosides and the disialoganglioside fraction of F-11 cells following mild base treatment, respectively. The plate was developed with solvent system C, and gangliosides were revealed by spraying with the resorcinol−hydrochloric acid reagent.

Table 1: Ganglioside Composition of F-11 Neuroblastoma Cells[a]

	F-11 cells (% ± SD) (6)	transfected F-11 cells (% ± SD) (4)
GM3	36.8 ± 3.2	41.2 ± 3.1
GM2	trace	trace
LM1	trace	trace
GM1	2.3 ± 0.3	3.0 ± 0.2
OAc-GD3	17.9 ± 0.6	4.8 ± 0.3
GD3	27.0 ± 1.3	40.3 ± 0.3
GD1a	4.3 ± 1.6	2.6 ± 1.3
GD2	0.9 ± 0.2	0.7 ± 0.6
GD1b	trace	trace
GT1b	2.6 ± 1.3	2.5 ± 0.5
GQ1b	nd[b]	nd[b]

[a] Number in parentheses is the number of individual experiments. [b] nd, not determined.

immunostaining using an anti-Gb4 rabbit polyclonal antibody (data not shown).

Ganglioside Composition of F-11 Neuroblastoma Cells. The content of gangliosides in F-11 cells was estimated to be 0.82 ± 0.08 μg (mean ± SD, $n = 6$) of lipid-bound sialic acid (NeuAc) per mg of protein. F-11 cells contained three major lacto-series gangliosides, including GM3 (37% of the total gangliosides), GD3 (27%), and O-acetylated GD3 (18%), which were characterized by comigration with authentic samples of these gangliosides (Figure 2, lane 4, from top to bottom) (Table 1). The structures of these gangliosides were confirmed as follows. The bands corresponding to O-acetylated GD3 and GD3 were recovered in the disialoganglioside fraction (Figure 2, lane 5; see Materials and Methods). After treatment with 0.3 N NaOH in methanol at 40 °C for 1 h, the band corresponding to the authentic sample of O-acetylated GD3 disappeared (Figure 2, lanes 6 and 7). Identification of the major disialogangliosides, GD3 and O-acetylated GD3 was further achieved by the HPTLC overlay method using the mouse monoclonal antibodies R24 and D1.1, which are specific to GD3 and O-acetylated GD3, respectively (Figure 3). The ganglioseries gangliosides were characterized by *in situ* sialidase treatment of the total gangliosides on the HPTLC plate. Following this treatment, cholera toxin B-subunit recognized four minor gangliotetraose gangliosides, GM1 (2% of the total gangliosides), GD1a (4%), GD1b (<1%), and GT1b (2.6%), which comigrated with authentic samples of these gangliosides (data not shown). Only trace amounts of GD2 were detected using the mouse monoclonal antibody, 3F8, which is specific to GD2 (Saito et al., 1995) (data not shown). These data indicate that F-11 cells express at least four gangliotetraose gangliosides and a gangliotriaose ganglioside, GD2, as a minor component (Table 1).

Liquid Secondary Ion Mass Spectrometry. Three major gangliosides, GM3, O-acetylated GD3, and GD3, and a minor ganglioside, GD2, were isolated by preparative TLC from the mono- and disialoganglioside fractions of F-11 neuroblastoma cells, respectively (see Materials and Methods). These gangliosides comigrated with the respective authentic standards (Figure 3d,e). Their carbohydrate struc-

