G_{D3}, A PROMINENT GANGLIOSIDE OF HUMAN MELANOMA

Detection and Characterization by Mouse Monoclonal Antibody*

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We have previously described (1) a mouse IgG3 monoclonal antibody (AbR_{24}) with a high degree of serological specificity for cultured human melanoma cells. All melanoma cell lines and two astrocytomas examined were positive for the heat-stable cell surface antigen detected by this antibody. Although choroidal melanocytes and brain had low levels of the antigen, a wide variety of other cells and tissues were unreactive. Three other monoclonal antibodies (Abs C_5, I_{24}, and K_9), having a similar restricted specificity, were derived from the same fusion. These antibodies showed the same strong reactivity with melanomas and lack of reactivity with epithelial cells, but had a slightly wider specificity range in that they also reacted weakly with MOLT-4 (a T cell line), leukocytes, and some fetal tissues.

In this communication, we identify the antigen detected by AbR_{24} as G_{D3}, a previously characterized disialoganglioside.[1] In comparison with other cells and tissues, melanomas have high levels of G_{D3}.

Materials and Methods

Tissue Culture. For derivations and culture of melanoma and other cells see refs. 1–4. Normal and malignant human tissue was obtained from surgical or postmortem specimens.

Monoclonal Antibodies. Mouse monoclonal antibodies AbR_{24}, AbC_5, AbI_{12}, and AbN_9 have been previously described (1). AbR_{24} and AbC_5 are IgG3 antibodies and AbI_{12} and AbN_9 are IgG2b and IgG1 antibodies, respectively.

Glycolipids. G_{D3} was a generous gift of Dr. Y.-T. Li, Tulane University, New Orleans (5). G_{M3} and G_{M2} were kindly provided by Dr. S. Kundu and Dr. D. M. Marcus, Baylor University, Houston, TX. G_{M1}, G_{D1a}, G_{T1} were purchased from Supelco Inc., Bellefonte, PA. Lactosylceramide was purchased from Glycolipid Biochemical Co., Birmingham, AL.

Serological Assays for Melanoma Cell Surface Antigens. Reactivity of AbR_{24} and AbC_5 with cell surface antigens of melanoma cells was determined with cultured cells growing in the wells of Microtest plates (Falcon 3034, Falcon Labware, Oxnard, CA) using an erythrocyte rosetting method (3) in which indicator cells are human O erythrocytes (RBC) to which *Staphylococcus*

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[1] *Abbreviations used in this paper:* C-M: chloroform-methanol; FBS: fetal bovine serum; PBS, phosphate-buffered saline; PA-MHA: protein A mixed hemagglutination assay; NANA: *N*-acetylneuraminic acid; TLC: thin-layer chromatography; GG, gamma globulin; Gal: D-galactose; Glc: D-glucose; GalNAc: N-acetyl-D-galactosamine; Cer: ceramide; G_{M1}: βGal 1 → 3 GalNAc 1 → 4 βGal[3 ← 2 NANA] 1 → 4 Glc-Cer; G_{M3}: NANA 2 → 3 βGal 1 → 4 Glc-Cer; G_{D3}: NANA 2 → 8 NANA 2 → 3 βGal 1 → 4 Glc-Cer; G_{D1a}: NANA 2 → 3 βGal 1 → 3 GalNAc β 1 → 4 Gal [3 ← 2 NANA] Glc-Cer; G_{M2}: βGalNAc 1 → 4 βGal [3 ← 2 NANA] Glc-Cer. G_{T1a}: NANA 2 → 8 NANA 2 → 3 βGal 1 → 3 GalNAc β1 → 4 Gal [3 ← 2 NANA] Glc-Cer. (Nomenclature of Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10:**613).

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aureus protein A is conjugated (PA-MHA). AbI_{12} and AbN_9 were assayed using a modification of this method in which rabbit anti-mouse Ig-conjugated indicator cells were used (IgG-MHA).

Enzyme Treatment. Melanoma cells growing as monolayers in microtest plates as described above were washed with Hanks' balanced salt solution (HBSS, Microbiological Associates, Walkersville, MD) and then treated with *Vibrio cholerae* neuraminidase (Calbiochem-Behring Corp., La Jolla, CA) or β-galactosidase (Type VII; Sigma Chemical Co., St. Louis, MO) using 1 U/well in 10 μl of HBSS. After incubation for 1 h at 37°C, the cells were washed four times with phosphate-buffered saline (PBS)-2% gamma globulin (GG)-free fetal bovine serum (FBS) and assayed for reactivity with antibody using the PA- or IgG-MHA assays.

Isolation of Glycolipids. Glycolipids were isolated initially by a modification of the method of Saito and Hakomori (6), and separated into neutral and acidic fractions by DEAE-Sephadex chromatography (7). Acidic glycolipids (gangliosides) were subsequently isolated directly from chloroform-methanol (C/M) extracts by DEAE-Sephadex chromatography and alkaline hydrolysis (7). In brief, cells were homogenized in C/M (2:1) and after filtration were re-extracted with C/M (1:1). After evaporating and redissolving the extract in C/M (1:2), it is filtered, evaporated, and dialyzed against distilled ice water for 24 h in the cold. After dialysis, samples were evaporated, dissolved in C-M/H_2O (30:60:8), and applied to a DEAE-Sephadex column (equilibrated with C-M/0.8 M Na acetate) (30:60:8). After washing the column with C/M/H_2O (30:60:8), acidic lipids were eluted with C/M/0.8 M Na acetate (30:60:8), evaporated, and dialyzed as before. The acidic fraction was then hydrolyzed with 0.1 N NaOH in methanol for 3 h at 37°C, dialyzed against cold water (48 h), evaporated, and dissolved in C/M (4:1). The solution was applied to a Biosil A column that had previously been washed with C/M (4:1). After eluting impurities with C/M (4:1), gangliosides were eluted with C/M (1:2).

