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A Monoclonal Antibody Recognizes an *O*-Acylated Sialic Acid in a Human Melanoma-associated Ganglioside*

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David A. Cheresh‡§, Ajit P. Varki¶‖, Nissi M. Varki‡, William B. Stallcup, Joel Levine**, and Ralph A. Reisfeld‡**

*From the ‡Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037, ¶The Cancer Center, University of California at San Diego, San Diego, California 92037, and the **Molecular Neurobiology Laboratory, Salk Institute, La Jolla, California 92037*

Monoclonal antibody D1.1 originally prepared against the B49 cell line derived from a rat brain tumor was shown to react with a ganglioside present in fetal rat brain. We have found that this antigen is also present in human malignant melanoma tumors as well as many melanoma cell lines. The ganglioside from human melanoma cell lines migrates between G_{M1} and G_{M2} on one-dimensional thin layer chromatography. Analysis by two-dimensional thin layer chromatography with intermediate ammonia treatment suggests that the ganglioside contains one or more base-labile *O*-acyl esters. Mild base hydrolysis under conditions known to remove *O*-acyl esters results in complete loss of antigenic reactivity. Thus, the alkali-labile moiety is a critical component of the epitope recognized by the antibody. Analysis of the sialic acids of total gangliosides from [6-^{3}H]glucosamine-labeled melanoma cells showed that approximately 10% of these molecules are *O*-acylated. Similar analysis of the purified ganglioside showed that greater than 30% of the sialic acids comigrated with authentic 9-*O*-acetyl-*N*-acetylneuraminic acid. The antibody did not cross-react with normal human skin melanocytes nor with any of a large number of normal human adult and fetal tissues. The antibody also did not react with numerous other malignant cell lines studied. These findings suggest that the antigenic epitope defined by antibody D1.1 contains an *O*-acylated sialic acid and may arise from aberrant *O*-acetylation occurring in human malignant melanoma cells.

Monoclonal antibodies to tumor-associated antigens have facilitated the characterization of molecular differences between tumors and normal cells and significantly advanced an understanding of the functional role of some of these antigens. However, most information gained thus far involves either protein or glycoprotein antigens, as they can be readily immunoprecipitated from tumor cell lysates.

Recent technological advances have made it possible to use monoclonal antibodies for the characterization of complex carbohydrate antigens on tumor cell surfaces. Several investigators have produced monoclonal antibodies directed to the carbohydrate portion of tumor cell-associated glycolipids (1–8). Monoclonal antibodies to the melanoma-associated ganglioside, G_{D2}[1] (7) and G_{D3} (2, 3), have been described. There are also descriptions of anti-glycolipid antibodies reactive with various other neoplastic tissues, including colon carcinoma (1) and neuroblastoma (5). In some cases, the antigenic epitopes defined by these antibodies have only subtle differences between them involving a single sugar residue or the type of sugar linkage.

The gangliosides are glycolipids that contain sialic acids. The elegant work of Schauer and others (9) has now clearly shown that the sialic acids are an extremely diverse group of sugars. This diversity is mostly generated by different types of *O*-substitutions (usually *O*-acetyl esters) at the 4-, 7-, 8-, and 9-hydroxyl positions of the parent molecule, neuraminic acid. Recent work indicates that the sialic acids of some gangliosides have *O*-acylation. This may have been missed by earlier studies because of the extreme lability of the *O*-acyl esters.

Recently, Levine *et al.* (10) described a monoclonal antibody D1.1 prepared against the rat B49 cell line which specifically recognizes a ganglioside on developing rat embryonic neuroectoderm. In this report, we now demonstrate that the ganglioside recognized by monoclonal antibody D1.1 is also found on human melanoma cells and contains an alkali-sensitive antigenic epitope which involves an *O*-acylated sialic acid residue.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Serological Reactivity of Monoclonal Antibody D1.1 with a Ganglioside Common to Rat Brain Tumor and Human Melanoma Cell Lines—As shown in Fig. 1, monoclonal antibody D1.1 specifically recognizes a ganglioside in B49 and M14 cells which migrates on thin layer chromatography between G_{M1} and G_{M2}. The M14 cells express this antigen as a doublet.