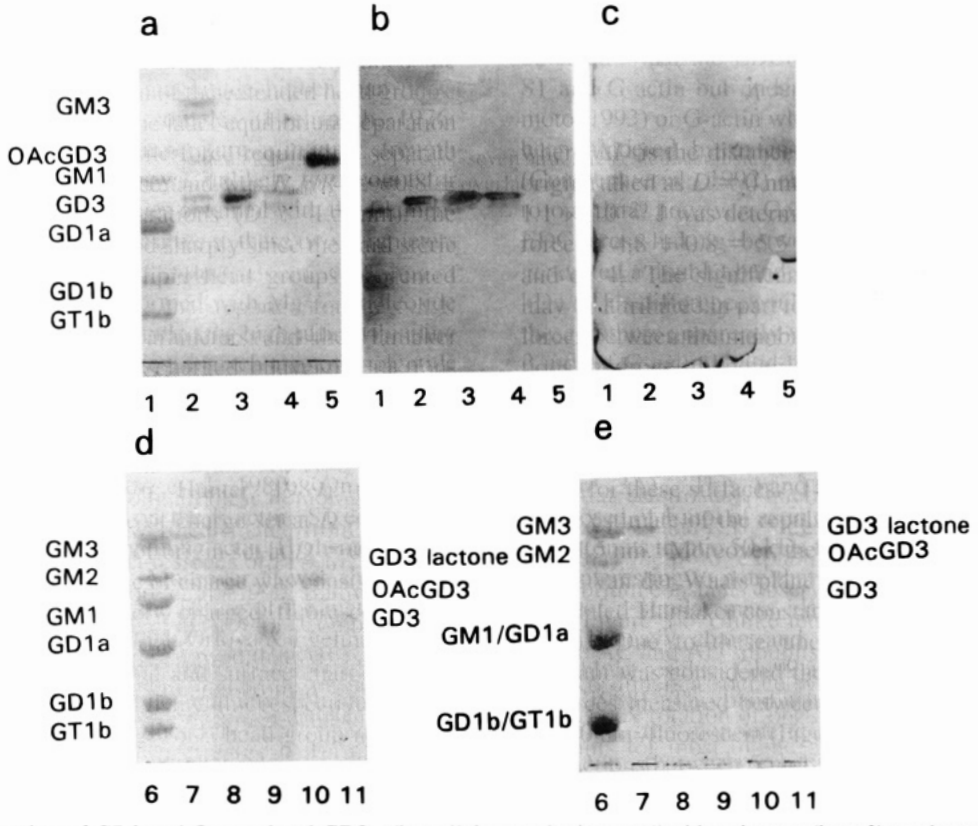


FIGURE 3: Identification of GD3 and O-acetylated GD3. (lane 1) human brain ganglioside mixture; (lane 2) total ganglioside pattern of F-11 neuroblastoma cells; (lane 3) standard GD3; (lane 4) disialoganglioside fraction of F-11 cells; (lanes 5 and 10) standard O-acetylated GD3 from bovine butter milk; (lane 6) GM3 from bovine adrenal medulla and human brain ganglioside mixture; (lanes 7−9) GM3, O-acetylated GD3, and GD3 isolated from F-11 cells, respectively; (lane 11) standard GD3-lactone (Maggio et al., 1990) and GD3 isolated from bovine butter milk (from top to bottom). Plates a−d were developed with solvent system C. Plate e was developed with solvent system D. Bands on plates a, d, and e were visualized by spraying with the resorcinol−hydrochloric acid reagent, and bands of plates b and c were immunostained with the mouse monoclonal antibody R-24, which is specific to GD3, and D1.1, which is specific to O-acetylated GD3, respectively.