Thin-layer Chromatography (TLC). Silica gel plates (Rediplates, Fisher Scientific Co., Pittsburgh, PA) were activated by heating at 120°C for 1 h. Solvents used for developing chromatograms were *N*-propanol/NH_4OH/H_2O, 60:9.5:11.5 (solvent 1) (8) and C/M/2.5 N NH_4OH, 60:40:9 (solvent 2). Once the solvent had migrated 12 cm from the origin, the plate was removed and air dried for 12–15 min at 110–120°C, cooled to room temperature, and sprayed with resorcinol-HCl (9). For preparative analysis, plates were dried at room temperature in an air flow hood for 2–3 h. Bands were visualized with iodine vapor, outlined, and silica gel scraped from the plate. The gel was then extracted twice with 40 ml of C/M/H_20 (50:50:15) with a small amount of Dowex 50 (Na^+). The suspension was centrifuged at 1,000 rpm for 15 min, and the solution filtered, evaporated, redissolved in C/M (4:1), and applied to a Biosil A column as described above. Impurities were eluted with C/M (4:1), and adsorbed gangliosides were then eluted with C/M (1:2).

Carbohydrate Analysis. Lipid-bound sialic acid in cell pellets was determined on C/M (2:1 and 1:2) extracts after hydrolysis in 0.1 N HCl at 80°C for 1 h as described by Warren (10). Sugars were analyzed after methanolysis (methanolic 1.0 N HCl at 100°C for 16 h) as their O-trifluoroacetates (11); *N*-acetylneuraminic acid was identified by the same procedure after methanolysis in 1.0 N HCl at 80°C for 1 h.

Serological Assays for Glycolipids

Passive hemmaglutination assay (12). Glycolipids (6 μg sialic acid) were dissolved, aliquoted into tubes (10 × 75 mm), and dried in a dessicator with P_2O_5 *in vacuo*. To each tube, 200 μl of PBS was added, the sides of the tube scraped, and the solutions sonicated for 15 min at 50°C. After transfer to a larger tube, 0.8 ml of PBS was added. The glycolipid solution was added slowly in a dropwise fashion to a 2% suspension of human O-RBC in PBS. After 1 h at 37°C with one mixing after 30 min, the cells were washed twice with PBS (12 ml each wash). Reactivity was tested by mixing a suspension of the treated RBC, and appropriately diluted AbR_{24} (50 μl each) in microtiter plates. After 1–2 h at 4°C, the agglutination reactions were scored visually.

Antibody inhibition assay. Glycolipids (6 μg sialic acid), dissolved in C-M (1:2), were aliquoted into tubes (6 × 50 mm) and dried as in the previous assay. AbR_{24} (1×10^4 to 2×10^4) was added (30 μl), and the tubes were vortexed and incubated for 30 min at room temperature and then for 30 min at 4°C. Tubes were centrifuged for 20 min at 2,000 rpm, and the supernatants removed and serially diluted. These samples were immediately transferred to formaldehyde-fixed SK-MEL-28 target cells. (The formaldehyde fixation was carried out by treating cells growing in the wells of microtest plates [Falcon 3034] with 0.33% HCHO in PBS. This treatment does not alter reactivity with AbR_{24} and provides a store of readily available target cells). Antibody reactivity was detected with the PA-MHA assay. Unabsorbed antibody served as a positive control.

GLYCOLIPID-MEDIATED IMMUNE ADHERENCE ASSAY (GMIA). A solution of glycolipids in 95% ethanol was added to the wells of microtest plates (Falcon 3034; 10 μl per well) and the plates were dried in a dessicator *in vacuo* with P_2O_5 for 45 min. Approximately 100 ng of lipid-bound sialic acid was found to be the optimal amount for efficient adsorption and maximal reactivity with antibody. Wells were then washed three times with PBS-2% GG-free FBS (10 ml/wash), and the plates were blotted with gauze. Diluted antibody (in PBS with 5% GG-free FBS) was added to the wells and incubated for 45 min at room temperature. Plates were blotted, washed four times with PBS-2% GG-free FBS, and blotted again. 10 μl of a 0.2% suspension of protein A-conjugated O-RBC were added to the wells. The plates were incubated for 30 min at room temperature. After blotting, the plates were washed twice with PBS-2% GG-free FBS, blotted once again, and read under the light microscope. Reactions were scored according to the proportion of the well covered by RBC. A test was read as negative when wells showed no adhering cells or only a thin ring of cells around the perimeter.

DETECTION OF SEROLOGICALLY-REACTIVE GLYCOLIPID AFTER SEPARATION BY TLC. Serological reactivity of glycolipids separated by TLC was tested using a modification of the method of Magnani et al. (13) in which ^{125}I-protein A was used to detect the bound antibody. After chromatography in solvents 1 or 2, thin-layer sheets were washed in PBS buffer containing 1% polyvinylpyrolidone and treated with AbR_{24} (1:1,500) for 6 h at 4°C. After washing in PBS, the plate was treated with ^{125}I-protein A (10 μCi/μg; 7×10^5 cpm/ml) prepared according to the procedure of Hunter and Greenwood (14). After standing for 12 h at 4°C, the plate was washed in PBS, air-dried, and exposed to X-Omat R film (Eastman Kodak Co., Rochester, NY) with a Cronex intensifier screen (Dupont Instruments, Wilmington, DE) for 2–6 hours.