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‖ Faculty Fellow of the John A. and George L. Hartford Foundation.

[1] Gangliosides are termed according to the nomenclature as previously described by Svennerholm (39).

[2] "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-3269, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

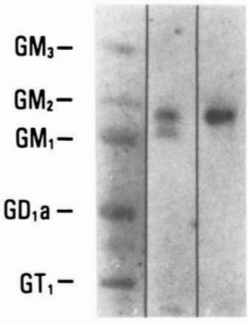

FIG. 1. **D1.1 reactivity with individual gangliosides.** Gangliosides were prepared from B49 and M14 cells and separated by chromatography on plastic-backed TLC plates as described. The plate was cut into strips. One strip was stained with resorcinol reagent, and a parallel sample from the same plate was treated with monoclonal antibody D1.1 and peroxidase-conjugated goat anti-mouse secondary antibody as described under "Experimental Procedures." The standards are as indicated.

TABLE I

Monoclonal antibody D1.1 reactivity with human cell lines

Reactivity of monoclonal antibody was determined by an enzyme-linked immunosorbent binding assay as measured by absorbance at 492 nm.

Neuroectodermal tumors		Other tumors	
Melanoma	Absorbance 492 nm	Bronchogenic carcinoma	
M14	0.206	CALU-1	—
M21	0.121	CALU-6	—
Melur	0.648	UCLA-P3	—
FM3	—[a]	T291	—
FM5	—	A427	—
FM7	—	Pancreatic carcinoma	
FM8	0.100	Panc-1	—
FM9	0.212	Lymphoma	
FOSS	0.121	HPB-A11	—
MS1	—	HSBR	—
FL2	0.074	Lymphoblastoid	
Neuroblastoma		L10	—
LAN-1	—	L14	—
LAN-2	—	LG2	—
LAN-5	—	3017	—
SK-N-MC	—	4098	—
SK-N-SH	—	PL3	—
Glioblastoma		Daudi	—
U-373 MG	—	Molt-4	—
U-87 MG	—		
U-138 MG	—		
Oat cell lung carcinoma			
NCI-H69	—		
T-293	—		

[a] —, Reactivities ≤0.020 were considered negative.

It has previously been shown that such doublets in melanoma-associated gangliosides are due to identical sugar moieties on ceramides containing fatty acids of variable length (19).

Reactivity of Monoclonal Antibody D1.1 with Human Tumor Cell Lines, Human Tumor Tissues, and Normal Adult and Fetal Tissues—The reactivity of monoclonal antibody D1.1 with various human cell lines was assessed in a solid phase enzyme-linked immunosorbent assay and was found to be restricted to melanoma since binding to various cell lines of neuroectodermal origin, lymphoblastoid cell lines, or tumors of other tissue origin was not detectable (Table I). The antibody expressed variable reactivity with the melanoma cell lines, binding to 7 out of 11 tested. The Melur cell line derived from a melanoma lesion metastatic to the brain was shown to have a relatively high reactivity with the antibody.

The reactivity of monoclonal antibody D1.1 against various tissues was initially tested by an immunoperoxidase assay on frozen tissues. Two of six melanomas screened showed a positive reactivity. One of these was a melanoma metastatic to an axillary lymph node. The vast majority of tumor cells showed positive staining. The other melanoma that showed a positive reaction was a primary nodular melanoma, whose depth of invasion was assessed to be level IV. Nests of melanoma cells, stained by the antibody, were seen to occasionally infiltrate the epidermis. Within the epidermal areas separated from the tumor by at least one rete peg, the normal melanocytes were negative. As shown in Table II, the monoclonal antibody D1.1 did not react with a wide variety of normal adult tissues, fetal tissues, and other malignancies when tested by the frozen section immunoperoxidase assay. In all cases, reactivities were compared to positive controls, W632, a monoclonal antibody directed against human Class I histocompatibility antigens HLA-A,B,C and 9.2.27, a monoclonal antibody which recognizes a melanoma-associated antigen (11, 12), and with P3X63 mouse myeloma protein which served as a negative control.

