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# Structural and Immunological Characterization of *O*-Acetylated $G_{D2}$

## EVIDENCE THAT $G_{D2}$ IS AN ACCEPTOR FOR GANGLIOSIDE *O*-ACETYLTRANSFERASE IN HUMAN MELANOMA CELLS*

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**We have shown previously that Golgi-enriched vesicles from the human melanoma cell line Melur can transfer [$^3$H]acetate from [*acetyl*-$^3$H]acetyl-CoA to endogenous $G_{D3}$ to form [*acetyl*-$^3$H]*O*-acetyl-$G_{D3}$ (Manzi, A. E., Sjoberg, E. R., Diaz, S., and Varki, A. (1990) *J. Biol. Chem.* 265, 13091–13103). Applying the same approach in the human melanoma cell line M21, label was found in [*acetyl*-$^3$H]*O*-acetyl-$G_{D3}$ and also in a species co-migrating with unsubstituted $G_{D3}$ on TLC. Both were sialidase-sensitive and alkali-labile, indicating incorporation as [$^3$H]*O*-acetyl esters on sialic acids. Immunological reactivity, sialidase sensitivity, chromatographic behavior, and the known ganglioside pattern of M21 cells suggested that the slower migrating species might be [*acetyl*-$^3$H]*O*-acetyl-$G_{D2}$. Sialic acids released from this labeled molecule by sialidase showed esterification with [$^3$H]acetate at both C7 and C9 hydroxyls. Lipid extracts from cells metabolically labeled with [$^3$H]galactose showed a corresponding ganglioside, which upon alkali treatment yielded a species migrating with $G_{D2}$. Analysis of purified ganglioside by high performance thin layer chromatography immuno-overlays, fast atom bombardment-mass spectrometry in positive and negative ion modes, periodate oxidation resistance, linkage analysis by permethylation and gas chromatography-mass spectrometry, and 500 MHz $^1$H NMR was consistent with the following structure:**

**9-*O*Ac-Neu5Ac$\alpha$2–8Neu5Ac$\alpha$2–3(GalNAc$\beta$1–4)**

**Gal$\beta$1–4Gluc$\beta$1–1′ceramide**

**Total gangliosides from M21 were analyzed by high performance thin layer chromatography immunooverlay with monoclonal antibodies D1.1, JONES, 27A, and 8A2, all known to, or suspected of reacting with 9-*O*-acetylated gangliosides. The first three bound well to 9-*O*-acetyl-$G_{D3}$ and a slower migrating 9-*O*-acetylated ganglioside, which was distinct from 9-*O*-acetyl-$G_{D2}$. Antibody 8A2 reacted weakly with purified 9-*O*-acetyl-$G_{D2}$ and strongly with two other 9-*O*-acetylated gangliosides migrating slower than 9-*O*-acetyl-$G_{D2}$. Thus, the family of *O*-acetylated gangliosides in melanoma cells is much more complex than previously appreciated.**

Gangliosides are a structurally heterogenous group of animal glycosphingolipids containing one or more glycosidically bound sialic acids (1–3). They are synthesized in the lumen of the Golgi apparatus by specific, sequentially acting glycosyltransferases (see Refs. 4–8 for examples). The finished products are transported to the cell surface, where they remain anchored via their hydrophobic ceramide tails. The hydrophilic oligosaccharide chains extend extracellularly where, along with other glycosphingolipids, glycoproteins, and proteoglycans, they constitute the protective and interactive "glycocalyx" of the cell surface. Gangliosides have received much attention recently because of their possible role in cell surface recognition (9–14), growth regulation (15–22), immune modulation (23–28), and their expression in tumors as oncofetal antigens (29–31). Since certain gangliosides are selectively expressed in human melanoma and neuroblastoma, they have also become targets for immunotherapy of these cancers (32–36).

The sialic acids are a diverse group of anionic 9-carbon sugars, of which the commonest is *N*-acetylneuraminic acid (Neu5Ac)[1]. *O*-Acetylation is one of several possible modifications which combine to form over 30 known derivatives of Neu5Ac (37–41). *O*-Acetyl esters on ganglioside sialic acids are known to exist at the C4, 7, and 9 hydroxyl groups (31, 37, 42, 43). It is well documented that 9-*O*-acetylated sialic acids mediate adhesion of influenza C virus to cell surfaces (44, 45), antagonize the ability of sialic acids to regulate the alternate complement pathway (25), and inhibit hydrolysis by eukaryotic and prokaryotic sialidases (46, 47). 9-*O*-Acetylation of the sialic acids on gangliosides is intricately regulated in a spatial and temporal manner throughout the development of tissues derived from the neuroectoderm (48–57). It is postulated that 9-*O*-acetylated gangliosides, by mediating intercellular adhesion events, may be involved in organizing developing neuroectodermal tissues. This notion is corroborated by our study in which a 9-*O*-acetylsialic acid-specific esterase was expressed in transgenic mice in a tissue-specific manner, resulting in developmental abnormalities (58).

We have recently used Golgi-enriched membrane preparations from a human melanoma cell line to study the biosynthesis of 9-*O*-acetyl-$G_{D3}$[2] (43). Using this approach in another human melanoma cell line, M21, we discovered a novel alkali-

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac$_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid; Neu5,7Ac$_2$, 7-*O*-acetyl-*N*-acetylneuraminic acid; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; FAB, fast atom bombardment; MS, mass spectrometry; MAb, monoclonal antibody; HPTLC, high performance thin layer chromatography.

[2] The various ganglio series gangliosides discussed in this study are designated according to Svennerholm *et al.* (104).

labile, sialidase-sensitive species with a slower mobility than that of [*acetyl*-$^3$H]*O*-acetyl-$G_{D3}$ on high performance thin layer chromatography (HPTLC) plates. This paper describes the structural and immunological characterization of this novel ganglioside as 9-*O*-acetyl-$G_{D2}$, using a variety of techniques. It also reports the detection of several other *O*-acetylated gangliosides in melanoma cells. The significance of these findings to the biosynthesis, developmental regulation, and biology of *O*-acetylated sialic acids on gangliosides is discussed.

## EXPERIMENTAL PROCEDURES

### *Materials*

[*acetyl*-$^3$H]Acetyl-CoA was synthesized as described (59) and was determined to have a specific activity of 27 Ci/mmol. The following materials were obtained from the sources indicated: [6-$^3$H]galactose (15 Ci/mmol) and [1-$^{14}$C]glucosamine (55 μCi/mmol), American Radiolabeled Chemicals; *Arthrobacter ureafasciens* sialidase, Calbiochem; partially methylated alditol acetate standards, BioCarb; and pepstatin, leupeptin, and *N*-ethylmaleimide, Sigma. Diisopropyl flurophosphate from Aldrich was stored as a 100 mM stock solution in isopropyl alcohol at −20 °C. Influenza C virus was obtained from American Type Culture Collection. All chromatography solvents were of HPLC grade, and all other chemicals were of reagent grade.

### *Monoclonal Antibodies*

Monoclonal antibodies recognizing 9-*O*-acetyl-$G_{D3}$ were generously provided by Dr. C. Barnstable, Yale University School of Medicine (JONES); Dr. M. Farquhar, University of California, San Diego (27A); and Dr. W. Stallcup, La Jolla Cancer Research Foundation, (D1.1). Dr. R. Reisfeld, Scripp's Clinic and Research Foundation, La Jolla, CA, kindly provided monoclonal antibodies MB3.6 and 14.G2A directed against $G_{D3}$ and $G_{D2}$, respectively. Dr. V. Lemmon, Case Western Reserve University, kindly provided monoclonal antibody 8A2, which reacts with several unidentified alkali-labile gangliosides in embryonic chicken brain(60).

### *Cell Culture*

The M21 cell line is a subclone of the human melanoma cell line UCLA-SO-M21 that expresses high levels of the disialoganglioside $G_{D2}$ (61). M21 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% fetal calf serum in a humidified environment containing 5% $CO_2$ at 37 °C.

### *Purification of O-Acetyl-$G_{D2}$ from M21 Cells Grown in Culture*

M21 cells were scraped from culture plates, pelleted at 2,000 rpm for 5 min, and washed three times in cold phosphate-buffered saline. Sixty P-150 culture plates (approximately $3 \times 10^8$ cells) yielded a packed cell volume of 7.5 ml. Total lipids were immediately extracted as described (1), the solvent removed under vacuum, and the residue resuspended in chloroform/methanol/water (30:60:8 v/v/v). At this point ~2 μCi of a [$^{14}$C]glucosamine-labeled disialoganglioside fraction from metabolically labeled M21 cells propagated in parallel was added to the total lipid extract as a tracer to follow the purification. The total lipid extract was fractionated into neutral and anionic species by DEAE-Sephadex chromatography as described (1), with some modifications. Monosialogangliosides were batch eluted with chloroform, methanol, 0.125 M ammonium acetate (30:60:8 v/v/v), whereas more anionic gangliosides were batch eluted with chloroform, methanol, 0.8 M ammonium acetate (30:60:8 v/v/v) for further purification. Solvent was removed under vacuum, and the residue was resuspended in water and dialyzed at 4 °C overnight against two changes of 2 liters of water. Gangliosides remaining in the dialysis bag were lyophilized, resuspended in chloroform/methanol/water (1:8:1 v/v/v), and further fractionated by HPLC on a TSK-DEAE-2SW column (43). Aliquots (2%) of each fraction were monitored by scintillation counting and lipid ELISA (see below). This allowed pooling of $G_{D2}$ fractions separately from $G_{D3}$ fractions. These pools were dried and resuspended in chloroform/methanol/water (66:31:3 v/v/v). *O*-Acetylated $G_{D2}$ was separated from $G_{D2}$ by HPLC on an Iatrobeads column (62) using a 90-min linear gradient from chloroform/methanol/water (66:31:3 v/v/v) to chloroform/methanol/water (52:39:9 v/v/v). Two percent of each fraction was monitored by liquid scintillation counting. With this particular solvent system, the Iatrobeads HPLC column mimics the ganglioside migration pattern on HPTLC plates. Thus, *O*-acetyl-$G_{D2}$ elutes prior to $G_{D2}$, and base-line resolution is typically achieved between the *O*-acetylated molecule and its nonsubstituted form. From such a preparation, 60–75 μg of pure *O*-acetyl-$G_{D2}$ was typically purified.

