Characterization of a hamster melanoma-associated ganglioside antigen as 7-*O*-acetylated disialoganglioside GD3

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Abstract We previously reported a hamster animal model of melanoma in which the tumor tissue expresses gangliosides GM3, GD3, and *O*-acetyl GD3. This ganglioside pattern is similar to that in human melanomas (Ren, S., A. Slominski, and R. K. Yu. 1989 *Cancer Res.* **49:** 7051). In this study, we isolated and purified these gangliosides using chloroform–methanol extraction, Folch partition, chromatographies on DEAE-Sephadex A-25, and Iatrobeads columns. The yields of gangliosides GM3, GD3, and *O*-acetyl GD3 were 44.1 mg, 19.6 mg, and 9 mg per 100 g of Ma melanotic melanoma tissues, respectively. The structures of these gangliosides were characterized by periodate oxidation, gas chromatographic (GC) analysis, fast-atom bombardment–mass spectrometry (FAB-MS), and nuclear magnetic resonance (NMR) studies. The structure of hamster melanoma *O*-acetyl GD3 is different from the 9-*O*-acetyl GD3 previously reported in human melanoma. The major fatty acids of this ganglioside are C16:0, C18:0, C20:0, C22:0, and C24:0 and the long-chain base is C18-sphingosine.—**Ren, S., T. Ariga, J. N. Scarsdale, Y. Zhang, A. Slominski, P. O. Livingston, G. Ritter, Y. Kushi, and R. K. Yu.** Characterization of a hamster melanoma-associated ganglioside antigen as 7-*O*-acetylated disialoganglioside GD3. *J. Lipid Res.* 1993. **34:** 1565–1572.

Supplementary key words 7-*O*-acetyl GD3 • fast-atom bombardment–mass spectrometry • nuclear magnetic resonance

Sialic acids are a diverse family of 9-carbon sugars found as the terminal monosaccharides on many vertebrate glycoconjugates (1, 2). Recently, there has been a growing interest in the occurrence, metabolism, and function of *O*-acetylated sialic acids of glycoproteins and gangliosides. The expression of *O*-acetylated sialic acid-containing glycoconjugates is known to be tissue-specific, developmentally regulated, and exhibits regional distribution in a variety of tissues; these glycoconjugates sometimes reappear as oncofetal antigens (3–10). The selective expression of *O*-acetylated disialoganglioside (*O*-Ac-GD3) is found in the embryonic adrenal gland, the mesonephros, and certain regions of the developing nervous system, but not in other parts of the fetus (3–6). Studies with the monoclonal antibody JONES have demonstrated that this unusual ganglioside shows a dorsal–ventral gradient across the developing retina, a distribution distinct from its precursor, GD3 (8–10). Similar discordance between GD3 and its *O*-acetylated counterpart is found in other areas of the developing central and peripheral nervous system (11–13).

O-Acetyl GD3 expressed in human melanoma is believed to be a potential melanoma-associated antigen (3, 4) and is a promising target for melanoma immunotherapy (3, 4, 6). We previously reported that hamster melanomas contained GM3, GD3, and *O*-acetyl GD3 as the major gangliosides (14), a pattern similar to that expressed in certain human melanomas. The expression of *O*-acetyl GD3 in melanoma cells may serve to modulate and provide selectivity for the critical events of cell metastasis and growth (14). In spite of such intriguing clues, little is known about the structure–function relationship of the *O*-acetylated gangliosides in various tissues. The spontaneous migration of the acetyl group at 7-position to 9-position in free sialic acid has been reported by different groups (15). Several lines of evidence indicate that the acetyl group in the *O*-acetylated GD3 of human melanomas is located at the 9-position of the terminal sialic acid moiety (4, 6, 16–18). Recently, Manzi et al. (19) presented in-

Abbreviations: Cer, ceramide; FAB-MS, fast-atom bombardment–mass spectrometry; NMR, nuclear magnetic resonance; HPTLC, high performance thin-layer chromatography; PBS, phosphate-buffered saline. The nomenclature for gangliosides is after that of Svennerholm (*J. Neurochem.* 1963. **10:** 613). The glycosphingolipid nomenclature follows that recommended by IUPAC-IUB (*J. Biol. Chem.* 1982. **257:** 3347).

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direct evidence for the existence of 7-*O*-acetyl GD3 together with 9-*O*-acetyl GD3 in human melanoma cell lines by analysis of the free sialic acid released from these gangliosides after neuraminidase treatment. More recently, we reported the presence in bovine buttermilk of a mixture of 9-*O*-acetyl, 7-*O*-acetyl, and 7,9-*O*-acetyl GD3 (20). To understand the function of acetyl groups during different cellular events, it is of fundamental importance to characterize the structures of *O*-acetyl GD3 from different tissues and human melanomas, and to compare these structures with those from bovine buttermilk. In the present paper, we describe the purification and characterization of the hamster melanoma-associated ganglioside antigen, 7-*O*-acetyl GD3.