Results

Alteration of AbR_{24} Serological Reactivity and Kinetics of Antigen Restitution after Neuraminidase Treatment of SK-MEL-28. After treatment with neuraminidase (*Vibrio cholerae*), SK-MEL-28 melanoma cells no longer reacted with AbR_{24} in PA-MHA assays (Table I). Reactivity with AbC_5 (an antibody with a serological specificity similar to that of AbR_{24} [1]) was also lost. Reactivity with AbN_9 and AbI_{12}, which recognize serologically unrelated determinants on glycoproteins of SK-MEL-28, was unaffected by neuraminidase. Enzyme-treated cells did not show nonspecific reactivity with either protein A- or anti-mouse Ig-indicator cells. β-Galactosidase had no detectable effect on the reactivity of SK-MEL-28 cells with AbR_{24} or AbC_5 (Table I). These results

TABLE I

Effect of Neuraminidase and β-Galactosidase on the Reactivity of Monoclonal Antibodies with SK-MEL-28 Melanoma Cells

Antibody*	IgG class*	Heat sensitivity of antigens*	Untreated‡	Neuraminidase treated‡	β-Galactosidase treated‡
			Percent positive cells		
R_{24}	IgG3	Stable	100	<10	100
C_5	IgG3	Stable	100	<10	100
N_9	IgG1	Sensitive	100	100	100
I_{12}	IgG2b	Sensitive	100	100	100

* From Dippold et al. (1). AbN_9 precipitates a glycoprotein antigen with a molecular weight of 150,000 and AbI_{12} precipitates a glycoprotein antigen with a molecular weight of 95,000.

‡ Results of direct tests with PA- or Ig-MHA assays.

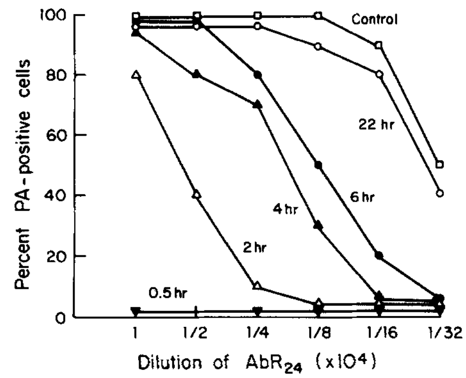

FIG. 1. Time course for the re-expression of AbR_{24}-reactive antigen on SK-MEL-28 cells after neuraminidase treatment. Assay: PA-MHA.

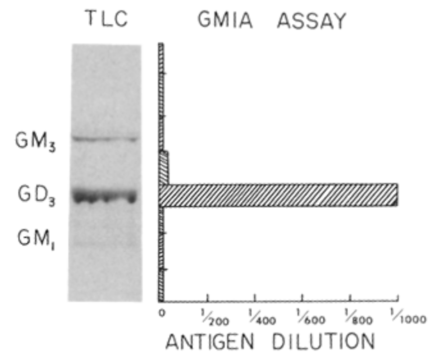

FIG. 2. Localization of AbR_{24}-reactive glycolipid on thin layer chromatograms using glycolipid-mediated immune adherence (GMIA) assay. Acidic glycolipids from SK-MEL-28 cells were separated by TLC in solvent 1. Silica gel bands (1 cm wide) were scraped from the plate, extracted with C-M (1.2), and assayed for antigens by GMIA as described in the text.

show that sialic acid constitutes an important part of the antigenic determinant recognized by antibodies AbR_{24} and AbC_5.

Serological reactivity of AbR_{24} with SK-MEL-28 remained undetectable for 30 min after neuraminidase was removed and replaced with minimal essential medium (MEM)-FBS. Continued incubation in this medium at 37°C resulted in a partial return of AbR_{24} reactivity after 2 h and complete recovery of serological reactivity after 22 h (Fig. 1).

Isolation of AbR_{24}-reactive Antigen from SK-MEL-28 Melanoma Cells and Its Identification as G_{D3} Ganglioside. Glycolipids were isolated from cultured melanoma cells (SK-MEL-28) by C-M extraction and Florisil chromatography of their acetates as described by Saito and Hakomori (6), and the glycolipid preparation was fractionated into neutral and acidic components by DEAE-Sephadex chromatography. Inhibitory activity against AbR_{24} antibody (assayed with PA-MHA) was found to reside entirely in the acidic glycolipid fractions.

In subsequent experiments, acidic glycolipids from SK-MEL-28 cells were isolated directly by fractionating the C-M extract on DEAE-Sephadex (6) and eliminating contaminating phospholipids by alkaline hydrolysis. Individual gangliosides in this