### *Isolation of Golgi-enriched Vesicles from M21 Cells*

All manipulations were done at 4 °C. 20 P-150 plates corresponding to approximately $10^8$ M21 cells were harvested by trypsinization, washed, pelleted, and resuspended in 0.25 M sucrose, 20 mM $KP_i$ (pH 7.0) (5 ml/ml of packed cells) containing 1 mM diisopropyl fluorophosphate, and 1 μg/ml leupeptin, pepstatin, and *N*-ethylmaleimide (buffer A). Cells were disrupted by nitrogen cavitation (1,000 p.s.i., 5 min/ml of cell suspension), and the homogenate was collected in a 50-ml polystyrene tube. Nuclei and unbroken cells were removed by centrifugation at 500 rpm for 10 min. The postnuclear supernatant was adjusted to 1.2 M sucrose and transferred to the bottom of an ultracentrifuge tube. Seven milliliters of 1.1 M sucrose followed by 10 ml of 0.25 M sucrose was carefully layered on top of the postnuclear supernatant. Each sucrose solution contained 20 mM potassium phosphate (pH 7.0). This discontinuous gradient was centrifuged at 85,000 × *g* (SW 28 Beckman rotor, 25,000 rpm) for 1.5 h. A "light membrane" fraction was isolated from the 0.25/1.1 M sucrose interface, pelleted at 100,000 × *g*, and resuspended in buffer A without protease inhibitors for *in vitro* labeling.

### *Labeling of M21 Golgi-enriched Vesicles*

Golgi-enriched preparations isolated as described above were labeled immediately following isolation with 15 μCi of [*acetyl*-$^3$H]acetyl-CoA in a total volume of 400 μl. After labeling at 24 °C for 20 min and quenching with 3 ml of ice-cold buffer A, the labeled vesicles were reisolated by pelleting at 100,000 × *g* for 30 min. The supernatant was removed, the pellets washed three times with water, transferred to glass conical tubes, and lyophilized overnight. Radiolabeled lipids were extracted from the dry pellets as described (43).

### *HPLC Analysis of Radiolabeled Golgi-enriched Total Lipid Extracts*

Total lipid extracts were applied to a TSK-DEAE-2SW HPLC column and fractionated with a 72-min linear gradient of chloroform/methanol/$H_2O$ (1:8:1 v/v/v) to chloroform, methanol, 1.2 M $NH_4$ acetate (1:8:1 v/v/v) as described (43). Aliquots (2%) of eluting fractions were monitored by lipid ELISA as described (43) using monoclonal antibodies JONES, MB3.6, and 14.G2A directed against *O*-acetyl-$G_{D3}$, $G_{D3}$, and $G_{D2}$ respectively (50, 63, 64), and by liquid scintillation counting. Relevant fractions were pooled, dried, resuspended in water, and desalted by dialysis or Sep-Pak chromatography (65), as indicated.

### *HPTLC*

Silica Gel 60 glass or aluminum-backed HPTLC plates were activated at 110 °C for 20 min. Samples were spotted and developed in chloroform, methanol, 0.02% $CaCl_2$ (60:40:9 v/v/v). Gangliosides were detected by resorcinol/HCl as described (66). Radiolabeled gangliosides were detected by spraying HPTLC plates with EnHance (Du Pont-New England Nuclear), drying, and submitting the HPTLC plate to fluorography. Saponification of *O*-acetyl esters on prespotted gangliosides was achieved by placing the HPTLC plate in a sealed ammonia vapor chamber overnight as described (67). After sufficient aeration to remove ammonia vapors, an equivalent amount of ganglioside was spotted in an adjacent lane for direct comparison.

### *Release and Purification of Sialic Acids*

Sialic acids were released from purified gangliosides by incubation with 5–10 mUnits of *A. ureafasciens* sialidase in 100 μl of 100 mM sodium acetate (pH 5.5) containing 0.5% taurocholate. Complete release was typically observed after an overnight incubation at 37 °C. Released sialic acids were collected by dialysis into 10 ml of water using 1,000 molecular weight cutoff dialysis tubing and were purified further by ion exchange chromatography on Dowex 50 AG 1-X8 (hydrogen form) followed by Dowex 3-X4A (formate form) as described (59, 68). These conditions are known to avoid destruction of *O*-acetyl esters and appreciable migration of 7-*O*-acetyl esters (59). Sialic acids from gangliosides oxidized with periodate were released with 0.1 N HCl at 80 °C for 2 h and purified as described above with the omission of the dialysis step.

*HPLC Analysis of Released Sialic Acids*

Released sialic acids from [*acetyl*-$^3$H]*O*-acetyl-$G_{D2}$ were analyzed by a Dionex CarboPac column as described (69). Fractions were monitored by liquid scintillation counting. Sialidase-released sialic acids from unlabeled *O*-acetyl-$G_{D2}$ were derivatized with 1,2-diamino-4,5-methylenedioxybenzene and analyzed by reversed phase HPLC as described (70). Truncated C8 analogues of sialic acid resulting from periodate oxidation were separated from Neu5Ac by ion suppression AX-5 HPLC as described (43). Separation was achieved with an isocratic elution of acetonitrile, water, 0.25 M monobasic sodium phosphate (68:21:11 v/v/v). Fractions were collected every 0.5 min, and elution was monitored by scintillation counting.

*Periodate Oxidation*

*O*-Acetyl-$G_{D2}$ and $G_{D2}$ were isolated from one P-150 plate of M21 cells metabolically labeled with [1-$^{14}$C]glucosamine as described above omitting the DEAE-Sephadex step. 10,000 cpm of each ganglioside was suspended in 1 ml of phosphate-buffered saline (pH 7.0) with and without 3 mM sodium metaperiodate. The oxidations were allowed to proceed for 1 h on ice in the dark after which they were quenched with a 100-fold molar excess, relative to periodate, of glycerol. The aldehydes produced by oxidation of sialic acid side chains were reduced by adding of 2 ml of 0.8 M sodium borohydride in 0.01 M NaOH and incubating for 2 h at room temperature. Reduction was quenched by the addition of glacial acetic acid dropwise until liberation of hydrogen ceased. Methanol (1 ml) was added to each reaction and dried under vacuum to remove borate complexes. This methanol dry-down was repeated three times. The dried reaction mixtures were resuspended in 0.02% Triton X-100, sonicated, and dialyzed overnight using 1,000 molecular weight cutoff dialysis tubing. The addition of detergent increased final yields 3-fold. Gangliosides remaining in the dialysis bag were dried, resuspended in 0.1 N HCl, and heated at 80 °C for 2 h to release sialic acids. To saponify any remaining *O*-acetyl esters the reaction solutions were made to 0.1 N NaOH and incubated at 37 °C for 30 min. The released sialic acids were purified and separated by AX-5 HPLC as described above.

*Methylation Analysis*

Twenty micrograms of putative *O*-acetyl-$G_{D2}$ or $G_{D2}$ was permethylated according to the method of Hakomori and co-workers (71) and isolated by dialysis (1,000 molecular weight cutoff) and lyophilization. Half of each permethylated ganglioside sample was submitted to acetolysis/hydrolysis by heating at 80 °C for 6 h in 0.5 N $H_2SO4$ in 90% acetic acid (72). The reaction mixture was passed over a 1-ml column of Dowex 3-X4A (acetate form) and washed with methanol as described by (71). The dried eluate was reduced with $NaBD_4$, and partially methylated sugars were acetylated as described (71). The remaining 50% of the permethylated gangliosides was submitted to methanolysis as described by Fukuda *et al.* (73). Separation of partially methylated, partially acetylated alditols was performed using a Hewlett-Packard 5890-A gas chromatograph and a DB-5 (J&W Scientific) capillary column (0.25 × 30 m). The column temperature was programmed at 20 °C/min, from 50 to 150 °C, and then at 4 °C/min to 250 °C, maintaining this temperature constant for a further 10 min. The eluate was analyzed by electron impact mass spectrometry using a VG Analytical 70-SE mass spectrometer, with an 11-250J data system. The eluate was continuously monitored by scanning from 40 to 400 milliunits at 1 s/decade, with an interscan time of 0.5 s. Separation of partially methylated, partially acetylated neuraminic acid methyl glycosides was achieved using the same type of column connected to a Finnigan Mat gas chromatograph-mass spectrometer with a computerized data system and increasing the column temperature from 50 to 230 °C at 20 °C/min and maintaining this temperature constant for a further 30 min. Identification of sugar derivatives was achieved by comparison of retention times and mass spectra with those known for standard compounds (73–75). Quantitation was done by multiplying the total area by the correction factor obtained for each type of methylated sugar from a known standard processed in the same manner. gas chromatography-MS analysis were carried out at La Jolla Cancer Research Foundation, La Jolla, CA.

*Fast Atom Bombardment-Mass Spectrometry*

*Positive Ion Mode (Imperial College, London)*—The sample (10 μg) was dissolved in methanol (10 μl) and a 1-μl aliquot was added to the thioglycerol matrix. Spectra were obtained using a VG analytical ZAB-HF mass spectrometer fitted with an M-scan FAB gun operated at 10 kV. Data were recorded on oscillographic chart paper and manually counted.

*Negative Ion Mode (La Jolla Cancer Research Foundation)*—9-*O*-Acetyl-$G_{D2}$ (8 μg) and $G_{D2}$ (12 μg) were dissolved in 5 μl of $CHCl_3$/MeOH (1:1 v/v), loaded onto the stainless steel FAB target, and evaporated to dryness. The residue was redissolved in 1 μl of hexamethylphosphoric triamide), and 1 μl of glycerol was added. Spectra were obtained using a VG analytical 70-SE mass spectrometer fitted with a cessium ion gun operated at 20 kV, with an emission current of 4 μA. Mass range $m/z$ 200–2500 was scanned at 25 s/decade, with an interscan time of 3 s, at an ion source accelerating potential of 6 kV, and a resolution of 1,000. Cessium iodide was used as standard for mass calibration. All data were acquired and processed on a VG 11–250J data system. Spectra shown in Fig. 8*B* represent the averaged data from five scans.

*$^1$H NMR Spectroscopy*

Fifty micrograms of ganglioside, either putative *O*-acetyl-$G_{D2}$ or $G_{D2}$, was deuterium exchanged by repeated drying under vacuum from deuterated methanol. The sample was then dissolved in 400 μl of deuterated dimethyl sulfoxide/$D_2O$ (98:2) for $^1$H NMR analysis. $^1$H NMR spectra were obtained with a Varian Unity 500 (500 MHz) instrument. Spectra were recorded at 25 °C, and resonances are indicated relative to tetramethylsilane (referenced to the HOD peak at 4.76 ppm). A spectral width of 4 kHz was used together with a pulse angle of 5.5 μs (45°). A total of 1,200 free induction decays were accumulated.