MATERIALS AND METHODS

Materials

High-performance TLC plates (nano-plates, 10 cm × 20 cm) were purchased from E. Merck, Darmstadt, Germany. DEAE-Sephadex A-25 and Iatrobeads were from Pharmacia Fine Chemicals, Uppsala, Sweden, and Iatron Laboratories, Inc., Tokyo, Japan, respectively. Anhydrous pyridine, hexamethyldisilazane, and trimethylchlorosilane were purchased from Pierce Chemical Co., Rockford, IL. All other chemicals were of HPLC or analytical grade. The authentic standard gangliosides, GM3, GD3, and *O*-acetyl GD3 were purified from bovine buttermilk in our laboratory (20).

Purification of gangliosides

Transplantable Bomirski Ma melanotic melanomas in Syrian golden hamsters were used (21). Bomirski Ma melanomas were implanted subcutaneously into the right and left regions of 10 male Syrian golden hamsters, 3 months old, as described previously (14). Tumor-bearing animals were killed by cervical dislocation under ether narcosis. Melanoma tissues were dissected, freed from necrotic and connective tissues, rinsed several times in PBS, and combined. The tumor tissues were stored at −70°C until extracted. The melanoma tissues (100 g) were cut into small pieces with scissors and then homogenized in 50 ml of cold distilled water. The total lipids were extracted with 10 volumes of chloroform–methanol (C–M, 2:1, v/v) by stirring overnight. After filtration, the residues were re-extracted with 10 volumes of C–M 1:2 (v/v) and 10 volumes of solvent A (C–M–water (W), 30:60:8, v/v) by stirring for 2 h at each extraction. The combined lipid extracts were adjusted to the ratio of solvent A and then applied to a DEAE-Sephadex A-25 column (90 cm × 2.0 cm, i.d.) (22). The column was washed with 10 volumes of solvent A to remove neutral lipids. The acidic lipids were eluted in a stepwise manner with a solvent system of 1000 ml each of solvent A containing 0.05 M, 0.2 M, and 0.8 M sodium acetate, respectively. The recovered mono-, di-, and polysialoganglioside fractions were evaporated to dryness and dialyzed against distilled water for 2 days to remove salts. The mono- and disialoganglioside fractions were applied to an Iatrobeads column (100 cm × 2.0 cm, i.d.) and individual gangliosides were eluted by a continuous gradient made from 1000 ml each of chloroform–methanol–0.5% $CaCl_2 \cdot 2H_2O$ (70:30:3 and 35:65:5, v/v). Final purification of *O*-acetyl GD3 was achieved by Iatrobeads column chromatography (110 cm × 0.4 cm, i.d.) with continuous gradient elutions of 200 ml each of C–M–W (70:30:3 and 35:65:5, v/v) (20). The purity of *O*-acetyl GD3 was examined by high-performance thin-layer chromatography (HPTLC) (E. Merck, Darmstadt, Germany) with three different solvent systems: *a)* n-propanol–water 80:20 (v/v); *b)* chloroform–methanol–0.5% $CaCl_2 \cdot 2H_2O$ 55:45:10 (v/v); *c)* chloroform–methanol–2.5 N ammonium hydroxide 65:35:5 (v/v). Gangliosides were visualized by spraying with the resorcinol-HCl reagent (23) followed by heating at 95°C for 30 min.

Compositional analysis

Compositional analysis was carried out by gas–liquid chromatography (GLC) (24). A ganglioside sample, containing about 50 μg of sialic acid, was subjected to methanolysis with 1 ml of 3% methanolic hydrochloride for 18 h at 75°C in order to determine neutral sugars, sialic acids, and fatty acids. After methanolysis, fatty acid methyl esters were extracted with n-hexane, and the extract was evaporated and dissolved in 50 μl of n-hexane. An aliquot of this solution was injected into a GLC column of HP5 with a head pressure of 25 psi (cross-linked 5% PhMe Silicone, 25 m × 0.2 mm × 0.5 um film thickness) and maintained at 270°C. The lower methanolic layer was evaporated under a stream of nitrogen and dried in vacuo. The dried residue was then treated with 100 μl of hexamethyldisilazane–trimethylchlorosilane–pyridine 1.3:0.8:1 (v/v) at 60°C for 5 min to effect the formation of *O*-trimethylsilyl derivatives of saccharides. An aliquot of the reaction mixture was injected onto the HP5 column programmed at 3°C/min from 230 to 270°C.