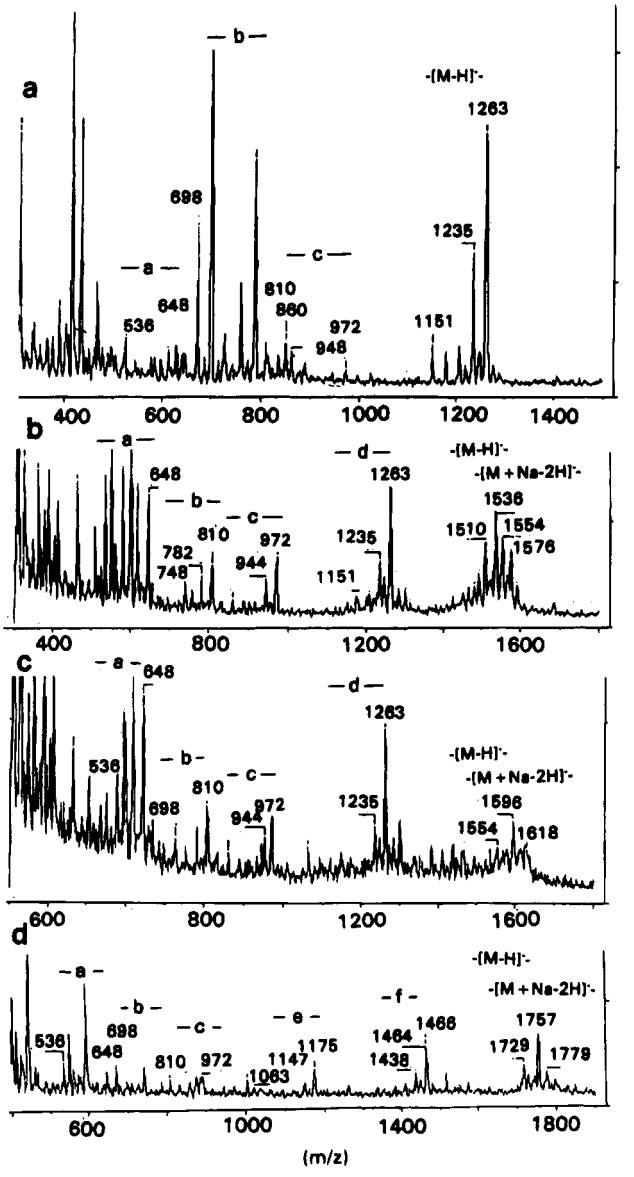
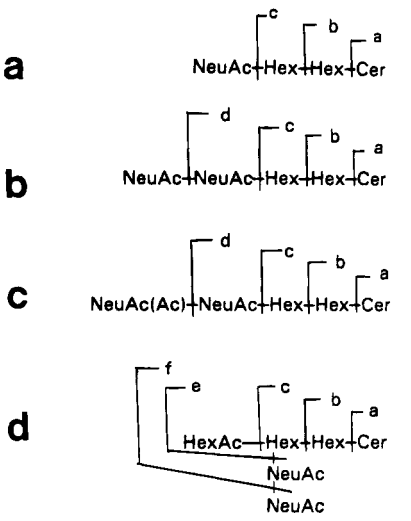


FIGURE 4: Liquid secondary ion mass spectrometry of gangliosides GM3 (a), GD3 (b), O-acetylated GD3 (c), and GD2 (d) isolated from F-11 neuroblastoma cells.

tures were further confirmed by negative ion LSI mass spectrometry as shown in Figure 4. The mass spectra of these gangliosides revealed the presence of prominent dehydrogenated molecular ions having C18 sphingosine and various fatty acids with chain lengths ranging from C16:0 to C24:1, which were composed of the major fatty acids, C22:0, C24:0, and C24:1. Fragmentation diagrams of these gangliosides are shown in Chart 1. All spectra demonstrated the fragment ions that are consistent with the molecular species of ceramide (Cer) (a, *m/z* 536 and 648), Cer-hexose (Hex) (b, *m/z* 698 and 810), and Cer-Hex-Hex (c, *m/z* 860 and 972). These ion groups are of diagnostic value in determining the structure of the lactose core in the molecule. In the mass spectrum of GM3, the prominent molecular ions $[M - H]^-$, *m/z* 1151, 1235, and 1263 corresponding to Cer-Hex-Hex-*N*-acetylneuraminic acid (NeuAc), provided information on the molecular weights of GM3 molecular species with C18 sphingosine and fatty acids, C16:0, C18:0, and C24:0, respectively (Figure 4a). These ions were also present in the spectra of GD3 and O-acetylated GD3 (Figure 4b,c). The quasi-molecular ions $[M - H]^-$ for GD3 and O-acetylated