*HPTLC Immuno-overlay*

HPTLC immuno-overlay was carried out as described (76). Ganglioside samples were fractionated on aluminum-backed Silica Gel 60 HPTLC plates as described above and immersed in 0.4% polyisobutylmethacrylate by diluting a 2% polyisobutylmethacrylate solution in chloroform with hexane (hexane, 2% polyisobutylmethacrylate in chloroform (84:16 v/v)) for 1 min. The plates were allowed to dry thoroughly and immersed overnight at 4 °C, in appropriate dilutions of primary antibody in phosphate-buffered saline containing 1% bovine serum albumin. The plates were washed three times for 3 min each in phosphate-buffered saline with 1% bovine serum albumin, after which appropriate secondary antibody (goat anti-mouse IgM or IgG) conjugated to horseradish peroxidase was added and incubated for 1 h at 4 °C. The washing process was repeated, and the plates were developed with *o*-phenylenediamine as described (77).

## RESULTS

*Labeling of Golgi-enriched Vesicles from M21 Cells with [acetyl-$^3$H]-Acetyl-CoA Results in the Formation of [acetyl-$^3$H]O-Acetyl-$G_{D3}$ and a Slower Migrating Alkali-labile Species*—The Melur melanoma cell line is known to express the gangliosides $G_{M3}$, $G_{D3}$, and *O*-acetyl-$G_{D3}$, whereas M21 cells are known to express in addition $G_{M2}$ and $G_{D2}$ (43, 63). We have confirmed these overall ganglioside expression patterns in the two cell lines by positive ion FAB-MS analysis (data not shown). Golgi-enriched vesicles were obtained from M21 cells by nitrogen cavitation followed by isolation of a light membrane fraction from a discontinuous sucrose density gradient as described under "Experimental Procedures." Vesicles isolated in this manner are typically enriched 12–15-fold in the Golgi marker GlcNAc(β1–4)galactosyltransferase. Ganglioside *O*-acetyltransferase activity in these preparations is detergent-labile and cannot be stimulated by the exogenous addition of $G_{D3}$.[3] Thus, biosynthesis of *O*-acetylated gangliosides in these preparations requires the endogenous acceptors in intact vesicles.

Golgi-enriched vesicles from M21 cells were incubated with [*acetyl*-$^3$H]acetyl-CoA, total lipids were extracted and fractionated by HPLC using a TSK-DEAE column as described (43). Fractions were monitored by solid phase lipid ELISA using MAbs JONES and 14.G2A directed against 9-*O*-acetyl-$G_{D3}$ and $G_{D2}$, respectively (50, 64). Gangliosides elute from

[3] E. R. Sjoberg and A. Varki, unpublished observations.

this column in a highly reproducible manner. *O*-Acetylated species typically elute from this column just prior to the elution of the non-substituted parent ganglioside. Not surprisingly the majority of the labeled acetate was incorporated into unidentified neutral and anionic sialidase-resistant species. However, two sialidase-susceptible peaks of radioactivity were found (see Fig. 1). The second of these co-elutes with 9-*O*-acetyl-$G_{D3}$ as indicated by reactivity to the MAb JONES. The first elutes one fraction prior to the peak of 14.G2A reactivity, corresponding with the predicted elution position of an *O*-acetylated derivative of $G_{D2}$.

Melanoma gangliosides typically appear as doublets in HPTLC analysis, with each species arising from fatty acid heterogeneity in the ceramide moiety (31, 78). When the sialidase-sensitive fractions eluting from the TSK-DEAE column were pooled separately and analyzed by HPTLC, the peak corresponding to the putative *O*-acetyl-$G_{D2}$ migrated as a doublet approximately in the position of unsubstituted $G_{D3}$ (Fig. 2). Upon alkali treatment, the doublet was completely destroyed, indicating that [$^3$H]acetate, donated from [*acetyl*-$^3$H]acetyl-CoA, was incorporated as a base-labile *O*-acetyl ester.

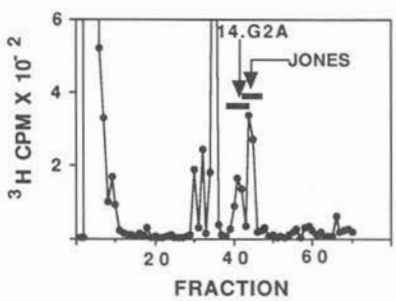


FIG. 1. **DEAE-HPLC profile of total lipid extract from [$^3$H] acetyl-CoA-labeled Golgi-enriched vesicles.** Golgi-enriched vesicles from M21 melanoma cells were isolated and labeled with [$^3$H] acetyl-CoA. Total lipids were extracted and applied to a TSK-DEAE-2SW HPLC column as described under "Experimental Procedures." Aliquots (20 $\mu$l) from each 1-ml fraction were monitored for radioactivity and for reactivity with MAbs 14.G2A and JONES directed against $G_{D2}$ and 9-*O*-acetyl-$G_{D3}$, respectively. Antibody-reactive fractions are indicated by *solid bars*. In each case, fractions with the highest reactivity are indicated by *arrows*.

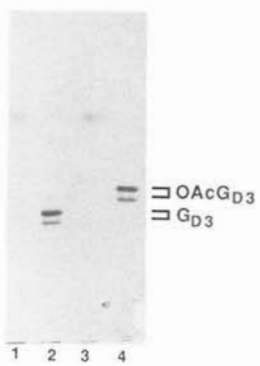


FIG. 2. **HPTLC of DEAE-HPLC-purified products from [$^3$H] acetyl-CoA-labeled Golgi-enriched vesicles from M21 melanoma cells.** Radioactive fractions from the DEAE-HPLC profile illustrated in Fig. 1, reacting with MAb 14.G2A (fractions 41–43) and those reactive with JONES (fractions 45–47), were pooled separately and analyzed by HPTLC. *Lanes 1* and *2* represent fractions 41–43; *lanes 3* and *4* represent fractions 45–47. 1,000 cpm was loaded in each *lane*. *Lanes 1* and *3* were prespotted and saponified with ammonia vapors prior to development. The migration positions of standard $G_{D3}$ and 9-*O*-acetyl-$G_{D3}$ from the human melanoma cell line Melur (43) run in parallel are indicated. *OAc*, *O*-acetylated.

*$^3$H-Acetyl Esters Are at the C7 and C9 Hydroxyls of Sialic Acid Residues*—When the putative [*acetyl*-$^3$H]*O*-acetyl-$G_{D2}$ was treated with sialidase, greater than 80% of the label was released, confirming the incorporation of [$^3$H]acetate into sialic acid. When the released sialic acids were analyzed by HPLC, both 7- and 9-*O*-acetylated Neu5Ac was observed. Fig. 3 demonstrates that 10–15% of the sialidase-sensitive radioactivity is associated with 7-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,7Ac$_2$), whereas the remainder is in 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$). Thus the putative [*acetyl*-$^3$H]*O*-acetyl-$G_{D2}$ is synthesized with either 7- or 9-*O*-acetyl esters. Although 4-*O*-acetylated Neu5Ac has a retention time identical to that of Neu5,7Ac$_2$ in this HPLC system, such residues are completely resistant to *A. ureafasciens* sialidase (37, 79). Similar results are obtained with DEAE-HPLC-purified [*acetyl*-$^3$H]*O*-acetyl-$G_{D3}$ from these preparations (data not shown). In either case there was no evidence for di-*O*-acetylated sialic acids. Thus, the general mechanism of ganglioside *O*-acetylation in M21 cells is similar to two other systems which we have previously examined: *O*-acetylation of gangliosides in the Melur melanoma cell line (43) and *O*-acetylation of *N*-linked glycans in rat liver Golgi (59, 80). Recently, *O*-acetyldisialosylparagloboside has also been shown to co-migrate with $G_{D3}$ in HPTLC systems (81). However, it is highly unlikely that [$^3$H]acetate is being esterified to disialosylparagloboside in our ganglioside *O*-acetyltransferase assay since we have been unable to detect expression of disialosylparagloboside in M21 cells (data not shown). In our earlier study of the Melur melanoma cell line (43), we also detected an alkali-stable, sialidase-sensitive product of *in vitro* labeling that was shown to be re-*N*-acetylated $G_{D3}$. However, this phenomenon appears to be variable in expression and was not detected in the *in vitro* labeling of Golgi-enriched vesicles derived from the M21 cell line used in this study.

Taken together, the data indicate that the slower migrating, alkali-labile product of the Golgi labeling reaction might be [*acetyl*-$^3$H]*O*-acetyl-$G_{D2}$ and suggest that $G_{D2}$ could be an acceptor for ganglioside *O*-acetyltransferase. More rigorous proof for the stucture of this novel ganglioside is presented below.

*O-Acetyl-$G_{D2}$ Is Also Detected in Metabolically Labeled M21 Cells*—If [*acetyl*-$^3$H]*O*-Acetyl-$G_{D2}$ can be formed *in vitro*, we reasoned that *O*-acetyl-$G_{D2}$ should be expressed by M21 cells. To determine this, we metabolically labeled M21 cells with [6-$^3$H]galactose for 3 days. This label was chosen because it is incorporated well into galactose and glucose, two sugars common to both $G_{D3}$ and $G_{D2}$. Total lipids were extracted

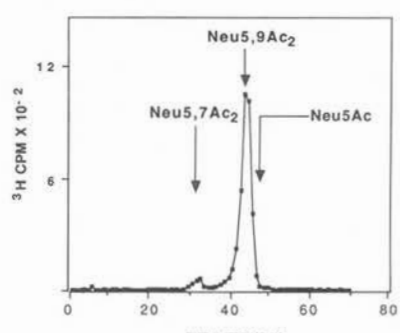


FIG. 3. **HPLC analysis of sialic acids from DEAE-HPLC-purified putative [$^3$H-*acetyl*]*O*-acetyl-$G_{D2}$.** The putative [$^3$H-*acetyl*]*O*-acetyl-$G_{D2}$ was isolated from [$^3$H]acetyl-CoA labeled M21 Golgi-enriched vesicles by DEAE-HPLC as illustrated in Figs. 1 and 2. Sialic acids from fractions 41–43 were released with sialidase, purified, and analyzed by Dionex HPLC using a CarboPac PA-1 column. The elution position of standards is indicated.

from the metabolically labeled cells and analyzed by HPTLC with and without prior alkali treatment (see Fig. 4). Although the ganglioside expression pattern of M21 extracts is complex, the autoradiogram indicates an alkali-labile doublet consistent with the HPTLC migration of the putative [*acetyl*-$^3$H]*O*-acetyl-$G_{D2}$. The percentage of the putative *O*-acetyl-$G_{D2}$ converted to $G_{D2}$ upon alkali treatment was quantitated by scanning densitometry. Alkali treatment decreases the label in the GD3/OAc$G_{D2}$ area by 26%, whereas the amount of $G_{D2}$ increases by a corresponding amount. Assuming that the specific activity of each ganglioside is approximately equal, this indicates that the putative *O*-acetyl-$G_{D2}$ represents approximately one-fourth of the $G_{D2}$ fraction. However, this may be an underestimate since M21 cells also express a small amount of *O*-acetyl-$G_{D3}$, which would be converted to $G_{D3}$ upon alkali treatment. Regardless of the exact percentages, we were able to use metabolically labeled M21 gangliosides to follow the purification of the putative *O*-acetyl-$G_{D2}$ as outlined below.