Fast-atom bombardment (FAB)–mass spectrometry

Negative ion fast-atom bombardment–mass spectra (FAB-MS) of native glycolipids were obtained by a TSQ 70 triple-stage quadrupole mass spectrometer (Finnegan MAT Inc., San Jose, CA) equipped with a FAB ion source. Xenon gas was used at 8 KV as the ionization beam. A ganglioside sample, 30 μg, was dissolved in 10 μl of C–M 1:1 (v:v), and the solution (about 1 μl) was applied to a stainless-steel sample holder. The sample was analyzed as described previously (24).

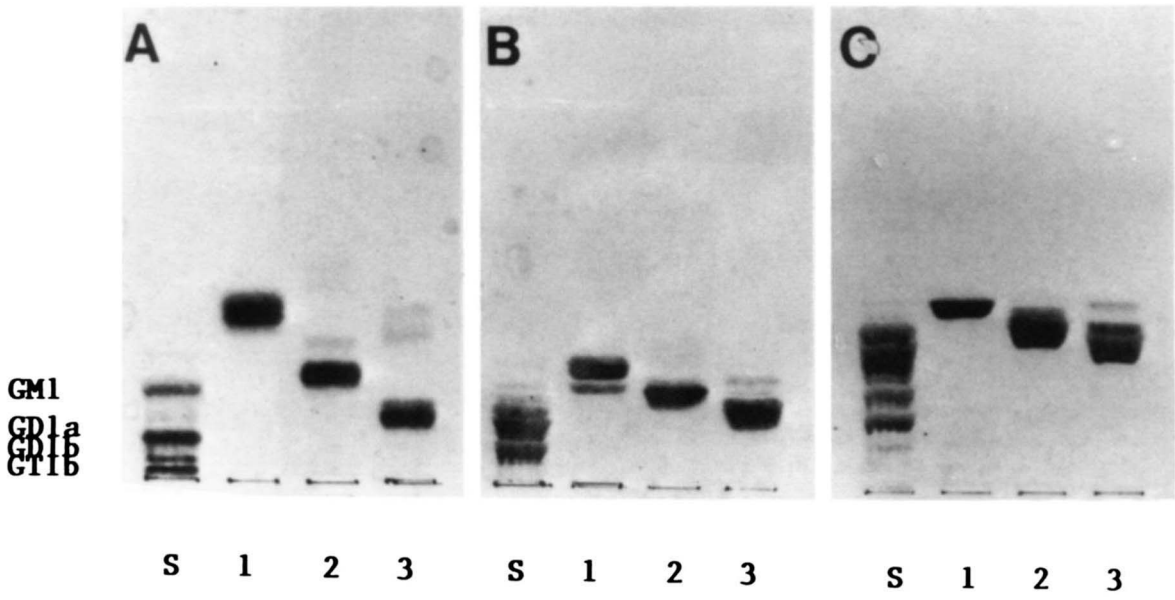

Fig. 1. Thin-layer chromatogram of the isolated gangliosides from Ma melanotic melanoma tissues in different developing solvents. Panel A: chloroform–methanol–0.5% aq.$CaCl_2$ • $2H_2O$ 55:45:10 (v/v/v); B, chloroform–methanol–2.5 N NH_4OH 65:35:10 (v/v/v); C, n-propanol–water 80:20 (v/v). Lanes 1, 2, and 3 represent purified GM3, *O*-acetyl GD3, and GD3, respectively. Each lane contains 4 μg of gangliosides. The bands were visualized by spraying with the orcinol–H_2SO_4 reagent.

Analysis of acetylation position by periodate oxidation

Ganglioside samples, containing 2–12 μg sialic acids, were evaporated under a stream of nitrogen, and then dried in vacuo. The residue was dissolved in 100 μl of PBS (pH 7.2). For de-*O*-acetylation, 100 μl of 0.2 M NaOH was added and the sample was kept at 4°C for 30 min to remove the *O*-acetyl group. Then, 100 μl of 0.2 M HCl was added for neutralization. For other samples, including the blanks, 200 μl of 0.1 M NaCl was added. All samples were oxidized by 200 μl of 2.5 mM sodium metaperiodate (blanks were treated with 200 μl PBS), and the mixtures were kept at 4°C for 30 min in the dark. The oxidation was stopped by adding 100 μl of 0.154 M sodium arsenite, followed by addition of 400 μl of acetylacetone solution (25). The mixtures were incubated at 60°C for 10 min, and diluted with 1.5 ml of double distilled water. The relative fluorescence [lambda max (excitation) = 410 nm, lambda max (emission) = 510 nm, spectrum = 470–570nm] of each sample was measured against the blanks.