The Melanoma-associated Ganglioside Is Alkali-labile—Melanoma gangliosides were isolated and subjected to two-dimensional TLC with or without intermediate exposure to alkali in order to determine if the TLC migration of the ganglioside recognized by monoclonal antibody D1.1 was consistent with the migration of *O*-acylated sialic acid-bearing gangliosides, as previously described (15). In Fig. 2 (*left*), 14 gangliosides are depicted that were resolved by two-dimensional TLC without intermediate alkali treatment. The *arrow* corresponds to the position of the D1.1 antigen, which was identified by the immunostaining technique on a parallel two-dimensional TLC plate and shows the corresponding doublet migrating between the ganglioside standard G_{M1} and G_{M2} (Fig. 2, *lower*). When the TLC plates were exposed to ammonium hydroxide vapors prior to initiation of migration in the second dimension (Fig. 2, *right*), the D1.1 ganglioside demonstrated retarded migration (*arrow*). This behavior is similar to the migration pattern of a previously described *O*-acylated ganglioside (15), suggesting the D1.1 ganglioside contains an *O*-acyl ester. The

TABLE II

Reactivity of monoclonal antibody D1.1 using immunoperoxidase assay with frozen tissue

All tissues were cut, stained, and examined as described under "Experimental Procedures."

Melanotic tissues		Fetal tissues	
Melanoma	+ (2/6 tested)	Spleen	–
Intradermal nevus	–	Fetal skin	–
Normal skin melanocytes	–	*Liver*	–
Normal adult tissues		Lung	–
Brain cortex	–	Colon	–
Cerebellum	–	Kidney	–
Midbrain	–	Malignancies	
Spleen	–	Oat cell carcinoma (lung)	–
Liver	–	Neuroblastoma	–
Lung	–	Astrocytoma	–
Colon	–	Glioblastoma multiform	–
Kidney	–	Breast adenocarcinoma	–
		Colon adenocarcinoma	–
		Lung adenocarcinoma	–

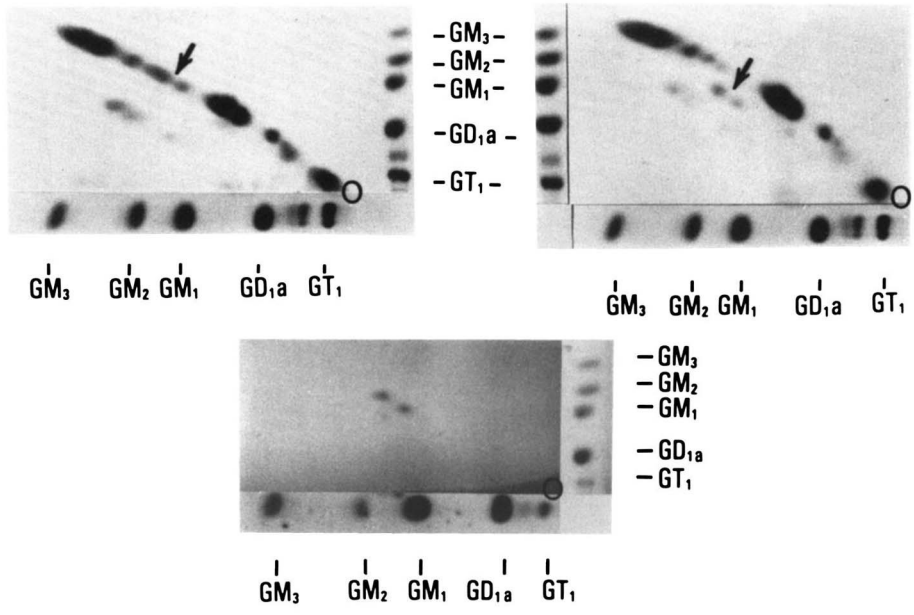

FIG. 2. **Two-dimensional TLC for identification of alkali-labile gangliosides.** Purified gangliosides from M14 cells were subjected to two-dimensional TLC without intermediate base hydrolysis (*left*) and with intermediate base hydrolysis (*lower*). Gangliosides were visualized with resorcinol reagent or by immunostaining using D1.1 overlay assay (*right*). *Arrows* correspond to the position of the ganglioside recognized by monoclonal antibody D1.1. The origin of the chromatogram is *encircled*. The first dimension was run from right to left.