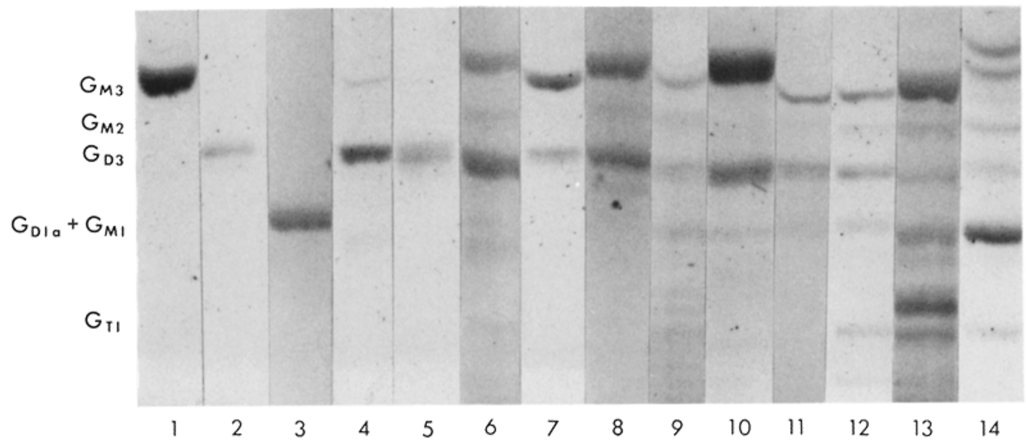

FIG. 3. TLC of acidic glycolipid fractions from a number of cell lines and tissues. Lane 1, G_{M3}; 2, G_{D3}; 3, G_{M1}; 4, SK-MEL-28 melanoma cell line; 5, AbR_{24}-reactive antigen isolated from SK-MEL-28; 6, SK-MEL-37 melanoma cell line; 7, SK-MEL-64 melanoma cell line; 8, MeW melanoma cell line; 9, SK-MEL-13 melanoma cell line; 10, melanoma (surgical specimen); 11, MOLT-4 T cell line; 12, mouse eye; 13, SK-RC-7 renal cancer cell line; 14, adult human brain. Gangliosides were separated in solvent 1 and visualized with resorcinol-HCl.

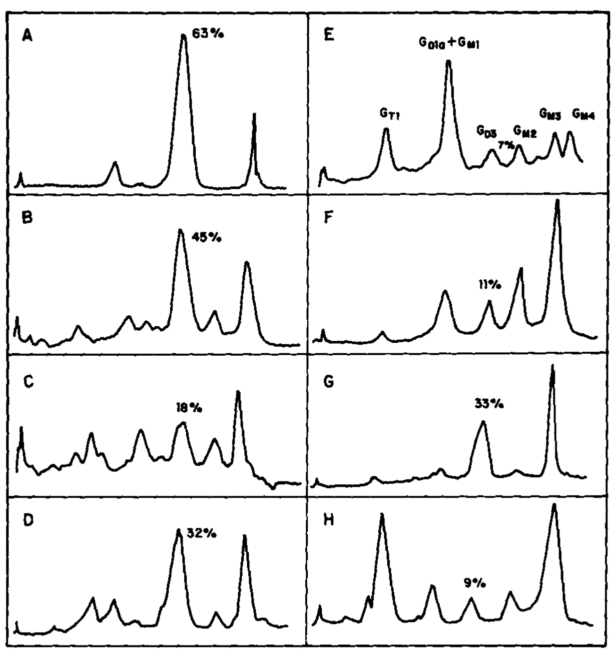

FIG. 4. Densiometric tracings of thin layer chromatograms of gangliosides from melanomas and other cells. A, SK-MEL-28 melanoma cell line; B, SK-MEL-37 melanoma cell line; C, SK-MEL-13 melanoma cell line; D, melanoma (surgical specimen); E, adult human brain; F, Raji B cell line; G, MOLT-4 T cell line; H, SK-RC-7 renal cancer cell line. The amount of G_{D3}, as percentage of total ganglioside fraction, was calculated from the areas of the peaks and is indicated in each panel.

TABLE II

*Reactivity of AbR_{24} with gangliosides isolated from various cell lines and tissues as determined by four serological test systems**

Source of gangliosides	Passive hemagglutinin‡	Inhibition§	GMIA‖	^{125}I-PA TLC¶
Melanoma (surgical specimens)				
MEL-MU	+	+	+	+
MEL-JI	+	+	+	+
MEL-LO	+	+	+	
Melanoma cell lines				
SK-MEL-13		+	+	
SK-MEL-21		+	+	
SK-MEL-28	+	+	+	+
SK-MEL-31		+	+	
SK-MEL-37	+	+	+	
SK-MEL-64			+	
SK-MEL-93		+	+	
MeWo	+	+	+	+
Carcinoma cell lines				
Renal				
SK-RC-7	−	−	−	+
SK-RC-11			−	
Bladder				
253J		−	−	
T-24		−	−	+
RT-4		−	+	+
Lung				
SK-LC-LL	−		−	+
Cervix				
ME-180	−		−	+
Colon				
HT-29	−		−	
Other cells and tissues				
Astrocytoma cell lines				
AJ			+	
AS		−		
MOLT-4 (leukemia cell line)		+	+	+
Raji (lymphoma cell line)		−	+	
Brain				
Bovine	−	−	−	+
Mouse	−	−	−	+
Fish		−	−	
Human (adult)	−	−	−	+
Human (fetal 10-wk)		+	+	
Human (fetal 12- and 22-wk)		−	−	+
Choroid (bovine)	−	+	+	
Eye				
Mouse	−	+	+	+
Fish		−	−	+
Liver				
Mouse		−	−	+
Human (fetal)	−	−	−	
Human (adult)		−	−	

TABLE II—*Continued*

Source of gangliosides	Passive hemagglutinin‡	Inhibition§	GMIA‖	^{125}I-PA TLC¶
Spleen				
Mouse		−	−	
Human (fetal)		−	−	
Human (adult)	−	−	−	+
Muscle (fetal human)		−	+	+
Kidney				
Mouse		−	−	+
Human (adult)	−	−	−	+
Heart (mouse)	−	−	−	+
Thymus (mouse)	−	−	−	
Lung				
Mouse	−	−	−	
Human (fetal or adult)	−	+	+	+
Umbilicus			+	
Erythrocytes				
Human (A & B)			+	
Human (O)			−	
Horse	−	−	−	+
Sheep		−	−	
Cat		−	+	
Placenta (human)	−	−	−	+
Gangliosides				
R_{24}-reactive glycolipid	+	+	+	+
G_{D3}			+	+
G_{M1}	−	−	−	−
G_{M2}	−	−	−	−
G_{M3}	−	−	−	−
G_{D1a}	−	−	−	−
G_{T1}	−	−	−	−

* Cells and tissues are human in origin unless indicated.