*Purification of Putative O-Acetyl-$G_{D2}$ Expressed in M21 Cell Cultures*—We developed a purification scheme to obtain quantities of the putative *O*-acetyl-$G_{D2}$ sufficient for physical characterization. As described under "Experimental Procedures," a 7-ml cell pellet was lipid extracted, and a disialoganglioside fraction from M21 cells metabolically labeled with [$^{14}$C]Glucosamine was added as a tracer to follow the purification. The extract was fractionated by DEAE-Sephadex and DEAE-HPLC. The elution of the DEAE-HPLC column was followed by lipid ELISA with MAbs directed against $G_{D2}$, $G_{D3}$, and 9-*O*-acetyl-$G_{D3}$. This allowed collection of a "$G_{D2}$ pool" free of 9-*O*-acetyl-$G_{D3}$ and $G_{D3}$. Final purification by Iatrobeads HPLC (data not shown) allowed separation of unmodified $G_{D2}$, and yielded about 75 $\mu$g of pure, putative *O*-acetyl-$G_{D2}$. As illustrated in Fig. 5, the purified ganglioside migrates as a doublet between bovine brain $G_{D1a}$ and $G_{M1}$ and co-migrates with $G_{D3}$ from human melanoma cells. Alkali converted this doublet to one that co-migrates with authentic $G_{D2}$ from M21 cells. The purified ganglioside was also treated with sialidase, the released sialic acids purified, derivatized with 1,2-diamino-4,5-methylenedioxybenzene, and analyzed by HPLC with flurometric detection (data not shown). The ratio of $Neu5,9Ac_2$ to Neu5Ac was approximately 1:1, suggesting that only one of the two sialic acids is *O*-acetylated. Unlike the material from labeled Golgi vesicles, $Neu5,7Ac_2$ was not detected in this material. This is not surprising since the 7-*O*-acetyl group can easily undergo spontaneous migration to the relatively stable C9 position (68, 82).

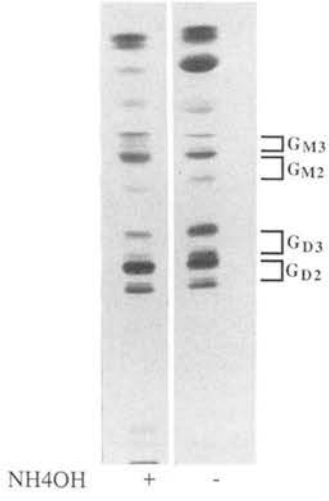


FIG. 4. **HPTLC analysis of [$^3$H]galactose-labeled total lipid extract from M21 melanoma cells.** M21 cells (~5 × $10^6$) were metabolically labeled with 50 $\mu$Ci of [6-$^3$H]galactose as described under "Experimental Procedures." Lipids were extracted, and 20,000 cpm of tritium/lane was spotted onto an HPTLC plate. One of the lanes was saponified prior to chromatography by exposure to ammonia vapors. The migration position of ganglioside standards isolated from the human melanoma cells run in parallel are indicated.

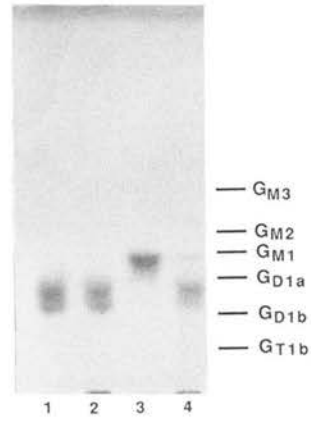


FIG. 5. **HPTLC analysis of the purified *O*-acetyl-$G_{D2}$ isolated from M21 melanoma cells.** *O*-Acetyl-$G_{D2}$ and $G_{D2}$ were isolated from M21 cells as described under "Experimental Procedures" and analyzed by HPTLC before and after alkali treatment. After development, the plates were dried and sprayed with resorcinol to detect gangliosides. *Lanes 2* and *4* were saponified prior to chromatography by exposure to ammonia vapors; *lanes 1* and *2*, 2 nmol of purified $G_{D2}$; *lanes 3* and *4*, 1 nmol of purified *O*-acetyl-$G_{D2}$. Standard gangliosides as indicated were from bovine brain, with the addition of $G_{M3}$ and $G_{M2}$.

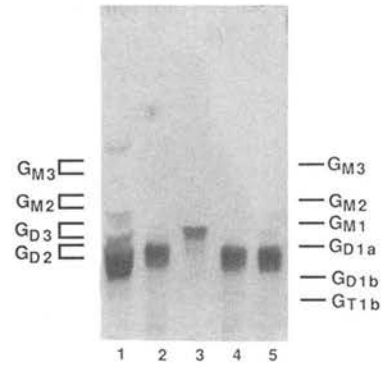


FIG. 6. **Monoclonal antibody reactivity with purified *O*-acetyl-$G_{D2}$.** Samples from M21 cells were spotted onto HPTLC plates, chromatographed, dried, and gangliosides reactive with MAb 14.G2A detected as described under "Experimental Procedures." *Lane 1*, M21 total gangliosides (15 nmol of total sialic acid); *lanes 2* and *3*, purified *O*-acetyl-$G_{D2}$, 0.5 nmol; *lanes 4* and *5*, purified $G_{D2}$, 1 nmol. *Lanes 2* and *4* were saponified by exposure to ammonia vapors prior to chromatography. Ganglioside standards were chromatographed in parallel and detected with resorcinol: *left*, M21 ganglioside doublets; *right*, bovine brain gangliosides with added $G_{M3}$ and $G_{M2}$. The purified samples tend to migrate a little faster than the corresponding compounds in the total extract.

*Monoclonal Antibody 14.G2A Reacts with Putative O-Acetyl-$G_{D2}$*—MAb 14.G2A (IgG3) is reported to be specific for the disialoganglioside $G_{D2}$ (64). If the ganglioside we purified was indeed *O*-acetyl-$G_{D2}$, alkali treatment should render it reactive to MAb 14.G2A. As illustrated in Fig. 6, two doublets in the M21 total ganglioside extract react with 14.G2A. The lower doublet migrates with $G_{D2}$, and the upper doublet co-migrates with $G_{D3}$. However, the purified putative *O*-acetyl-$G_{D2}$ reacts strongly with 14.G2A even without alkali treatment. Upon saponification, this doublet disappears, and a doublet appears migrating with $G_{D2}$. The migration of purified $G_{D2}$ is not

affected by the saponification procedure. This provides immunological evidence that the alkali-labile ganglioside from M21 cells is structurally related to $G_{D2}$. This analysis also confirms the absence of $G_{D2}$ contamination in the purified preparations used for the subsequent structural analysis.

*Methylation Analysis to Determine the Core Structure and Linkage Sequence*—Linkage analysis by permethylation and gas chromatography-mass spectrometry was carried out on the putative *O*-acetyl-$G_{D2}$ in parallel with $G_{D2}$ isolated from the same cells, as described under "Experimental Procedures." Since the strongly basic conditions of permethylation destroy *O*-acetyl esters, the alditol acetates and their relative ratios resulting from the putative *O*-acetyl-$G_{D2}$ should be identical to $G_{D2}$. The relevant regions of the total ion chromatograms of the putative *O*-acetyl-$G_{D2}$ corresponding to the products of methanolysis and acetolysis are reproduced in Fig. 7, *A* and *B*. Peaks from the total ion chromatograms were identified by diagnostic fragmentation patterns of individual mass spectra and by comparison of elution times with those of standards (not shown). As illustrated in Fig. 7*B*, acetolysis of the permethylated purified ganglioside yielded the following partially methylated alditol acetates: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol-[1-$^2$H]; 1,5-di-*O*-acetyl-3,4,6-tri-O-methyl-2*N*-acetamido-2*N*-methyl-2-deoxyglucitol-[1-$^2$H]; and 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol-[1-$^2$H]. The relative ratios of these products were very similar to that of $G_{D2}$, approximately 1:1:1. For information on the sialic acid linkages, we subjected the permethylated ganglioside to methanolysis, which is known to preserve sialic acids (83). The total ion chromatogram (see Fig. 7*A*), yields 4,7,8,9-tetra-*O*-methylneuraminic acid methyl ester methyl glycoside and 4,7,9-tri-*O*-methylneuraminic acid methyl ester methyl glycoside, in approximately equal amounts, again very similar to $G_{D2}$. This is indicative of the presence of two sialic acids, in which the internal sialic acid residue is substituted at the C8 hydroxyl group.

*FAB-MS Analysis of Putative 9-O-acetyl-$G_{D2}$*—The positive FAB spectrum of the intact ganglioside is shown in Fig. 8*A*. The major ion clusters correspond to *O*-acetylated $G_{D2}$ having a 16:0 acyl chain and are interpreted as *m/z* 1,671 (*m/z* 1,689 minus water), 1,689 ($[M+H]^+$), 1,706 ($[M+NH4]^+$), 1,711 ($[M+Na]^+$), and 1,727 ($[M+K]^+$). Lesser ions include *m/z* 1,799 ($[M+H]^+$, 24:1 acyl chain), 1,801 ($[M+H]^+$, 24:0 acyl chain), 1,821 ($[M+Na]^+$, 24:1 acyl chain), 1,823 ([M+Na], 24:0 acyl chain), 1,907 (M+H+thioglycerol]$^+$, 24:1 acyl chain). Very minor signals at *m/z* 1,629 (*m/z* 1,647 minus water), 1,647 ($[M+H]^+$), and 1,669 ($[M+Na]^+$) are attributed to contaminating $G_{D2}$ lacking the *O*-acetyl group.