Nuclear magnetic resonance analysis

Samples were prepared for ^1H NMR analysis as described previously (26). Briefly, each sample (2 mg) was dissolved in 0.5 ml of D_2O and lyophilized to remove exchangeable protons. The residue was dissolved in 0.5 ml of dimethyl sulfoxide-d_6/D_2O 98:2 (v/v). NMR spectra were obtained on an NMR spectrometer operating at ^1H frequencies of 300 MHz.

RESULTS

Three major gangliosides A, B, and C were obtained from the hamster melanotic melanoma tissues and purified to homogeneity as revealed by HPTLC shown in **Fig. 1.** Gangliosides A, B, and C co-migrated with authentic GM3, GD3, and *O*-Ac-GD3 from bovine buttermilk (20), respectively. As previously reported (14), the Bomirski hamster Ma melanoma tissue contained 0.23 μmol of lipid-bound sialic acid/g wet weight. Gangliosides A, B, and C accounted for 49%, 35%, and 16% of the to-

TABLE 1. Sugar and fatty acid compositions of gangliosides isolated from Bomirski Ma melanotic melanoma

Composition	Ganglioside Species		
	GM3 (A)	GD3 (B)	*O*-Acetyl GD3 (C)
Sugar			
Glucose	1	1	1
Galactose	1.05	1.09	1.09
Sialic acid	1.08	1.93	1.84
Fatty acid			
C16:0	3.5	3.8	10.9
C18:1	trace	1.6	4.7
C18:0	2.2	12.5	17.2
C20:0	9.5	12.3	11.3
C21:0	trace	trace	trace
C22:1	trace	trace	trace
C22:0	31.5	29.6	25.3
C23:0	8.4	1.4	trace
C24:1	7.9	10.8	7.0
C24:0	36.5	27.3	21.8

tal lipid-bound sialic acid, or 44 mg, 19.6 mg, and 9 mg per 100 g of wet tissues, respectively. **Table 1** shows the compositional analysis of carbohydrates and fatty acids of gangliosides A (GM3), B (GD3), and C (*O*-acetyl GD3). Ganglioside A contained 1 mole each of glucose (Glc), galactose (Gal), and sialic acid (SA). Gangliosides B and C contained Glc, Gal, and SA in a molar ratio of 1:1:2. The major fatty acids of ganglioside C were C18:0, C20:0, C22:0, and C24:0.

Fig. 2 shows the negative ion-FAB-mass spectra and the fragmentation diagrams of gangliosides GM3, GD3, and *O*-acetyl GD3. Among the mass spectra, ganglioside