‡ Passive hemagglutination with glycolipid-coated RBC. AbR_{24} was used at a dilution of 1:100; a minimum of 5 μg of G_{D3} could be detected.

§ Inhibition of PA-MHA reactivity of R_{24} antibody (1:80,000) with SK-MEL-28 target cells. Results were scored positive (+) when the degree of rosetting was reduced to <20%. At this dilution, AbR_{24} could be completely inhibited by 2 μg of G_{D3}.

‖ A reaction was considered positive when >50% of the surface area of the well was covered by a lawn of protein A-conjugated RBC. AbR_{24} was used at a dilution of 1:1000. With this amount of antibody, ~25 ng of G_{D3} could be detected.

¶ ^{125}I-PA-TLC. In this procedure 6 μg of lipid-bound NANA was separated by TLC, and the plate treated with AbR_{24} (1:1500) and ^{125}I-protein A. Reactive components were detected by autoradiography. This procedure can detect ~10–25 ng of G_{D3}.

mixture were isolated by preparative TLC in solvent 1 (8). By scraping a series of silica gel bands from the plates and extracting the glycolipids, the antigenic activity was located in the major acidic glycolipid band that migrated just above G_{M1} and G_{D1a} (Fig. 2). In the data presented in Fig. 2, the antigenic activity of fractions was measured by the GMIA assay. Similar results were obtained by antibody inhibition tests using the PA-MHA assay with AbR_{24} and SK-MEL-28 target cells.

The isolated AbR_{24}-reactive glycolipid was identified as NANA(2 → 8)NANA

(2 → 3)Galβ(1 → 4)Glc-ceramide (G_{D3}) by the following criteria: (*a*) carbohydrate analysis of the purified glycolipid showed that it contained glucose, galactose, and *N*-acetylneuraminic acid (NANA) in a ratio of 1.0:1.09:2.11, with only a trace (< 0.1) of hexosamine; (*b*) partial hydrolysis of the ganglioside with *Vibrio cholerae* neuraminidase (3 h at 37°C) resulted in the formation of two components comigrating on thin-layer chromatograms with G_{M3} and lactosylceramide; (*c*) the purified melanoma glycolipid co-migrated with authentic G_{D3} in TLC (Fig. 3); and (*d*) AbR_{24} reacted with authentic G_{D3}, but not with any of the other standard gangliosides tested (see below).

Distribution of G_{D3} in Melanoma and Nonmelanoma Cell Lines and in Normal and Malignant Tissues

TLC PATTERNS OF GANGLIOSIDES FROM VARIOUS SOURCES. Total ganglioside fractions were prepared from a large variety of tumor cell lines, fresh tumors, and normal tissues. When these extracts were fractionated by TLC and the gangliosides detected using the resorcinol reagent, it became evident that melanomas have a characteristic pattern of gangliosides. In all the melanoma cell lines examined, glycolipids co-migrating with G_{D3} and G_{M3} were prominent acidic glycolipids, with G_{D3} being the major component in many of these cell lines (Figs. 3 and 4). G_{D3} was also a prominent ganglioside in extracts of mouse eye and bovine choroid. With the exception of MOLT-4 (a T cell line), none of the other cells or tissues had G_{D3} as the major component. Extracts of fresh melanoma tumors gave ganglioside patterns resembling SK-MEL-28, with G_{D3} and G_{M3} predominating (Fig. 3). Most melanoma cell lines gave this simplified pattern, but some showed a more complex profile in which higher gangliosides were detected in appreciable amounts (Figs. 3 and 4). G_{D3} constituted 18–63% of the total ganglioside fraction in the melanoma cell lines examined (Fig. 4). Most melanoma cell lines and specimens had values in the 30–50% range. These values compared with 7% in adult human brain, 9% in a renal cancer cell line (SK-

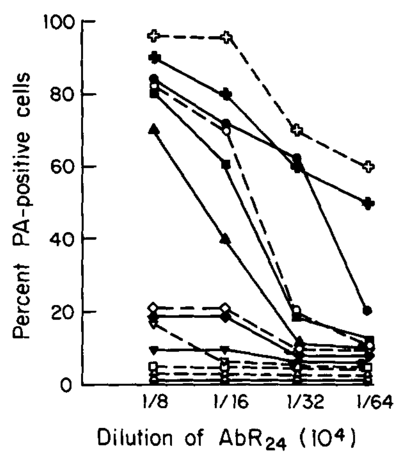

FIG. 5. Inhibition of reactivity of AbR_{24} with SK-MEL-28 melanoma cells by acidic glycolipid fractions from a variety of cell lines and tissues. Assay: PA-MHA. ✥, AbR_{24} control; ✚, adult human spleen; ●, adult human liver; ○, teleost eye; ■, SK-RC-7 renal cancer cell line; ▲, adult human brain; ◇, MeWo melanoma cell line; ◇, SK-MEL-29 melanoma cell line; ▽, SK-MEL-37 melanoma cell line; ▼, mouse eye; □, MOLT-4 T cell line; △, melanoma (surgical specimen); ◒, SK-MEL-28 melanoma cell line.