Negative ion FAB-MS was also carried out on underivatized *O*-acetyl-$G_{D2}$ and $G_{D2}$. When triethanolamine was used as the matrix (31) very minor molecular ions were obtained from 10 μg of ganglioside. Isobe *et al.* (84) recently introduced the use of hexamethylphosphoric triamide to obtain carbohydrate sequence information of native gangliosides in a neutral matrix by negative FAB-MS. Using this matrix, we obtained clearer molecular ions. Illustrated in Fig. 8*B* are the molecular ion regions for *O*-acetyl-$G_{D2}$ and $G_{D2}$. The ions can be assigned as follows: *m/z* 1,690 ($[M-H^+]^-$), 1,711 ($[M-2H^++Na^+]^-$), and 1,728 ($[M-2H^++K^+]^-$), containing a 16:0 acyl chain; 1,756 ($[M-2H^++K^+]^-$), containing an 18:1 acyl chain; 1,799 ($[M-H^+]^-$), 1,821 ($[M-2H^++Na^+]^-$), containing a 24:1 acyl chain. The following signals from the molecular ion region of $G_{D2}$ can be assigned: *m/z* 1,647 ($[M-H^+]^-$), containing a 16:0 acyl chain; 1,675 ($[M-H^+]^-$), containing an 18:0 acyl chain; 1,756 ($[M-H^+]^-$), 1,778 ($[M-2H^++Na^+]$), containing a 24:1 acyl chain. The slight discrepancy between the numbers from the positive and negative ion mode studies can be explained by

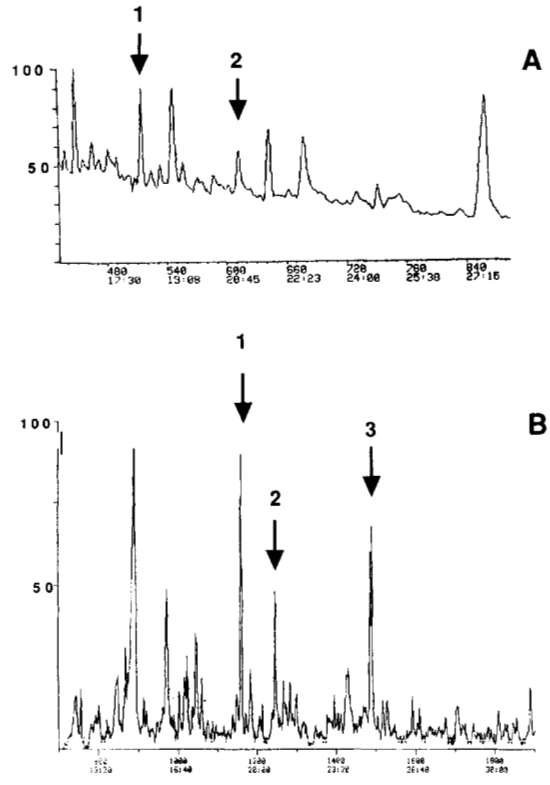


FIG. 7. **Gas chromatography-mass spectrometry of *O*-acetyl-$G_{D2}$.** Purified *O*-acetyl-$G_{D2}$ was permethylated and subjected to either methanolysis or acetolysis. *Panel A* displays the relevant portion of the total ion chromatogram from the products of methanolysis. Peaks corresponding to sialic acids are indicated with *numbered arrows*. *Arrow 1*, 4,7,8,9,tetra-*O*-methylneuraminic acid methyl ester methyl β-glycoside; *arrow 2*, 4,7,9,tri-*O*-methylneuraminic acid methyl ester methyl β-glycoside. *Panel B* displays the relevant portion of the total ion chromatogram of the alditol acetates derived from acetolysis. *Arrow 1*, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol-[1-$^2$H]; *arrow 2*, 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2*N*-acetamido-2*N*-methyl-2-deoxyglucitol-[1-$^2$H]; *arrow 3*, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol-[1-$^2$H]. The remaining peaks represent non-carbohydrate contaminants.

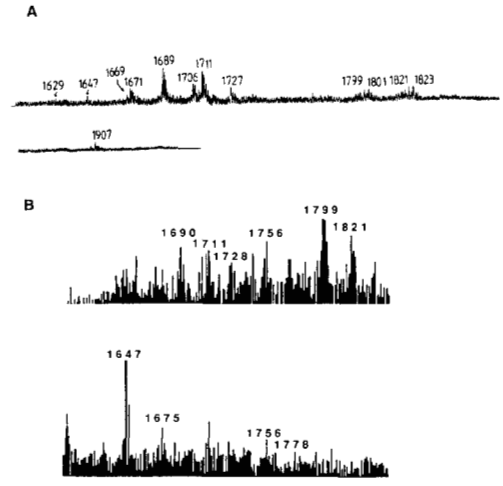


FIG. 8. **Positive and negative ion fast atom bombardment mass spectra of *O*-acetyl-$G_{D2}$.** *Panel A* displays the molecular ion region of the positive FAB mass spectrum of *O*-acetyl-$G_{D2}$ isolated from M21 cells. *Panel B* displays the negative ion FAB mass spectra, showing the molecular ion regions of *O*-acetyl-$G_{D2}$ (*upper*) and $G_{D2}$ (*lower*) isolated from M21 cells. The signals are discussed under "FAB-MS analysis."

use of manual counting or computer-generated numbers in each case. Apparent discrepancies between the relative ratios of ceramides between the positive (16:0>24:1) and negative ion (24:1>16:0) modes can be explained by the fact that the samples analyzed by the two different methods were isolated and purified separately. During final purification from the Iatrobeads HPLC column, we observed partial fractionation according to ceramide species, with the more hydrophobic species eluting earlier. In pooling to avoid $G_{D2}$ contaminants we likely enriched for d18:1-24:1 ceramides in some preparations.

Next we tried to elucidate whether the *O*-acetyl group is esterifying the inner or outer sialic acid residue of *O*-acetyl-$G_{D2}$. Shown below is the predicted negative-ion FAB-MS fragmentation pattern for *O*-acetyl-$G_{D2}$ with ceramide containing C16:0, C18:0, C24:0, and C24:1 fatty acyl chains.

| | | |
|---|---|---|
| 1467 | 1175 | 810 |
| 1465 | 1173 | 808 |
| 1383 | 1091 | 726 |
| 1355 | 1063 | 698 |

9-*O*Ac-Neu5Ac-*O*-Neu5Ac-*O*-(HexNAc-*O*-)Hex-*O*-Hex-*O*-ceramide

Although the major fragment ions resulting from β-cleavage were present and corresponded well with the expected fragmentation indicated above, a high level of chemical noise made the conclusive assignment of some of the fragment ions difficult (data not shown). This was particularly problematic in the low mass regions of the spectra. However, the following predicted fragment ions were noted: the *O*-acetyl-$G_{D2}$ gave fragments at *m/z* 1,468, 1,466, 1,356, and 1,384, which correspond to M-(Neu5Ac+42); *m/z* 1,175, 1,173, 1,092, and 1,064, corresponding to M-(2Neu5Ac+42); *m/z* 811, 809, 726, and 699, for molecules with d18:1-24:0, d18:1-24:1, d18:1-18:0, and d18:1-16:0 ceramides. The $G_{D2}$ control gave the same fragmentation, differing only in the molecular ion signals, indicating the the only differences were at the nonreducing terminus.

Taken together, the FAB-MS data indicate that the new ganglioside differs from $G_{D2}$ only in 42 mass units, corresponding to the mass of an acetyl ester. The negative ion FAB spectra indicates that the additional acetyl group esterifies the outer sialic acid residue of $G_{D2}$ resulting in the sequence: Neu5AcOAc-Neu5Ac-(HexNAc)-Hex-Hex-Cer. However, because of high chemical noise in the intermediate and low mass regions of the spectra, it was difficult to be certain about this. To provide corroborating evidence for esterification of the terminal sialic acid residue, we subjected $G_{D2}$ and *O*-acetyl-$G_{D2}$ to periodate oxidation as described below.

*O-Acetyl-$G_{D2}$ Is Resistant to Periodate Oxidation*—Mild periodate oxidation of glycosidically linked sialic acid followed by borohydride reduction results in the formation of C7 and C8 analogues of sialic acid, truncated in the glycerol side chain (85). Substitution of the C8 or C9 hydroxyl groups of sialic acid results in resistance to periodate oxidation (86). Since the inner sialic acid of $G_{D2}$ is glycosidically linked via its C8 hydroxyl, only the outer sialic acid residue is susceptible to periodate oxidation. However, if the acetyl ester on *O*-acetyl-$G_{D2}$ esterifies the C9 hydroxyl group of the outer sialic acid, the molecule should be completely resistant to mild periodate oxidation. To demonstrate this resistance, we metabolically labeled M21 cells. [6-$^3$H]Glucosamine could not be used since it is incorporated at the 9 position of Neu5Ac and would be released as formaldehyde upon periodate oxidation (87). We therefore used [1-$^{14}$C]glucosamine which we have previously demonstrated to be incorporated into the C4 position of Neu5Ac (43). $^{14}$C-Labeled *O*-acetyl-$G_{D2}$ and $G_{D2}$ were purified as described above. Equivalent cpm of $G_{D2}$ and *O*-acetyl-$G_{D2}$ were treated with and without 3 mM periodate on ice at neutral pH in the dark for 1 h. After reduction of the resulting aldehydes, sialic acids were released by acid hydrolysis, saponified, purified, and analyzed by HPLC as illustrated in Fig. 9. It is evident that $G_{D2}$ treated with periodate yields truncated C8 Neu5Ac and Neu5Ac in an approximate 2:1 ratio. This is probably because the C7/C8 analogues of Neu5Ac are more susceptible to acid hydrolysis than Neu5Ac (85). In marked contrast, sialic acids from periodate-treated *O*-acetyl-$G_{D2}$ gave virtually no C8 analogue. Analysis of sialic acids released from negative controls in which the gangliosides are treated with buffer alone reveals only Neu5Ac (data not shown). This evidence together with the negative ion FAB-MS data clearly demonstrate that the acetyl ester of *O*-acetyl-$G_{D2}$ resides on the glycerol side chain of the terminal sialic acid.