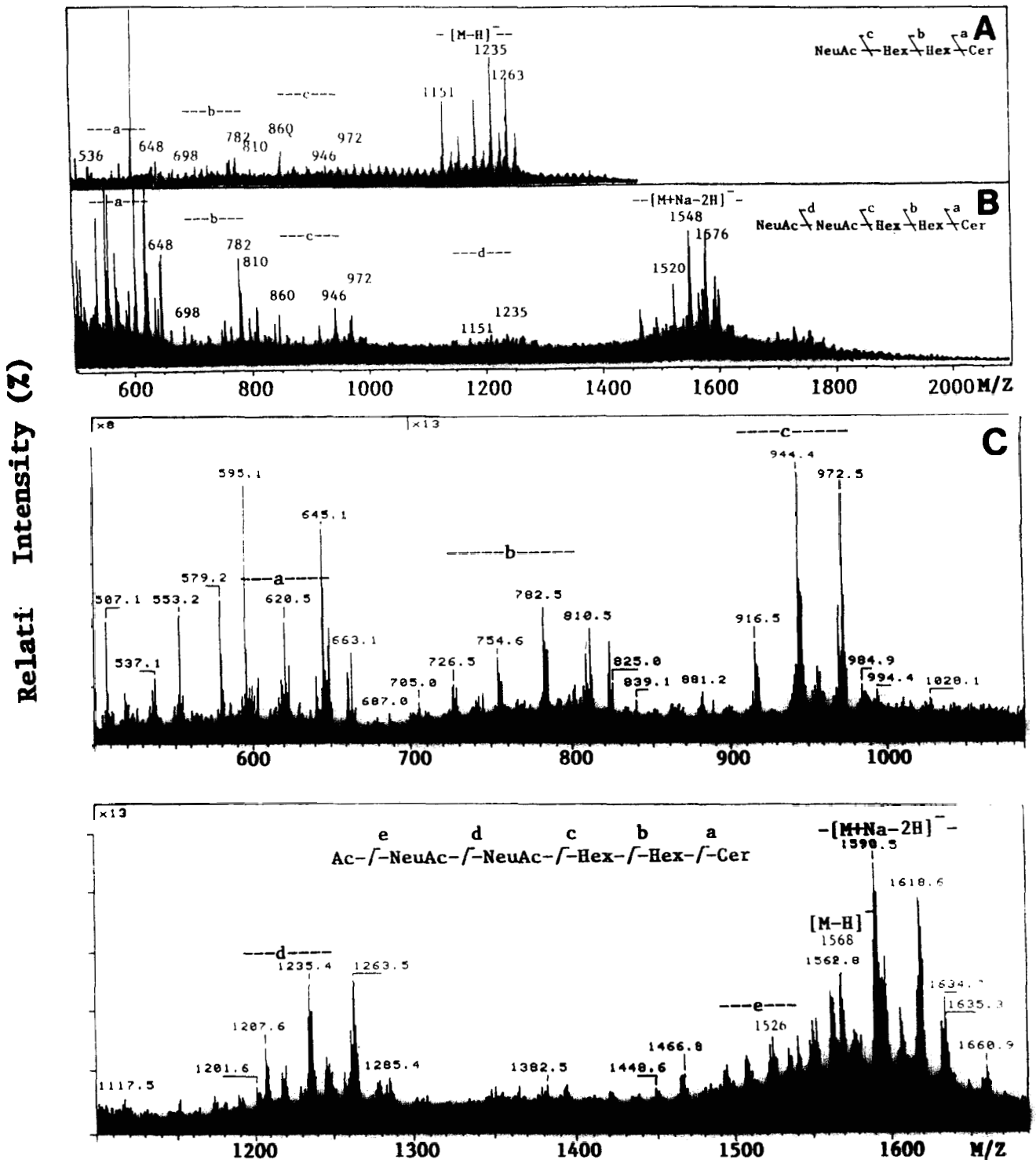

Fig. 2. Negative ion-FAB mass spectra and fragmentation diagrams of purified GM3 (A), GD3 (B), and *O*-Ac GD3 (C)

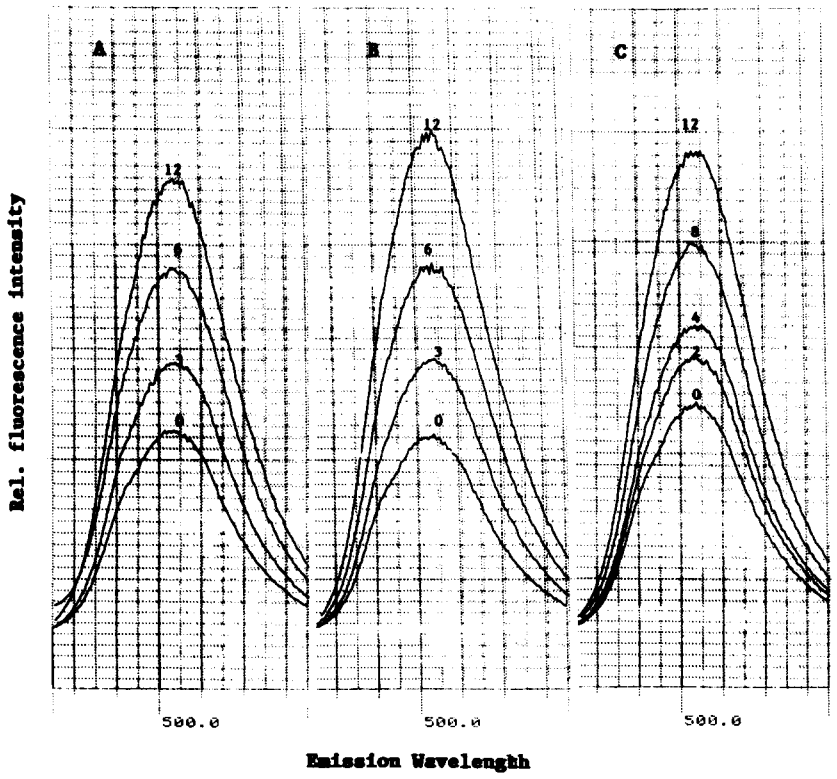

Fig. 3. Fluorescence spectra of gangliosides GD3 and *O*-acetyl GD3 containing 2–12 μg of sialic acid residues. Panel A represents *O*-acetyl GD3; B, base-treated *O*-acetyl GD3; C, ganglioside GD3. The spectra were recorded at 410 nm (excitation) and 470–570 nm (emission).