Chart 1: Fragmentation Diagrams of Gangliosides in F-11 Neuroblastoma Cells



GD3, however, were shifted by 291 and 333 mass units from those observed for GM3, respectively, in which an additional NeuAc and mono-O-acetylated NeuAc molecule is attached to GM3 (Figure 4b,c). In addition to the dehydrogenated molecular ions $[M - H]^-$, the adduct ions $[M + Na - 2H]^-$ were also detected in the spectra of all of these disialoganggliosides. The fragment ions corresponding to the elimination of an acetyl group and an *N,O*-diacetylneuraminic acid unit from O-acetylated GD3 molecular ions are detected at *m/z* 1554 and 1263 (ion group d), respectively, which are consistent with mono-O-acetylated GD3 ganglioside with C18 sphingosine and fatty acid C24:0 (Figure 4c). The mass spectrum of GD2 revealed the presence of five sugars, as indicated by the presence of seven major ion groups, consistent with the molecular species of Cer (ion group a), Cer-Hex (b), Cer-Hex-Hex (c), Cer-Hex-Hex-hexosamine (HexAc) (e, *m/z* 1063 and 1175), and Cer-Hex-Hex-NeuAc (f, *m/z* 1354 and 1466) and two quasi-molecular ions, consistent with Cer-Her-Hex (NeuAc-NeuAc)-HexNAc, $[M - H]^-$ and $[M + Na - 2H]^-$. The prominent dehydrogenated molecular ions at *m/z* 1729 and 1757 should be consistent with GD2 and C18 sphingosine and fatty acids C22:0 and C24:0, respectively (Figure 4d). The adduct ion corresponding to $[M + Na - 2H]^-$ was also detected at *m/z* 1779, which contained C18 sphingosine and fatty acid C24:0.

Immunocytochemical Localization of GD3 and O-Acetylated GD3. Figure 5 shows the immunocytochemical localization of GD3 and O-acetylated GD3 using the mouse monoclonal antibodies R24 and D1.1, respectively. F-11 cells were 100% immunoreactive against these antibodies. Immunoreactivity was almost confined around the cell body and the long varicose processes; there was no difference in the localization between GD3 and O-acetylated GD3 at the light microscopic level.

Ganglioside Composition of Transfected F-11 Cells. Figure 6 shows the ganglioside composition of F-11 cells transfected with the *O*-acetylesterase gene from influenza C virus. The total lipid-bound sialic acid was 0.86 ± 0.2 μg/mg of protein, which was similar to that of the native F-11 cells. In contrast to the parent F-11 cells, however, the transfected cells contained predominantly GM3 and GD3. There were no significant changes in the content of GM3,

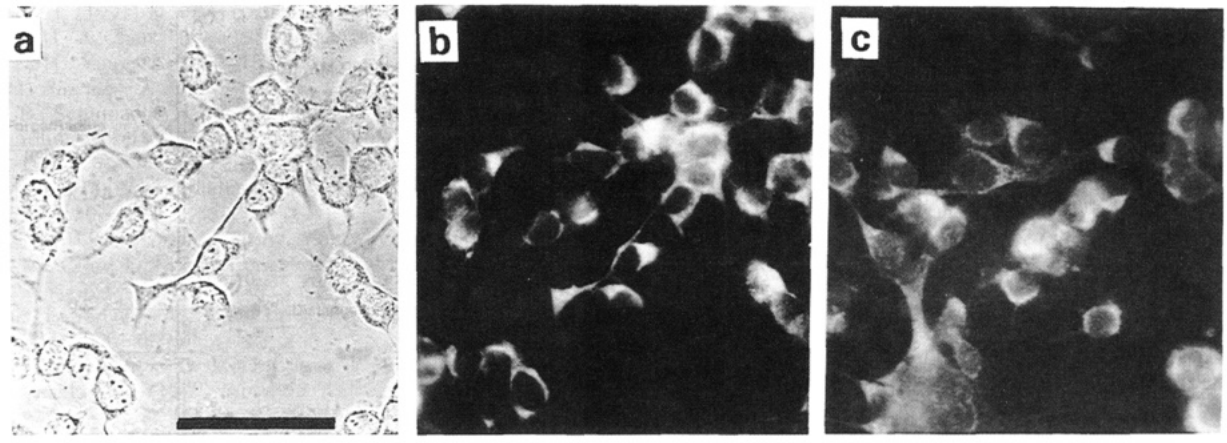


FIGURE 5: Immunocytochemistry of F-11 neuroblastoma cells with the monoclonal antibodies R-24 and D.1.1: (a) phase contrast microscopy; (b) anti-GD3 amntibody (R-24); (c) anti-O-acetylated GD3 antibody (D.1.1). F-11 cells were 100% immunoreactive against these antibodies. Immunoreactivity was almost confined around the cell body and the long varicose processes. Bar indicates 100 μm.