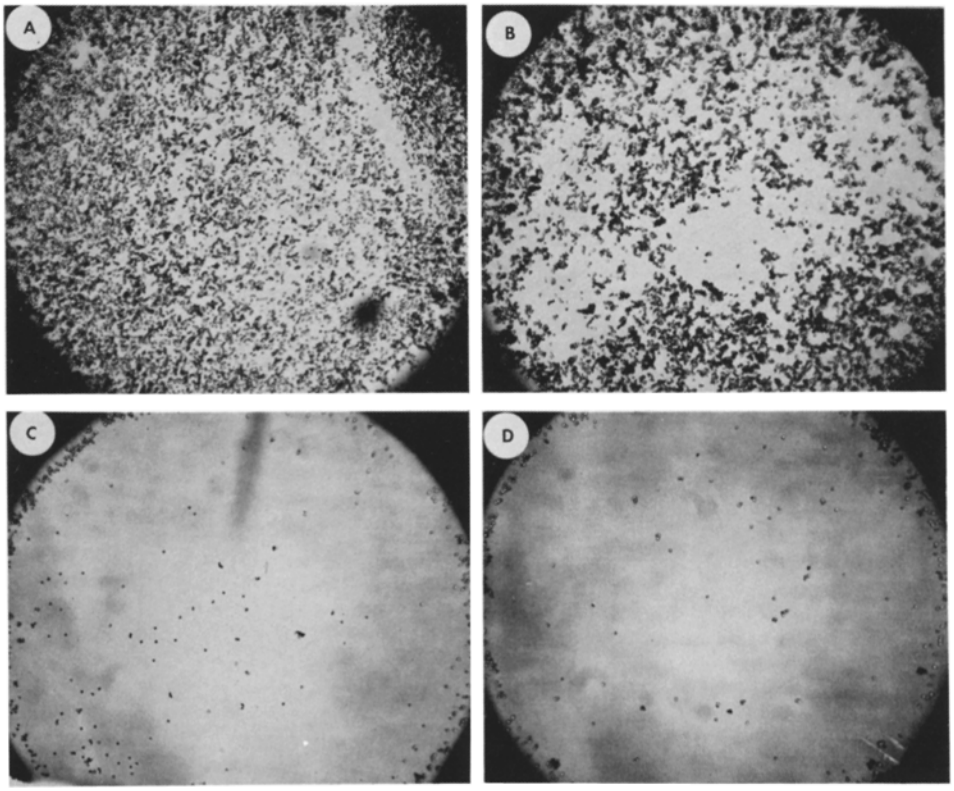

FIG. 6. GMIA assay using AbR_{24}. (A) AbR_{24}-reactive glycolipid isolated from SK-MEL-28 melanoma cell line; (B) G_{D3} ganglioside; (C) no ganglioside; (D) G_{M2} and G_{M3} ganglioside mixture. Antibody: AbR_{24} (1:1000).

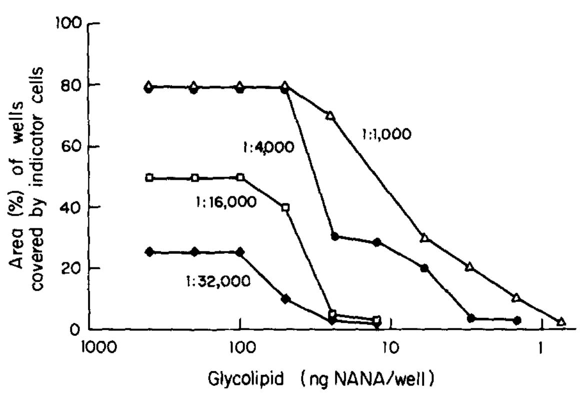

FIG. 7. Detection of G_{D3} ganglioside by AbR_{24} in GMIA assays. AbR_{24} dilutions are indicated in the figure.

RC-7), 11% in RAJI cells (a Burkitt's lymphoma), and 33% in MOLT-4 cells (Fig. 4). In terms of the serological reactivity of AbR_{24}, it is important to note that melanomas, in addition to having higher proportions of G_{D3} in their glycolipid fraction, also have higher total ganglioside levels. This is evident from a determination of the levels of

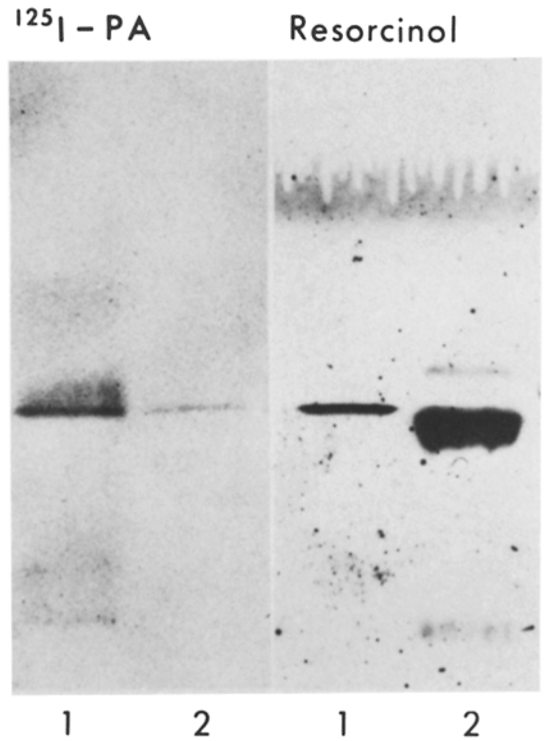

FIG. 8. Detection of G_{D3} ganglioside on TLC plates by reactivity with AbR_{24} and ^{125}I-protein A. Right side: gangliosides visualized with resorcinol-HCl reagent; left side: gangliosides reacting with AbR_{24} and ^{125}I-protein A. Lane 1: AbR_{24}-reactive ganglioside from SK-MEL-28; 2: gangliosides extracted from adult human brain. Solvent 2.

lipid-bound sialic acid in a number of cell lines. In melanomas, the values ranged from 0.039 to 0.063 μmol/0.1 ml cells (determined on nine lines). RAJI, MOLT-4, and renal cancer cells (three lines) had lipid-bound sialic acid values of 0.011 ± 0.003, 0.013 ± 0.006, and $0.025 - 0.029$ μmol/0.1 ml cells, respectively.