A (GM3) revealed four ion groups, and gangliosides B (GD3) and C (*O*-acetyl GD3) revealed five ion groups that arose from ceramide and ceramide-bearing fragments. These groups are of diagnostic value in determining the structure of gangliosides. In the mass spectrum of GM3, the prominent molecular ions $[M+Na-2H]^-$ *m/z* 1151, 1179, 1207, 1235, and 1263 provide information on the molecular weights of GM3 molecular species with C18-sphingosine and C16:0, C18:0, C20:0, C22:0, and C24:0 fatty acids, respectively. These ions are also present in the spectra of GD3 and *O*-acetyl GD3. The quasi-molecular ions for GD3 and *O*-acetyl GD3, however, are shifted by 313 and 355 mass units from those observed for GM3, respectively (Fig. 2), suggesting the presence of an additional sialic acid residue and monoacetylated sialic acid residue in GD3 and *O*-acetyl GD3, respectively. For example, the ions at *m/z* 1540, 1568, and 1596 in *O*-acetyl GD3 correspond to monoacetyl GD3 ganglioside with C18-sphingosine and C20:0, C22:0, and C24:0 fatty acids, respectively, and 1562, 1590, and 1618 to quasi-molecular ions $[M+Na-2H]^-$ (Fig. 2). The fragment ions corresponding to the elimination of an acetyl group and an *O*-acetyl sialic acid unit from *O*-acetyl GD3 molecular ions are detected at *m/z* 1498, 1526, and 1554 (ion group e), *m/z* 1207, 1235, and 1263 (ion group d), respectively. The ions *m/z* 916, 944, and 972 (ion group c), *m/z* 754, 782, 810 (ion group b), and *m/z* 592, 620, and 648 (ion group a) are ceramide dihexoside, ceramide monohexoside, and ceramide, respectively (Fig. 2). The presence of

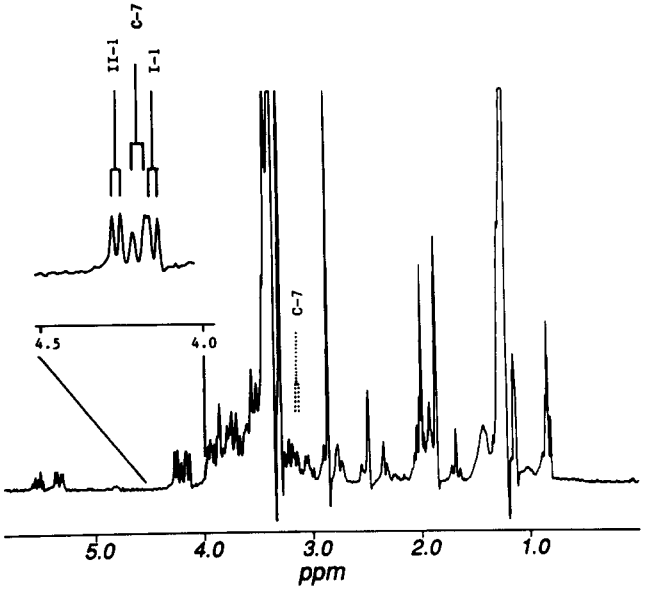

Fig. 4. Partial 300 MHz-NMR spectrum of 7-*O*-acetyl GD3 (7-*O*-Ac GD3) purified from hamster melanoma in dimethyl sulfoxide-d_6/D_2O 92:2 (v/v).