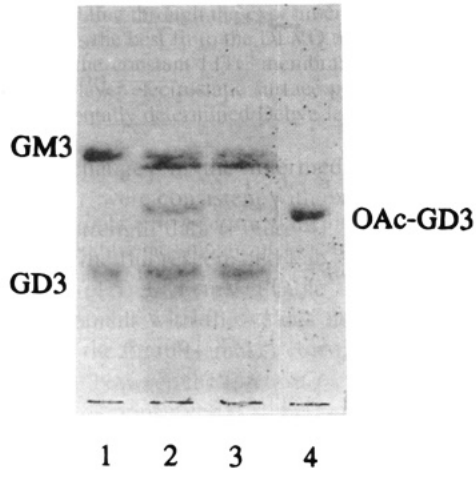


FIGURE 6: Gangliosides in transfected F-11 neuroblastoma cells. (lane 1) authentic samples of GM3 from bovine adrenal medulla (Ariga et al., 1982) and GD3 from bovine butter milk; (lane 2) gangliosides in F-11 cells, based on 2.1 mg protein; (lane 3) gangliosides in transfected F-11 cells, based on 1.5 mg of protein; and (lane 4) authentic sample of *O*-acetyl GD3 from bovine butter milk. The bands of plate were visualized by spraying the resorcinol−hydrochloric acid reagent. The plate was developed with solvent system C.

GM1, GD1a, GD2, and GT1b. Most interestingly, the amount of O-acetylated GD3 was significantly decreased from 18 to 5% of the total gangliosides, and represented a 26.7% reduction of that in the control cells. On the other hand, the level of GD3 was increased from 27 to 40%, which represented a 149.3% increase of the value in the control cells (Table 1). There were no significant changes in neutral glycolipid composition in the transfected cells (data not shown). Morphological changes of F-11 cells transfected by *O*-acetylesterase gene are shown in Figure 7. The transfected cells appeared to have enlarged soma size as well as to be more differentiated as evidenced by the elongated neurites and cell processes.

DISCUSSION

The F-11 cell line is a fusion product of embryonic rat dorsal root ganglion (DRG) cells with mouse neuroblastoma cell line N18TG-2 (Platika et al., 1985). When this study was initiated, we expected to find its GSL composition to be somewhat reminiscent of the two parental cells. Results from this present investigation revealed a number of interesting and unexpected features. With respect to neutral GSLs, F-11 cells contained four major neutral globo-series species, i.e., GlcCer, LacCer, Gb3, and Gb4, and Gb4 as the most prominent component accounting for about 40% of the total neutral GSLs. This unusually high concentration of Gb4 is surprising as few mature cell lines exhibit such an accretion of a single GSL component. It is well-known that Gb4, or P-antigen, is a stage-specific embryonic carbohydrate antigen (SSEA-3); this GSL has been shown to be located on the primordial germ cells in mouse embryos (Tippett et al., 1986; Marani et al., 1986) and is synthesized in early chick embryos (Felding-Herbermann et al., 1986). An earlier immunocytochemical study using monoclonal antibodies demonstrated that rat DRG contained lacto- and globo-series GSLs that constitute the SSEA-1, SSEA-3, and SSEA-4 (Jessell & Dodd, 1985). Boland and Dingledine (1990) also reported the presence of SSEA-3 and SSEA-4 antigens in F-11 neuroblastoma cells by immunocytochemistry using specific monoclonal antibodies. Hence, Gb4 may be characteristic of proliferating, immature cells and may play an important role in cellular events during early stages of cellular differentiation.