three spots off the diagonal seen in both upper panels of Fig. 2 most likely represent gangliosides with very unstable lactone inner esters which represent artifact obtained during the ganglioside purification procedure.[3]

The retarded migration of this ganglioside after alkali treatment suggests that the de-*O*-acylated product may be related to or identical with G_{D3}, a known melanoma-associated ganglioside (2, 19), since it does co-migrate with G_{D3} (Fig. 2, *right*) after alkali treatment. The large doublet in the middle of this chromatogram has been previously shown to be the disialoganglioside G_{D3}.[4] In addition, the ganglioside recognized by monoclonal antibody D1.1 was also shown to contain 2 sialic acid residues since it did elute in the disialo fraction of a DEAE-Sepharose anion exchange column (data not shown).

The Antigenic Epitope Recognized by Monoclonal Antibody D1.1 Is Alkali-labile—Pretreatment of monoclonal antibody D1.1 with total melanoma gangliosides inhibited monoclonal antibody D1.1 binding to M14 cell targets as shown in Fig. 3. In order to determine whether the epitope recognized by this antibody involved the putative *O*-acylated sialic acid residue, gangliosides purified from melanoma cells were subjected to alkali treatment sufficient for de-*O*-acylation and then allowed to react with monoclonal antibody D1.1. As shown in Fig. 3, the alkali-treated gangliosides lose their ability to inhibit antibody binding to melanoma cells, suggesting that a component in the ganglioside extract containing an alkali-labile determinant was part of the antigenic epitope recognized by the antibody. In addition, alkali-treated gangliosides separated by TLC no longer react with the D1.1 antibody (Fig. 4, *left*). A control was employed to show that base treatment did not destroy other ganglioside antigens. Specifically, alkali treatment of gangliosides was unable to cause loss of reactivity of monoclonal antibody R24 (Fig. 4, *right*) which is known to react with the melanoma-associated ganglioside G_{D3} (2). Two additional anti-ganglioside monoclonal antibodies produced in our laboratory were also shown to retain their reactivity with alkali-treated melanoma gangliosides. One of these antibodies, MB.3.6 reacts with G_{D3}; the other, 126, has been shown to react with G_{D2}.[4]

Human Melanoma Cells Synthesize Gangliosides with O-

[3] G. Hunter, personal communication.

[4] D. A. Cheresh, A. P. Varki, and R. A. Reisfeld, manuscript in preparation.

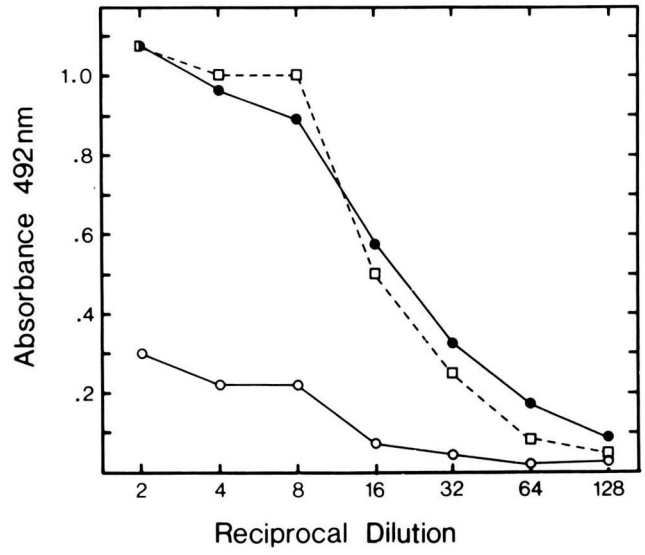

FIG. 3. **Competition of antibody binding by gangliosides.** Monoclonal antibody D1.1 was preincubated with alkali-treated or untreated melanoma gangliosides as described. The antibody was appropriately diluted (2 = 5 μg/ml) and assayed for reactivity with M14 cells as targets in an enzyme-linked immunosorbent binding assay as described. □, untreated antibody; ●, antibody preincubated with alkali-treated ganglioside; ○, antibody preincubated with untreated gangliosides.