DETECTION OF G_{D3} IN CELL LINES AND TISSUES USING ABR_{24} ANTIBODY. G_{D3} levels in a large variety of cells and tissues were estimated using R_{24} antibody. Four assay methods were used: (*a*) passive hemagglutination, (*b*) antibody inhibition, (*c*) a new method, GMIA, devised to combine the simplicity of the MHA method with the ability of glycolipids to adsorb to plastic, and (*d*) a method using ^{125}I-protein A to detect AbR_{24} reacting with G_{D3} on TLC chromatograms. The sensitivity of the assays varies considerably; the passive hemagglutination assay is the least sensitive, and the ^{125}I-PA method the most sensitive (Table II).

Using the least sensitive detection method (passive hemagglutination), G_{D3} could be detected in extracts of melanoma cell lines and melanoma tissue, but not in other sources (Table II). More sensitive assays (inhibition of PA-MHA and GMIA methods) showed that G_{D3} was detectable in a wider range of cells (bovine choroid, mouse eye, fetal and adult human lung, RAJI B-cell line, MOLT-4 T-cell line, RT-4 bladder cancer cell line, and AJ astrocytoma cell line). A typical inhibition experiment is presented in Fig. 5, and the data are summarized in Table II. Using the GMIA method, it was found that wells coated with R_{24}-reactive glycolipids from melanoma

(Fig. 6A) or authentic G_{D3} gave strongly positive reactions (Fig. 6B); some quantitative data on this reaction are shown in Fig. 7. Other purified glycolipids (G_{M1}, G_{D1a}, G_{M3}, and G_{M2}) were unreactive in this assay (Table II and Figure 6D). AbR_{24} added alone was also unreactive (Figure 6C). Application of this method to acidic glycolipids extracted from other cells gave approximately the same results as inhibition assays (Table II). In contrast to the restricted distribution of G_{D3} determined by these methods, the ^{125}I-protein A method detected G_{D3} in all the cells and tissues examined (Table II and Fig. 8). That the AbR_{24}-reactive component detected in these tissues and cells was in fact G_{D3} was indicated by its co-migration with authentic G_{D3} (in two solvent systems), and by the finding that another protein A-binding monoclonal antibody (AbI_{12}), detecting an unrelated glycoprotein specificity, was unreactive.

Discussion

Mouse monoclonal antibody R_{24}, which shows a high degree of serological specificity for cell surface antigens of melanoma cells, recognizes a disialoganglioside, G_{D3}. Past studies have shown that antibodies to gangliosides have been difficult to raise (15). This may have to do with the fact that most gangliosides are constituents of the species being immunized, and also because *in situ* sialidase activity may destroy ganglioside immunogenicity (16). In this regard, it might be significant that the mouse from which AbR_{24} was developed had been extensively immunized over a period of 6 mo with melanoma cells (SK-MEL-28) having a very high G_{D3} content. Two other monoclonal antibodies recognizing gangliosides have recently been described (17, 18). One reacts specifically with chicken neuronal cells and is directed against one of the higher gangliosides present in the G_Q fraction (17); the second is directed against human colon carcinoma and recognizes an as yet uncharacterized monosialoganglioside (18).

In this report, we show that G_{D3} is a prominent ganglioside in cultured melanoma cells and in melanoma tissue. When compared with other cells, melanoma cells also possess relatively high total ganglioside levels. As shown by others, G_{D3} is present in small amounts in most mammalian tissues, but it is a major ganglioside in the retina, where it comprises between 30 and 40% of the gangliosides (19). In adult human brain, G_{D3} represents ~8–10% of the total ganglioside content (19). Levels of G_{D3} may be higher in fetal brain; in fetal rat brain (15–17 d gestation) G_{D3} represents ~50% of the total ganglioside content, falling rapidly to ~10% by day 20 (20). Portoukalian and co-workers (21) have also reported that G_{D3}, identified by TLC and carbohydrate analysis, is a major constituent of melanomas. They showed that the proportion of G_{D3} varied from 31.0% to 57.2% of the ganglioside fraction in the four different melanoma specimens examined. From these results, as well as our own analysis, one can conclude that G_{D3} ganglioside is a prominent component of malignant melanoma. Whether normal melanocytes have high levels of G_{D3} is at present unclear. Normal choroidal melanocytes show weak reactivity with AbR_{24} in direct serological tests (titer 1:100) as compared with the strong reactivity of melanoma cells (titer of $1:5 \times 10^4$ to $1:5 \times 10^5$) (1). With the recent development of a method for culturing skin melanocytes (22), it will now be possible to make a direct comparison of the G_{D3} content of melanocytes and melanomas. Although a precise biological function for G_{D3} remains to be determined, it has been suggested that G_{D3} has a role in serotonin binding (23, 24).