Concerning gangliosides, F-11 neuroblastoma cells contained three major lacto-series gangliosides which account for 82% of the total gangliosides, i.e., GM3, GD3, and O-acetylated GD3. Only trace amounts of ganglio-series gangliosides, i.e., GM1, GD1a, GD1b, GT1b, GQ1b, and GD2, were detected by the highly sensitive HPTLC overlay method. Earlier studies on gangliosides in rat DRG demonstrated that b-series gangliosides such as GD1b, GT1b, and GQ1b were predominant species in addition to GM3, LM1, and GD1a (Hara et al., 1988; Ohsawa, 1990). GM1, GD3, and Fuc-GM1 were also present as minor gangliosides, with GD3 expressed in considerable amounts in cultured DRG (Ohsawa, 1990). Chou et al. (1989) reported the presence of several unique gangliosides containing blood group B determinant, i.e., $IV^3Gal,IV^2Fuc,II^3NeuAc,GgOse_4Cer$ (B-GM1), $IV^3Gal,IV^2Fuc,II^3NeuAc_2,GgOse_4Cer$ (B-GD1b) and $IV^3Gal,IV^2Fuc,GgOse_4Cer$ (B-asialo-GM1), in rat DRG. In the present study, we could not detect these fucosylated gangliosides, e.g., Fuc-GM1 and B-GM1 by the HPTLC overlay method using cholera toxin B-subunit or anti-B-GM1 monoclonal antibody (Ariga et al., 1987). In addition, the ganglioside pattern in F-11 cells was completely different from that of human dorsal root ganglion neurons,

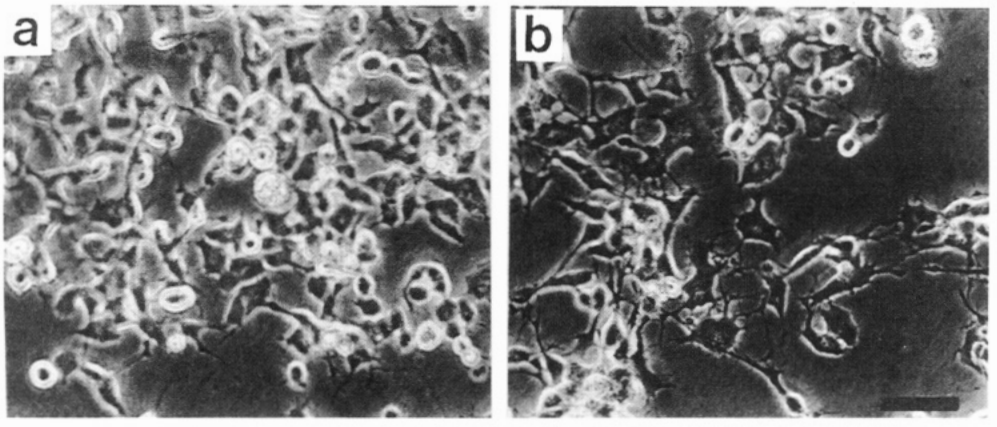


FIGURE 7: Morphological changes in transformed F-11 cells: (a) untreated F-11 cells; (b) transfected F-11 cells by *O*-acetylesterase gene. Bars indicates 50 μm (Reduced to 80% of original for publication). Morphologically, the transfected cells appeared to have elongated neurites and processes.

which contain LM1, GM1, GD1a, and GD1b as the major ganglioside components (Ariga et al., 1990), and that of the parent cell line (N18TG-2), which contains GM3 (5% of the total gangliosides), GM2 (38%), GD3 (1%), GM1 (19%), and GD1a (36%) (Dawson, 1979). Moreover, the ganglioside composition differed significantly from other related cell lines: e.g., a hybrid cell line, NCB-20, the clonal fusion product between N18TG-2 and Chinese hamster fetal brain cells; NBr-10A, a clonal cell line between N18TG-2 and Buffalo rat liver cells, in which GD1a is a predominant ganglioside (Yavin and Habig, 1984; Berry-Kravis and Dawson, 1985); SB21-B1, a clonal cell line between neuroblastoma NS20-6TG and mouse L parent lines, which contains GM3 and GM2 as the major gangliosides (Yavin & Habig, 1984). The expression of high levels of lactoseries gangliosides, especially GD3 and O-acetylated GD3, may reflect the fact that F-11 cells are originated from neuroblastoma cells.