Acylated Sialic Acids—In order to determine whether human melanoma cells propagated in long term tissue culture were synthesizing gangliosides containing *O*-acylated sialic acid residues, we metabolically labeled such cells with [6-^{3}H]glucosamine, a known precursor of sialic acids. We chose to study the Melur cell line because it was found to contain a relatively large amount of the antigen recognized by antibody D1.1.[4] Mixtures of gangliosides isolated from the Melur cell line, metabolically labeled with [^{3}H]glucosamine and [^{3}H]galactose, were shown to contain a relatively simple ganglioside pattern with the antigen recognized by monoclonal antibody D1.1 comprising approximately 10% of the total.[4] The Melur cells were labeled for 3 days in growth media containing 2 mCi of [^{3}H]glucosamine and 0.2 mCi of [^{3}H]galactose, and the total gangliosides were extracted as described above. The

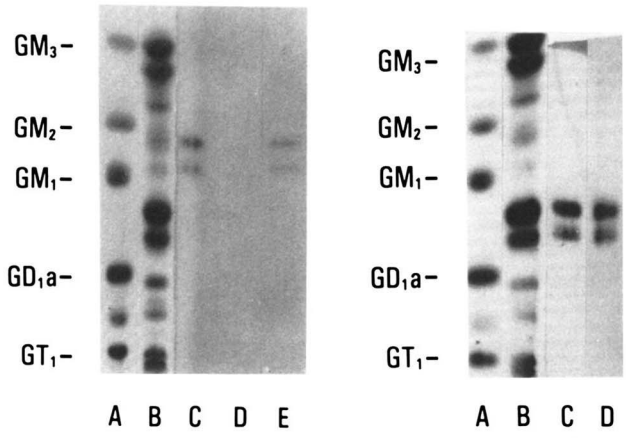

FIG. 4. **Effects of alkali treatment on antibody binding to gangliosides.** Melanoma gangliosides were separated by TLC, and strips of the plates were cut and either sprayed with resorcinol reagent or overlaid with various anti-ganglioside antibodies for serological detection of antibody reactivity as described under "Experimental Procedures." *Left: lane A*, ganglioside standards; *lane B*, M14 gangliosides (*lanes A* and *B* were visualized with resorcinol reagent); *lane C*, untreated M14 gangliosides; *lane D*, alkali-treated M14 gangliosides; *lane E*, non-alkali-treated control M14 gangliosides (*lanes C–E* were allowed to react with monoclonal antibody D1.1 in the overlay assay. *Right: lane A*, ganglioside standards; *lane B*, M14 gangliosides (*lanes A* and *B* were visualized with resorcinol reagent); *lane C*, alkali-treated M14 gangliosides; *lane D*, non-alkali-treated control M14 gangliosides (*lanes C* and *D* were allowed to react with monoclonal antibody R24 previously shown to recognize melanoma ganglioside G_{D3} (2).