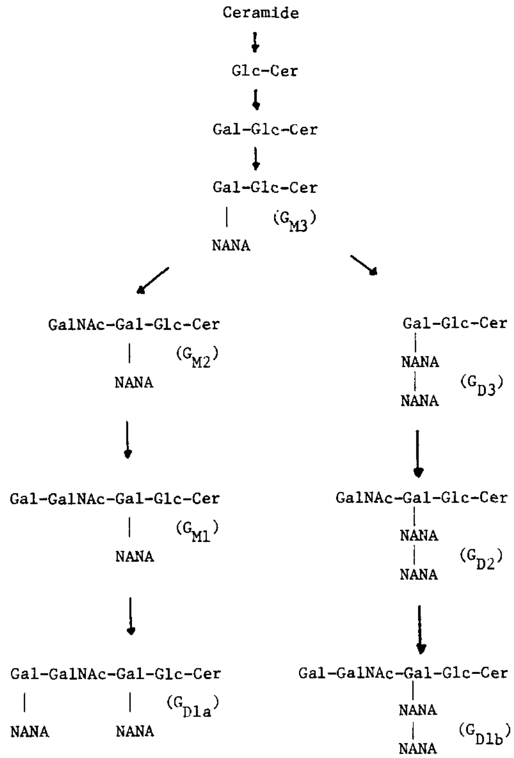

FIG. 9. Proposed pathways for the biosynthesis of gangliosides (modified after Yu and Ando [26]).

In examining the TLC patterns of the gangliosides isolated from different melanoma cell lines, we noticed considerable variation in the proportion of the various gangliosides. In most cell lines, G_{D3} and G_{M3} were the predominant gangliosides (Figs. 3 and 4). A few melanoma cell lines showed a more complex pattern, with G_{M2} and some higher gangliosides being better represented; whether these differences in ganglioside profiles correlate with biological characteristics (e.g., differentiation state) of the tumor needs to be determined. In general, melanomas exhibit a distinctive ganglioside profile. Of the other cells and tissues examined, only the T cell line MOLT-4 showed a similar profile, and this may be another example of antigens shared by T cells and cells of neuuroectodermal origin, eg., Thy-1 (25). Gangliosides derived from bovine choroid and mouse eye had more complex patterns, with G_{D3} being only one of three or four prominent components.

It is evident from the analysis of extracted glycolipids that the presence of G_{D3} ganglioside is by no means restricted to melanoma cells—it is ubiquitous. Yet using direct serological assays for cell surface antigens, only melanomas, choroidal melanocytes, and astrocytomas were reactive with AbR_{24} (1). Even using sensitive absorption tests, only normal brain of other cells and tissues tested absorbed AbR_{24}. A number of explanations for the apparent discrepancy between the serological finding and the biochemical data presented here can be suggested. First, it is possible that G_{D3} is not a cell surface constituent of most nonmelanoma cells. It is well established that G_{D3} is a biosynthetic precursor of other gangliosides (Fig. 9) and would therefore be located mainly within the cell, probably in the Golgi apparatus where the glycosyl transferases responsible for glycolipid synthesis are found (27, 28). As our biochemical studies were carried out on whole cells or tissues, the results are certainly compatible with this explanation. Another possibility is that G_{D3} is present at the cell surface of R_{24}-negative cells, but is not available for reaction with antibody. This phenomenon has been found with other cell membrane glycolipids, e.g., globoside is a major glycolipid of RBC membrane but RBC react only weakly with anti-globoside antibody (29). It is also possible, of course, that G_{D3} is not expressed on the surface of most nonmelanoma cells in amounts that are detectable by the serological tests used. It is important to note that the cell types that reacted with AbR_{24} in both direct and absorption tests have both a high lipid-bound sialic acid content and have G_{D3} as a prominent ganglioside.

What might be the mechanism of the accumulation of G_{D3} and G_{M3} in melanoma cells? One possible explanation is that melanoma cells have low levels of *N*-acetylgalactosaminyl transferase(s) that would result in the accumulation of the normal substrates for the enzyme(s), i.e., G_{M3} and G_{D3} (Fig. 9). In bovine thyroid, a single *N*-acetylgalactosamine-transferase is thought to act on both G_{D3} and G_{M3} to form G_{D2} and G_{M2} (28), and low levels of this enzyme in melanomas could explain the ganglioside pattern we observed. Among other explanations, it is possible that melanomas have high levels of β-*N*-acetylgalactosaminidase that would result in increased degradation of G_{M2} and G_{D2}, or that melanomas have elevated levels of certain sialyltransferases, resulting in increased synthesis of G_{D3} and G_{M3}. It is significant in this regard that melanoma patients have increased serum sialyltransferase levels (30). Enzyme levels in tumor tissue have not yet been studied, although the fact that the glycoproteins of human melanoma cell lines have increased sialylation as compared with the glycoproteins of other cell types (31) suggests increased activity of this enzyme in melanoma.

Summary

Mouse monoclonal antibody AbR_{24} has a high degree of specificity for human melanoma cells when tested on viable cultured cells using the protein A mixed hemagglutinin serological assay. The antigen detected by this antibody has been isolated from melanoma cells and shown to be G_{D3} ganglioside by compositional and partial structural analysis and by comparison with authentic G_{D3} in thin layer chromatography (TLC). AbR_{24} reacts with authentic G_{D3}, but not with any other ganglioside tested. Using TLC and reactivity with AbR_{24}, a wide range of cells and tissues was examined for the presence of G_{D3}. A new serological assay, termed glycolipid-mediated immune adherence, was devised for assaying the reactivity of AbR_{24} with gangliosides. Melanomas (cultured cells or tumor tissue) were shown to have G_{D3} and G_{M3} as major gangliosides. Other cells and tissues examined also contained G_{D3}, but usually only in low amounts. Melanomas (and MOLT-4, a T cell line) were characterized by a simplified ganglioside profile with G_{D3} and G_{M3} as major components. The apparent discrepancy between the ubiquitous presence of G_{D3} and the serological specificity of AbR_{24} for melanoma cells can be explained in terms of localization and concentration of G_{D3} in different cells.

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