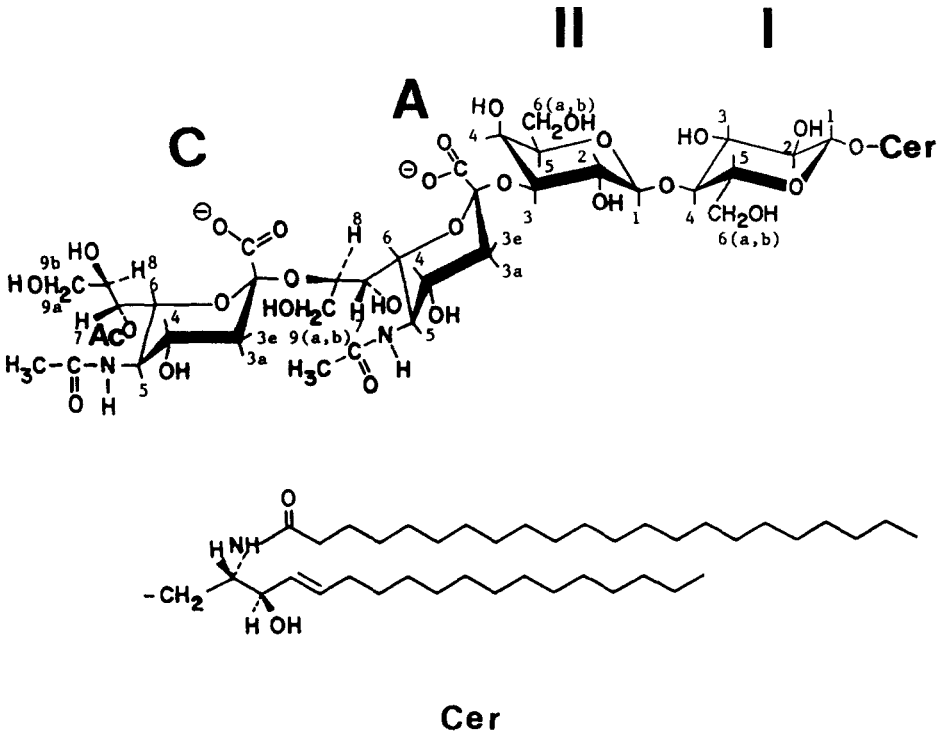

Fig. 5. The proposed structure of melanoma-associated ganglioside, 7-*O*-acetyl GD3, purified from hamster Ma melanotic melanoma.

ions, *m/z* 1207, 1235, and 1263 in *O*-acetyl GD3, which are identical to those presented in GM3 (spectrum A), suggests that the *O*-acetyl group must be attached to the terminal sialic acid residue of the molecule (Fig. 2).

In order to discriminate the position of *O*-acetyl group at C-8, C-9, or C-7 of the terminal sialic acid residue, *O*-acetyl GD3 was oxidized by mild periodate. For comparison, ganglioside GD3 and base-treated *O*-acetyl GD3 were similarly treated. The formaldehyde produced from the C-9 of the terminal sialic acid reacts with acetylacetone in the presence of ammonium acetate leading to a fluorogen. Ganglioside GD3, in which the terminal sialic acid residue is unsubstituted at the glycerol side chain, is assumed to give rise to one equivalent of formaldehyde (1 mol/mol GD3) (**Fig. 3**). *O*-Acetyl GD3 gave the same fluorescence intensity as the base-treated ganglioside GD3, as well as GD3 (Fig. 3). As 8-*O*-acetyl and 8,9-*O*-diacetyl GD3 are not supposed to be oxidized due to the lack of vicinal hydroxyl groups in the glycerol side chain (25), and 9-*O*-acetyl GD3 does not yield a formaldehyde upon oxidation, this result strongly indicates that the *O*-acetyl group is located at the 7-position of the terminal sialic acid than at 9- or 8- position.

To confirm the site(s) of *O*-acetyl substitution on the terminal sialic acid residue, the *O*-acetylated sample was analyzed by proton nuclear magnetic resonance (^{1}H NMR) (**Fig. 4**). The presence of a proton resonance with a small (1.5 Hz) splitting near 4.16 ppm coupled with our previous assignment data (20) strongly suggest the presence of *O*-acetyl substitution at the 7-position of the terminal sialic acid. The absence of a resonance at 4.82 ppm clearly indicates the absence of *O*-acetyl substitution at the 9-position of the terminal sialic acid residue. Resonances for the H3e proton of the internal and terminal sialic acid residues were found at 2.35 and 2.70 ppm, respectively. Resonances for the N-acetyl groups of the two α-D-NeuAc residues were observed at 1.88 and 1.89 ppm. An additional singlet at 2.01 ppm was attributable to the *O*-acetyl methyl protons. Based on these assignments and the additional data from HPTLC mobilities, periodate oxidation, mass spectral, and gas chromatographic analyses, we conclude that this monosubstituted ganglioside corresponds to 7-*O*-Ac GD3 (acetyl-*O*-7NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1'Cer). The molecular structure is shown in **Fig. 5.**