*500-MHz $^1$H NMR Spectroscopy of O-Acetyl-$G_{D2}$*—The postulated structure of 9-*O*-acetyl-$G_{D2}$ is shown in Fig. 10, along with the full 500-MHz $^1$H NMR spectrum obtained from 50 μg of purified material. Resolution enhanced anomeric and acetyl regions of putative *O*-acetyl-$G_{D2}$ (*panels D* and *B*) are compared directly with the spectra obtained from $G_{D2}$ isolated from the same cell line as a reference (*panels C* and *A*). Signals were assigned according to previously reported values in the literature for the solvent and temperature conditions used. The anomeric region of putative *O*-acetyl-$G_{D2}$ displays two anomeric protons at 4.284 ppm ($J_{1,2}$ = 6.8 Hz), and 4.130 ppm ($J_{1,2}$ = 7.0 Hz), which can be assigned to the H-1 units of βGal and βGlu, respectively. The corresponding signals from the $G_{D2}$ spectrum showed similar chemical shifts at 4.275 ppm ($J_{1,2}$ = 8.5 Hz) and 4.128 ppm ($J_{1,2}$ = 8.0 Hz). Thus the core region of the putative *O*-acetyl-$G_{D2}$, like $G_{D2}$, is likely to be Gal(β1-4)Glc(β1-1)Cer. This is consistent with the putative *O*-acetyl-$G_{D2}$ being sensitive to endoglycoceramidase

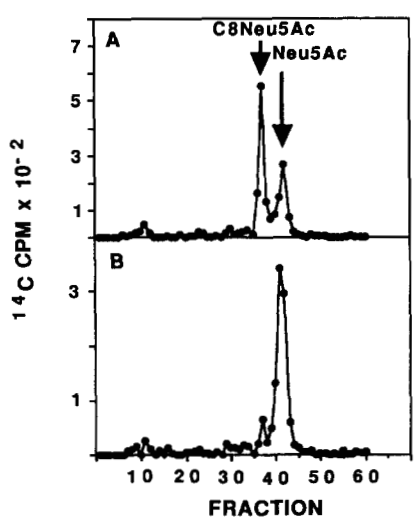


FIG. 9. **Periodate oxidation of $G_{D2}$ and *O*-acetyl-$G_{D2}$.** Labeled $G_{D2}$ and *O*-acetyl-$G_{D2}$ were isolated from M21 cells metabolically labeled with [$^{14}$C]glucosamine. Samples were treated with 3 mM periodate, reduced with borohydride, the sialic acids released by acid hydrolysis, purified, and analyzed by AX-5 HPLC as described under "Experimental Procedures." Sialic acid analogues were separated by isocratic elution with acetonitrile, water, 0.25 M monobasic sodium phosphate (68:21:11 v/v/v) at a flow rate of 0.5 ml/min for the initial 5 min increasing to 1 ml/min for the remainder of the run. Thirty-second fractions were collected. An internal standard of [9-$^3$H] Neu5Ac was added just prior to each chromatographic analysis to determine the elution position of Neu5Ac (not shown). *A*, $G_{D2}$ treated with 3 mM periodate; *B*, *O*-acetyl-$G_{D2}$ treated with 3 mM periodate.

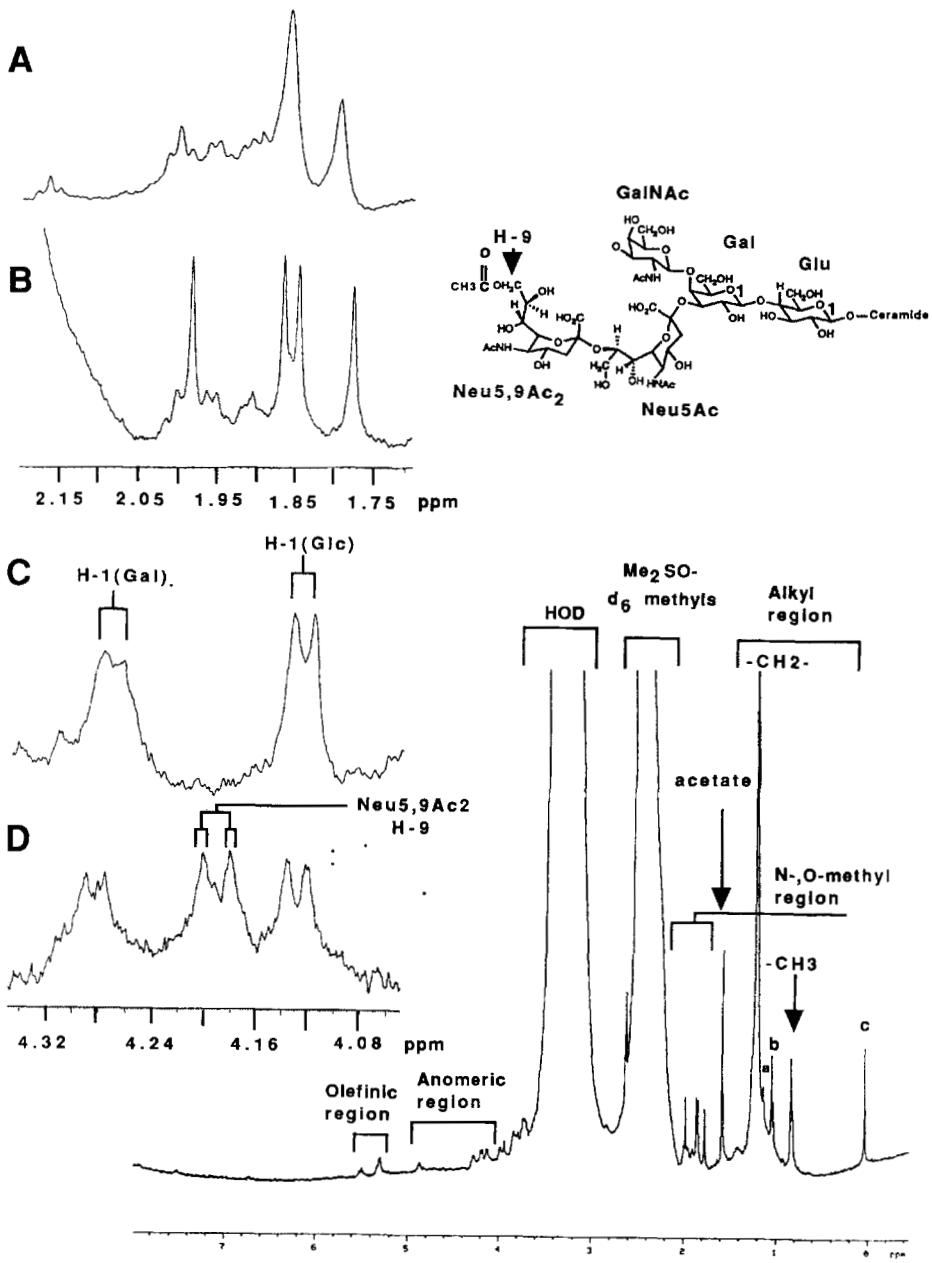


FIG. 10. **500 MHz $^1$H NMR spectrum of *O*-acetyl-$G_{D2}$ in deuterated dimethyl sulfoxide/$D_2O$ (98:2).** *Panel A* shows the resolution enhanced expansion of the acetyl region of $G_{D2}$ isolated from M21 cells. This is directly compared with *panel B*, which displays the resolution enhanced expansion of the acetyl region of *O*-acetyl-$G_{D2}$ showing the *O*-acetyl methyl singlet (1.982 ppm), two *N*-acetyl methyl singlets of Neu5Ac (1.864 and 1.845 ppm), and an *N*-acetyl methyl singlet of GalNAc (1.775 ppm). *Panel C* displays the resolution enhanced expansion of the anomeric region of $G_{D2}$ isolated from M21 cells. This is directly compared with *panel D*, which displays the resolution enhanced expansion of the anomeric region of *O*-acetyl-$G_{D2}$ showing H-1 of βgalactose (4.284 ppm), H-1 of βglucose (4.130 ppm), and H-9 of 9-*O*-acetylated sialic acid (4.193 ppm). *Peaks a, b,* and *c* arise from contaminants. The postulated structure of 9-*O*-acetyl-$G_{D2}$ is illustrated.

(data not shown), an enzyme whose preferential substrate specificity is dependent upon the presence of such a core region (88).

The anomeric proton of βGalNAc appears at $\delta = 4.882$ ($J_{1,2} = 8.5$ Hz) and around $\delta = 4.78$ for *O*-acetyl-$G_{D2}$ and $G_{D2}$, respectively. In the case of $G_{D2}$ the shape of the signal is poor, preventing exact determination of chemical shift and coupling constant. The GalNAc chemical shift in 9-*O*-acetyl-$G_{D2}$ is consistent with that previously assigned by Hirabayashi *et al.* (89) for a ganglioside presenting -3)GalNAc(β1–4)[Neu5Ac-(α2–3)]Gal(β1-, while the corresponding signal for $G_{D2}$ agrees better with the assigment made by Yu *et al.* (90) for GalNAc(β1–4)[Neu5Ac(α2–3)]Gal(β1–4)Glc(β1–1′)Cer ($G_{M2}$). There is a noticeable downfield shift (approximately 0.1 ppm) suffered by the anomeric proton of GalNAc in 9-*O*-acetyl-$G_{D2}$ compared with $G_{D2}$. The strong mutual interaction with hydrogen bonds between the βGalNAc and the sialic acid residues linked to the same unit has been proved recently by a combination of NMR and molecular mechanics analysis (91). In 9-*O*-acetyl-$G_{D2}$, the sialic acid unit vicinally linked to βGalNAc is in turn linked to another sialic acid via its C-8 hydroxyl group thereby precluding the possibility of such hydrogen bonding interactions through this group. The 9-*O*-acetyl group also changes the spatial presentation of the rest of the groups in the C7-C8-C9 tail and restrains flexibility. Integration of the three anomeric protons indicates a 1:1:1 ratio, as expected.