With respect to GD3 and O-acetylated GD3, they have been shown to be present in high concentrations in many tumor cells of neuroectodermal origin, e.g., glioma, medulloblastoma, neuroblastoma, melanoma, and teratoma cell lines (Ren et al., 1993; Yates et al., 1979; Miyatake & Ariga, 1981; Carubia et al., 1984; Honsik et al., 1986; He et al., 1989). GD3 has also been shown to be characteristic of immature neuroectodermal cells (Seyfried & Yu, 1985). O-Acetylated GD3 and GD3 are selectively expressed in the embryos and certain regions of the developing nervous system (Yu et al., 1988; Cheresh et al., 1984; Levine et al., 1984; Varki, 1992). In particular, the expression of O-acetylated gangliosides is known to be tissue-specific and developmentally regulated and sometimes to reappear as an oncofetal antigen (Ren et al., 1993; Cheresh et al., 1984; Schlosshauer et al., 1988, Varki, 1992, Chou et al., 1990). The abundance of GD3 and O-acetylated GD3 in many tumors of neuroectodermal origin should stimulate the development of new approaches for tumor diagnosis, vaccine construction, and tumor immunotherapy (Ritter et al., 1990).

The biological function of GD3 and O-acetylated GD3 is still obscure. However, it is apparent that the expression of these gangliosides is regulated by *O*-acetyltransferase and *O*-acetylesterase (Varki, 1992). Little is known about these enzymes and their subcellular localization. Influenza C virus expresses a surface protein, the hemagglutinin esterase, which binds specifically to O-acetylated sialic acids and cleaves O-acetyl esters from the sialic acids. However, under physiological conditions (37 °C and neutral pH), this protein functions only as a highly specific sialylate:*O*-acetylesterase (Herrler et al., 1985; Rogers et al., 1986). Recently, the specific influenza C esterase was expressed as a plasma membrane protein on the cell surface of an intact developing embryo, and the O-acetylated sialic acid residues are destroyed in the target tissues by influenza C virus esterase (Varki et al., 1991). It was not demonstrated, however, from the previous study whether O-acetylated GD3 was destroyed (Varki et al., 1991). In the present study, we provided direct evidence that transfection of F-11 cells with the viral *O*-acetylesterase gene could effectively downregulate the expression of O-acetylated GD3 (Figure 6, lane 3) with the concomitant increase in the level of GD3. This observation strongly suggests that the *O*-acetylesterase gene may induce the degradation of O-acetylated GD3 to GD3. As a result of these ganglioside changes, a profound effect appears to be produced on neurite initiation/elongation or cellular differentiation in the transfected F-11 cells. In this regard, it is further worth noting that GD3 has always been associated with differentiating cells that are metabolic active (Seyfried & Yu, 1985). Thus, the increase of GD3 in our cell line may very well underlie the observed morphological changes associated with the *O*-acetylesterase action. This notion is further supported by the very recent observation that an increased GD3 biosynthesis apparently can induce cholinergic differentiation with neurite sprouting in Neuro2a cells (Kojima et al., 1994). Regardless of the mechanisms, the use of the *O*-acetylesterase gene should represent a powerful means in manipulating the expression of O-acetylated gangliosides and facilitate further studies on the relationship between ganglioside metabolism and cellular differentiation/tumorigenicity.

Finally, in the present study, trace amounts of GD2 were also detected by the highly sensitive HPTLC overlay method and confirmed by negative ion LSI mass spectrometry (Figure 4d). GD2 is known to be widely expressed in neuroblastoma cells and has been postulated to be a marker for highly metastatic cells, such as melanomas, small-cell lung carcinomas, and certain brain tumors (Cheresh et al., 1986; Schengrund & Shochat, 1988; Cheung et al., 1993; Ye & Cheung, 1992). It has been advocated as a potential tumor marker for *in vivo* diagnostic purposes as well as targeting radiotherapy and immunotherapy (Cheung et al.,

1987, 1988; Mujoo et al., 1987). During the past decade, tumor-specific GSL markers have been used in the treatment of various human malignant tumors. For example, Livingston et al. (1987) attempted to treat patients with melanomas using passive immunization with monoclonal antibodies against tumor-specific gangliosides and by direct immunization with ganglioside vaccines. Elucidation of the heterogeneity of tumor cell GSL composition should influence future investigations regarding tumor diagnosis and therapeutic management.

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