DISCUSSION

O-Acetylated ganglioside GD3 is the first example of an *O*-acetylated ganglioside in human tissues (3, 4, 6). Since Cheresh et al. (3, 4) reported that the monoclonal antibody D1.1 recognized 9-*O*-acetyl GD3 on human melanoma cells, which was characterized by alkaline treat-

ment, immunostaining and periodate oxidation, several groups of investigators reported that the alkali-labile ganglioside migrating between GM1 and GM2 and reacting with mAb D1.1 was 9-*O*-acetyl GD3 (5, 14, 27). Thurin et al. (6) found that a glycolipid antigen detected by a monoclonal antibody (ME 311) was 9-*O*-acetyl GD3 based on data from proton NMR and fast-atom bombardment-mass spectrometry. Constantine-Paton et al. (28) found that the antigen recognized by JONES antibody is 9-*O*-acetylated ganglioside GD3 depending on its mobility on HPTLC, lability to alkali treatment, and immunostaining with monoclonal antibodies. Neither antibody is specific for GD3 9-*O*-acetylated on the terminal sialic acid. We have identified reactivity of D1.1, JONES, and ME311 with synthetic 9-*O*-acetyl GD3 which is acetylated on the subterminal sialic acid (18), with the hamster melanoma *O*-acetyl GD3 shown here to be acetylated at the 7 position of the terminal sialic acid, and with *O*-acetyl GD2 of melanoma (29) and neuroblastoma (30). In addition to mAbs D1.1 and JONES, the receptor-destroying enzyme of influenza C virus, sialate *O*-acetylesterase (31), and cancer antennarius lectin may confirm the presence of 9-*O*-acetylation on the sialic acid residue of human melanoma although the specificity of these reagents for 9-*O*-acetylated GD3 has yet to be firmly established (17). Recently, Drazba, Dierce, and Lemmon (32) reported that an antigen in the developing chick retina recognized by the monoclonal antibody 8A2 was an *O*-acetylated ganglioside. More recently, we found four *O*-acetylated gangliosides, 9-*O*-acetyl GD3, 7-*O*-acetyl GD3, 7,9-*O*-diacetyl GD3, and *O*-acetyl GT3, in bovine buttermilk (20). In the present study, the purified *O*-acetyl GD3 from the hamster melanotic melanoma tissue had the same mobility on the TLC plate as 7- or 9-*O*-acetylated ganglioside from bovine buttermilk (20, 27). This *O*-acetyl GD3 was converted to GD3 after mild alkali treatment, and identified by reaction with mAbs D1.1 and JONES (14). Interestingly, the NMR data suggest that the *O*-acetylated ganglioside from hamster melanoma tissues is 7-*O*-acetylated GD3 based on the chemical shift information we published previously (20). It should be noted that other workers published similar NMR spectra and assigned the ganglioside from human melanoma tissues as 9-*O*-acetyl GD3 (6, 16); however, some of these previous experiments have relied extensively on poorly resolved resonances in the ^{1}H NMR spectra, which would be difficult in solving the resonance assignments for these compounds (20). Our data for the 7-position is different from that reported by Manzi et al. (19) who found that the acetyl group located at both 7- and 9-positions because of nonenzymatic migration of acetyl group from the 7- to 9-position. This discrepancy could be due to the neuraminidase treatment and analysis of free *O*-acetylated sialic acid in their procedure, which could facilitate migration of the acetyl group from 7- to 8- and then to the 9-position (15). It is interesting to note that the 7-*O*-acetyl GD3 was relatively stable, since we did not find any degradation of the 7-*O*-acetyl GD3 during purification; no evidence of degradation in the ammoniacal developing system at room temperature was observed (Fig. 2).

9-*O*-Acetyl GD3 was reported to be present in human melanoma tissues (4), in germinal cells of the central nervous system (5), rat retina (8), in other developing neural tissues (9–12), and in bovine buttermilk (27). Since the hydroxyl groups can be *O*-acetylated at different positions, 4-, 7-, 8-, and 9-, in the terminal sialic acid residue of ganglioside GD3 (33), it is possible that different *O*-acetyl GD3 species can be generated in different tissues. Other *O*-acetylated gangliosides include 4-*O*-acetyl-GD3 from equine erythrocytes (34, 35), 9-*O*-acetyl GT1b from mouse brain (36), and 9-*O*-acetyl GT3 from chicken embryonic retina (37) and cod brain (38); however, definitive structures of some of these gangliosides have never been rigorously established. In this investigation, we demonstrate for the first time that the major *O*-acetylated ganglioside in hamster melanotic melanoma is 7-*O*-acetyl GD3. It is relatively rare to observe *O*-acetylation at C-7 of the sialic acid residue in gangliosides as well as in free sialic acids (33). This unique structure, 7-*O*-acetyl GD3, appears to be an antigen specific to hamster melanoma and promises to be a useful target for melanoma immunotherapy.

Although we know that *O*-acetylated gangliosides are developmently regulated in murine embryogenesis (39), the functional significance of *O*-acetyl GD3 expression and the functional difference between 7-*O*-acetyl GD3 and 9-*O*-acetyl GD3 is not yet fully understood. Further studies are in progress to elucidate the function of the expression of 7-*O*-acetyl GD3 in the melanoma formation and progression.

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