The acetyl region of the *O*-acetyl-$G_{D2}$ spectrum displayed in *panel B* is characterized by the appearance of a methyl singlet at 1.982 ppm corresponding to an *O*-acetyl ester, downfield from two sialic acid *N*-acetyl methyl singlets at 1.864 and 1.845 ppm. The $G_{D2}$ acetyl region upon integration reveals two sialic acid *N*-acetyl resonances at 1.860 ppm. The methyl singlet present in the *O*-acetyl region from *O*-acetyl-$G_{D2}$ is absent from the corresponding $G_{D2}$ region, consistent with the presence of an *O*-acetyl ester. The integral requires that a single *O*-acetyl group (three protons) be present which agrees with the difference of 42 mass units found by FAB-

MS analysis. Both spectra show the presence of GalNAc *N*-acetyl methyl singlets at 1.775 ppm for *O*-acetyl-$G_{D2}$ and 1.794 ppm for $G_{D2}$. There is a downfield shifted signal at 4.19 ppm in the 9-*O*-acetyl-$G_{D2}$ spectrum that is clearly absent from the $G_{D2}$ spectrum. This signal was assigned to H-9a ($\delta$ = 4.203) and H-9b($\delta$ = 4.182) with $J_{9a,9b}$ = 10.5 Hz of 9-*O*-acetylated sialic acid (31, 92). There is no evidence for acetylation of C7 hydroxyls, such as was found in Golgi-enriched preparations labeled with [$^3$H-*acetyl*]acetyl-CoA. However, in those preparations only 10–15% was found to be 7-*O*-acetylated, which might not be detected in our NMR experiments. Additionally, the *in vitro* labeling reactions required much less manipulation of samples relative to our NMR experiments, which may have caused migration to the relatively more stable C9 hydroxyl (82). A signal observed in the spectrum of 9-*O*-acetyl-$G_{D2}$ at 3.941 ppm has been assigned to H-5 of the sialic acid (42, 92). A similar signal is observed in the spectrum of $G_{D2}$, but the influence of the residual HOD peak results in poor resolution.

Signals arising from the ceramide moiety of both gangliosides show the same chemical shifts and coupling constants. As expected the spectra are dominated by the signals of the alkyl region: $\delta$ = 1.214 ppm for the resonance of the methylene groups and $\delta$ = 0.833 for the terminal methyl groups of both alkyl chains (93). Integration of both signals yield a ratio 56:6, which agrees well with the expected value for a 1:1 mixture of 16:0 and 24:1 fatty acyl groups with a d18:1 sphingosine. The olefinic region shows a doublet of triplets at $\delta$ = 5.512 ppm and a doublet of doublets at $\delta$ = 5.309, each integrating for one proton, as expected for two methine protons of a trans double bond with neighboring methine and methylene groups, respectively, as found in C-4 and C-5 of sphingosine. These assigments are supported by previous reported spectra (93, 94). A triplet superimposing the signal at 5.309 ppm ($\delta$ = 5.298, $J$ = 5.0 Hz) was assigned to the isochronous methine protons of the cis double bond of the unsaturated fatty acyl group (93). From the spectrum of $G_{D2}$ it is possible to assign the allylic (doublet of triplets at 1.907 ppm), and $\alpha$-carbonyl methylene resonances (triplet at 2.001 ppm, $J$ = 6.0 Hz) of C-6 of sphingosine and C-2 of the fatty acyl group. An additional allylic resonance is found at $\delta$ = 1.950 ppm and was assigned to the methylene protons adjacent to the cis double bond of the fatty acyl group by comparison with previously reported data (93). The fact that this signal is observed rules out the possibility of hydroxylation of the fatty acyl group that would produce a downfield shift towards the HOD peak avoiding the observation. A very small signal at 7.509 ppm, in both spectra, corresponds to the amide proton of sphingosine. Since the region upfield from the HOD peak in the spectrum of 9-*O*-acetyl-$G_{D2}$ appears less affected than the correspondent region of $G_{D2}$, it is possible to detect in this case a doublet of doublets at 3.988 ppm, a doublet of doublets at 3.813 ppm, and a doublet of triplets at 3.73 ppm that agree well with the reported chemical shifts for the protons attached to C-1, C-3, and C-2 of sphingosine, respectively (93).

*9-O-Acetyl-$G_{D2}$ Does Not React with MAbs JONES, 27A, or D1.1*—Several MAbs directed against 9-*O*-acetyl-$G_{D3}$ have been derived by immunizing mice with cells arising from the neuroectoderm at various stages of development (31, 48–50, 77) or with antigens derived from glomerular tissues (95–97). These antibodies also react with other unidentified alkali-labile gangliosides with slower HPTLC migration relative to 9-*O*-acetyl-$G_{D3}$ (98). To clarify if these unknown gangliosides may be 9-*O*-acetyl-$G_{D2}$, we tested the reactivity of three MAbs known to detect 9-*O*-acetyl-$G_{D3}$ with total gangliosides from M21 cells and with purified 9-*O*-acetyl-$G_{D2}$. As shown in Fig. 11, all three MAbs react with M21 ganglioside antigens with identical HPTLC migration characteristics. The upper doublet, migrating between the standards $G_{M2}$ and $G_{M1}$, is by previous definition, 9-*O*-acetyl-$G_{D3}$. The slower migrating doublet is an unidentified ganglioside antigen with a slightly slower migration than 9-*O*-acetyl-$G_{D2}$. In contrast, none of these MAbs recognized purified 9-*O*-acetyl-$G_{D2}$. The band migrating above $G_{M3}$ is apparently nonspecific since it is observed consistently in immuno-overlays of total ganglioside extracts with all our antibodies. Additionally, it develops as a yellowish color rather than the deep brown color observed with specific binding.

*MAb 8A2 Defines Two Unidentified M21 9-O-Acetylated Gangliosides and Reacts Weakly with 9-O-Acetyl-$G_{D2}$*—MAb 8A2 was originally derived by immunization with embryonic day 14 chick optic nerve. It is directed against several alkali-labile, mild periodate-resistant gangliosides from embryonic chicken brain, with slower HPTLC migratory characteristics relative to 9-*O*-acetyl-$G_{D3}$ (60). As shown in Fig. 11, 8A2 reacts with two M21 ganglioside antigens migrating slower on HPTLC than 9-*O*-acetyl-$G_{D2}$. In agreement with the previous report (60) it does not react with 9-*O*-acetyl-$G_{D3}$. The faster migrating 8A2-reactive species shares an identical HPTLC migration position with the unidentified ganglioside species reacting with MAbs D1.1, JONES, and 27A and migrates slightly slower than 9-*O*-acetyl-$G_{D2}$. The second unidentified 8A2-reactive M21 ganglioside antigen has a migration position similar to bovine brain $GD_{1b}$. Although 8A2 did not detect 9-*O*-acetyl-$G_{D2}$ in M21 ganglioside extracts or by lipid ELISA of M21 gangliosides fractionated by DEAE HPLC (data not shown) we observed weak reactivity with purified 9-*O*-acetyl-$G_{D2}$ (see Fig. 11). Since a similar amount of 9-*O*-acetyl-$G_{D2}$ should be present in the M21 total ganglioside extract relative to the amount of purified material analyzed (0.5–1 nmol) it may be that impurities in the total extract interfere with detection. Regardless, 8A2 is certainly not highly reactive with 9-*O*-acetyl-$G_{D2}$ but instead defines two structurally unidentified 9-*O*-acetylated gangliosides from M21 cells, one of which may also be recognized by D1.1, JONES, and 27A in HPTLC immuno-overlays.

Inner ganglioside lactones can also cause alkali-sensitive

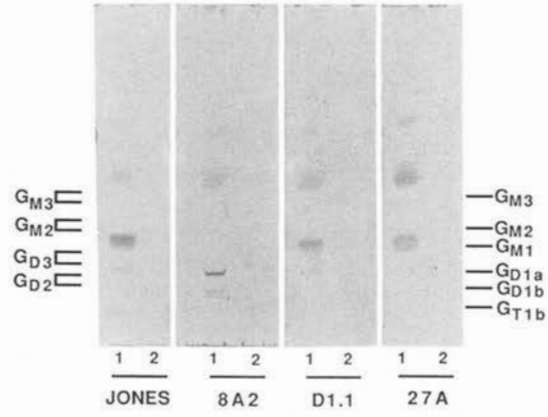


FIG. 11. **Immunoreactivity of M21 gangliosides and 9-*O*-acetyl-$G_{D2}$ with monoclonal antibodies directed against *O*-acetylated gangliosides.** M21 gangliosides (5 nmol of total sialic acid, *lanes* marked *1*) and purified *O*-acetyl-$G_{D2}$ (0.5 nmol, *lanes* marked *2*) were chromatographed in adjacent lanes on aluminium-backed HPTLC plates. Lanes were cut and overlayed with MAbs JONES, 8A2, D1.1, or 27A. Antibody-reactive gangliosides were detected as described under "Experimental Procedures." Standard M21 gangliosides (*far left*) and bovine brain gangliosides with the added $G_{M3}$ and $G_{M2}$ (*far right column*) were chromatographed in parallel lanes and detected by resorcinol.

shifts of HPTLC migration (65, 99, 100) and could be confused with *O*-acetylated compounds. To confirm that the reactivity of D1.1, JONES, 27A, and 8A2 with M21 gangliosides is dependent upon the presence of a 9-*O*-acetyl group, we treated the total ganglioside mixtures with influenza C virus, which is known to carry a 9-*O*-acetyl sialic acid-specific *O*-acetylesterase (44, 45). This treatment abrogated reactivity of all four antibodies with M21 gangliosides (data not shown).

## DISCUSSION

In the present study, we have purified and structurally characterized the novel tumor-associated ganglioside 9-*O*-acetyl-$G_{D2}$ by various radiochemical, physical, and immunological methods. Initial clues to the expression of 9-*O*-acetyl-$G_{D2}$ were obtained by studying the biosynthesis of ganglioside *O*-acetyl esters in Golgi-enriched vesicles from human melanoma cells. Since this assay system depends upon the transfer of [$^3$H]acetate from exogenously added [$^3$H-*acetate*]acetyl-CoA to endogenous acceptors, we could detect the expression of an *O*-acetylated ganglioside distinct from 9-*O*-acetyl-$G_{D3}$ by chromatographic procedures. The detection of 7- and 9-*O*-acetyl isomers of $G_{D2}$ lends merit to this facile and sensitive approach since our subsequent NMR studies were unable to detect the 7-*O*-acetyl isomer. This apparent discrepancy between newly synthesized *O*-acetyl esters and the bulk of *O*-acetylated gangliosides may be explained by intra- and extracellular pH differences. The Golgi apparatus, the presumed intracellular site of *O*-acetyl ganglioside biosynthesis, is known to be slightly acidic (101–103), favoring stability of 7-*O*-acetyl esters (68, 82). As newly synthesized 7-*O*-acetylgangliosides reach the cell surface, they should encounter an increase in pH. Since 7-*O*-acetyl esters on sialic acids are known to migrate to the more stable C9 position under mildly alkaline conditions, the increased cell surface pH may explain why 7-*O*-acetyl isomers of gangliosides have not been detected in either the current or previous NMR experiments (31). However, it is also possible that during the multistep purification of *O*-acetylated gangliosides the 7-*O*-acetyl esters may have migrated to the C9 hydroxyl position (82).

We have previously examined the biosynthesis of *O*-acetyl esters on $G_{D3}$ in human melanoma Golgi vesicles and on *N*-linked oligosaccharides in rat liver Golgi preparations (43, 59, 80). In both of these systems we found that acetyl groups were transferred to either C7 or C9 sialic acid hydroxyl groups. Thus, the biosynthesis of *O*-acetyl esters on $G_{D2}$ appears to be mechanistically similar to that for both $G_{D3}$ and *N*-linked oligosaccharides. Importantly, these results indicate that $G_{D2}$ as well as $G_{D3}$ is an acceptor for ganglioside *O*-acetyltransferase. At this time it is unknown if acetate is transferred to the sialic acids of $G_{D2}$ or $G_{D3}$ in a transmembrane reaction similar to the *O*-acetyltransferase of rat liver (80). We are investigating this matter further.

With the structural elucidation of 9-*O*-acetyl-$G_{D2}$, seven gangliosides have been reported to exist as 9-*O*-acetylated derivatives: $G_{D3}$, $G_{D2}$, $G_{T1b}$, $G_{Q1b}$, $G_{T3}$, disialosylparagloboside, and $G_{D1a}$ (31, 53, 67, 77, 81, 105, 106). Upon examination of this list, two patterns emerge. First, it is evident that all common B series gangliosides with the exception of $G_{D1b}$ have been now reported as 9-*O*-acetylated derivatives (see Fig. 12). Because of the known biosynthetic relationships of these parent molecules to one another (4, 5, 107), the expression of 9-*O*-acetylated $G_{D1b}$ can now be predicted (see Fig. 12). Second, the *O*-acetyl esters on almost all reported 9-*O*-acetylated gangliosides have been directly or indirectly demonstrated (9-OAc$G_{D3}$, 9OAc$G_{D2}$, 9OAc$G_{T1b}$ and 9OAc$G_{Q1b}$, 9OAc$G_{T3}$, and 9OAc-disialosylparagloboside) to be specifically located on a terminal α2–8-linked sialic acid originating from the internal core β1–4-linked galactose of lactosylceramide. Even in the case of $G_{Q1b}$, which carrys two distinct terminal α2–8-linked sialic acid residues, this specific pattern holds true (*i.e.* the α2–8-linked sialic acid arising from the outer Gal(β1–3)GalNAc sequence is not *O*-acetylated). This is in striking contrast to the *O*-acetyltransferase of rat liver, which primarily esterifies α2–6-linked sialic acid residues on *N*-linked oligosaccharides (59). This pattern suggests the existence of a specific ganglioside *O*-acetyltransferase recognizing only terminal α2–8-linked sialic acids originating from the internal β1–4-linked core galactose. Indeed, this may also explain our observation that *O*-acetylation of sialic acids is restricted to gangliosides in melanoma cells (43).

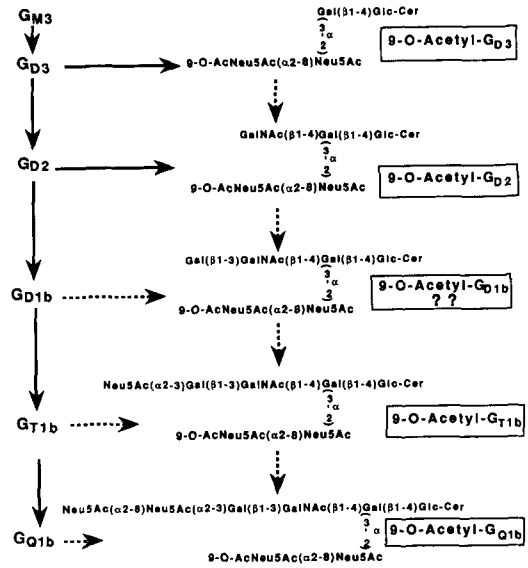


FIG. 12. **Reported and postulated 9-*O*-acetylated gangliosides of the B-series: proposed structural and biosynthetic relationships.** The four well characterized 9-*O*-acetylated gangliosides that have been reported in the literature are shown. The *solid arrows* indicate biosynthetic pathways reported in a variety of previous studies discussed in the text and some shown in this study. The *broken arrows* indicate possible biosynthetic reactions that need to be explored. The postulated ganglioside 9-*O*-acetyl-$G_{D1b}$ is shown in relation to the previously established ones.

The only exception to this pattern is a report of 9-*O*-acetylated $G_{D1a}$ (106), in which the 9-*O*-acetyl ester can only exist on a terminal α2–3 sialic acid. However, the structure of this putative 9-*O*-acetyl-$G_{D1a}$ was derived only by relative HPTLC mobilities, *Vibrio cholera* sialidase digestion and gas chromatography-MS of released sialic acids (106), without sequence or linkage analysis of the core structure by other techniques. It is possible that the 9-*O*-acetyl group in this case was actually on an α2–8-linked sialic acid of a different ganglioside, with a similar HPTLC mobility to $G_{D1a}$ after de-*O*-acetylation. Alternatively, there may be distinct ganglioside *O*-acetyltransferases, one specific for terminal α2–8-linked sialic acids and another specific for terminal α2–3 sialic acids.

Several findings from the immunological characterization of 9-*O*-acetyl-$G_{D2}$ are worthy of discussion. A MAb presumed to be specific for $G_{D2}$ (14.G2A) also reacted strongly with 9-*O*-acetyl-$G_{D2}$. This is in contrast to antibodies against $G_{D3}$, which do not typically react with its *O*-acetylated counterpart (29, 108). This finding may have important consequences since the Fab portion of 14.G2A has been used in diverse studies ranging from potential immunotherapy of melanoma to cellular adhesion (36, 109). For instance, an antibody with the same specificity as 14.G2A (14.18) was used to define

gangliosides enriched in focal adhesion plaques, implicating $G_{D2}$ as a molecule acting synergistically with cell adhesion proteins (109). Thus 9-*O*-acetyl-$G_{D2}$ may also be involved in cellular adhesion events. From a clinical standpoint, the structural elucidation of the novel tumor-associated antigen 9-*O*-acetyl-$G_{D2}$ represents a promising new target molecule for immunotherapy, particularly since its expression in tissues may be restricted to a greater extent than $G_{D2}$. In this regard, a MAb reacting specifically with chemically *O*-acetylated $G_{D2}$ has been recently created (110).

Several MAbs defining 9-*O*-acetylated gangliosides as developmentally regulated antigens now exist. Three of these MAbs, JONES, D1.1, and 27A, define 9-*O*-acetyl-$G_{D3}$ and also react with a slower migrating, alkali-labile species in HPTLC immuno-overlays. Since none of these three MAbs reacted with purified 9-*O*-acetyl-$G_{D2}$, this lower alkali-labile band is yet another 9-*O*-acetylated ganglioside of melanoma cells. We also tested a fourth MAb, 8A2, which reacts with several base-labile, periodate-insensitive gangliosides with slower HPTLC mobilities than 9-*O*-acetyl-$G_{D3}$ (60). Although it reacted very weakly with 9-*O*-acetyl-$G_{D2}$, 8A2 bound well to two 9-*O*-acetylated gangliosides in M21 cells with even slower mobilities on HPTLC. Thus, 8A2 defines two structurally unidentified 9-*O*-acetylated gangliosides from M21 cells, one of which may be the slower migrating species recognized by JONES, D1.1, and 27A. We are currently characterizing the structure of 8A2 antigens expressed by M21 cells.

Several observations suggest that 9-*O*-acetylated gangliosides are expressed in a temporally and spatially regulated manner during development of the central nervous system. For example, studies with the monoclonal antibody JONES demonstrate a dorsal-ventral gradient of 9-*O*-acetyl-$G_{D3}$ across the developing retina, superimposed upon a constantly expressed level of its precursor, $G_{D3}$ (49–51). In the developing central nervous system, high levels of 9-*O*-acetyl-$G_{D3}$ are regionally expressed, decreasing to lower levels postnatally (51, 56, 57). It has been generally assumed that ganglioside *O*-acetylation is regulated by the developmental expression of ganglioside *O*-acetyltransferase. However, the postnatal cessation of $OAcG_{D3}$ expression coincides with the appearance of more complex B-series gangliosides, such as $9OAcG_{T1b}$ and $9OAcG_{Q1b}$ in the adult central nervous system (53, 67). Thus, the loss of histochemical staining with $9OAcG_{D3}$-specific antibodies could represent an enhanced conversion to non-cross-reactive $OAcG_{D2}$ rather than a loss of ganglioside *O*-acetyltransferase activity. If this is true, temporal and spatial expression of *O*-acetylated gangliosides during development may be modulated not by the *O*-acetyltransferase but instead by regulating the *N*-acetylgalactosaminyltransferase which catalyzes the first committed step of B-series ganglioside biosynthesis. In this scenario ganglioside *O*-acetyltransferase could be constitutively expressed throughout, acting on all available B-series gangliosides to varying extents.

The structural characterization of 9-*O*-acetyl-$G_{D2}$ partially bridges the biosynthetic gap between 9-*O*-acetyl-$G_{D3}$ and 9-*O*-acetyl-$G_{T1b}$ and supports this hypothesis (Fig. 12). Also, the observation that $G_{D2}$ is a precursor for 9-*O*-acetyl-$G_{D2}$ sets a precedent for $G_{T1b}$ and $G_{Q1b}$ being precursors of their own 9-*O*-acetylated derivatives. Obviously, final proof of such biosynthetic and regulatory mechanisms for 9-*O*-acetylation of gangliosides in neuroectodermal tissues and tumors awaits the molecular characterization of the enzymes involved in the biosynthesis of these interesting gangliosides.

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*Note Added in Proof*—While this manuscript was under review a report postulating the existence of 9-*O*-acetyl$G_{D2}$ in neuroblastoma cells appeared (Ye, J. N., and Cheung, N.-K. V. (1992) *Int. J. Cancer* **50**, 197–201). Ye and Cheung inferred the presence of this 9-*O*-acetylated ganglioside partially on the basis of reactivity with MAb D1.1. The present report demonstrates that D1.1 is not reactive with 9-*O*-acetyl$G_{D2}$ but instead reacts with an uncharacterized 9-*O*-acetylated ganglioside migrating slightly below 9-*O*-acetyl $G_{D2}$. The neuroblastoma-associated ganglioside was purified by extraction from HPTLC plates. Thus, a possible explanation for this discrepancy is that reactivity to D1.1 observed by Ye and Cheung was brought about by a contaminating 9-*O*-acetylated ganglioside such as the uncharacterized species discovered in the current study.

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