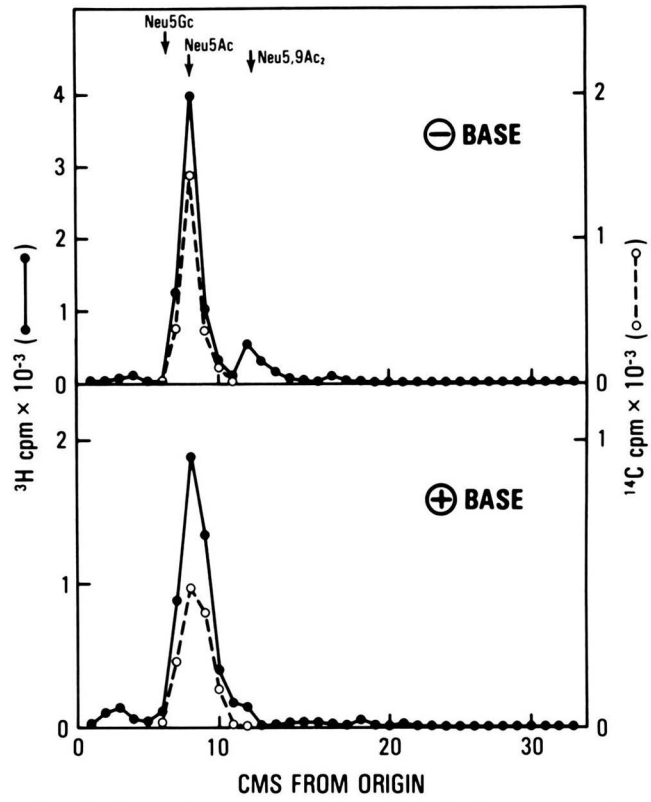

FIG. 5. **Paper chromatography of [^{3}H]sialic acids from human melanoma cell gangliosides.** Sialic acids were purified from the total gangliosides of [6-^{3}H]glucosamine-labeled Melur melanoma cells and subjected to paper chromatography along with an internal [^{14}C]*N*-acetylneuraminic acid standard, as described. The sample was spotted on the paper with (*lower*) or without (*upper*) prior base treatment to remove *O*-acyl esters. The positions of migration of the following standards are indicated: *Neu5Gc*, *N*-glycolylneuraminic acid; *Neu5Ac*, *N*-acetylneuraminic acid; and *Neu5,9Ac*$_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid.

sialic acids were released and purified as described under "Experimental Procedures." The conditions for release and purification were chosen to maximize recovery while minimizing losses of *O*-acyl groups (18). Approximately 30% of the total radioactivity was recovered in the Dowex 3-X4A eluate. These released purified sialic acids were then subjected to paper chromatography with an internal [^{14}C]*N*-acetylneuraminic acid standard. As shown in Fig. 5, (*upper*), approximately 87% of the [^{3}H]sialic acid released co-migrated with the [^{14}C]*N*-acetylneuraminic acid, while the remainder (13%) migrated faster in the same position as the *N*-acetyl-9-*O*-acetylneuraminic acid standard. When treated with base under conditions known to de-*O*-acylate sialic acids, the faster migrating peak almost completely disappeared (3% remaining), accompanied by a corresponding increase in the ^{3}H/^{14}C ratio of the major *N*-acylneuraminic acid peak. This strongly suggests that approximately 10% of the sialic acids contained in *total* gangliosides synthesized by these cells are *O*-acylated. In similar studies of the biosynthesis of sialic acids in murine Friend erythroleukemia cells, a radioactive peak with identical migration has been more completely characterized as being 9-*O*-acetyl-*N*-acetylneuraminic acid.[5] This finding increases the likelihood that alkali lability of the ganglioside recognized by antibody D1.1 is attributable to the presence of *O*-acylated sialic acids.

The Ganglioside Recognized by Monoclonal Antibody D1.1 Contains an O-Acylated Sialic Acid—In order to more directly demonstrate the presence of *O*-acylated sialic acids in the ganglioside in question, an aliquot containing 2×10^6 cpm of total gangliosides was chromatographed on one-dimensional TLC, and the region corresponding to the D1.1 ganglioside was scraped out and eluted. When an aliquot of the purified material was run on TLC and then studied by autoradiography, a double band was seen, corresponding to the location of the ganglioside recognized by the D1.1 antibody (data not shown).

Sialic acids were released from this ganglioside, purified, and subjected to paper chromatography as described above. As shown in Fig. 6, 32% of the total ^{3}H cpm migrated ahead of the [^{14}C]*N*-acetylneuraminic acid standard. This peak was eliminated by prior de-*O*-acylation with base (Fig. 6, *lower*), with a corresponding increase in the ^{3}H/^{14}C ratio in the non-*O*-acetylated peak. This directly demonstrates that the D1.1 ganglioside contains an *O*-acylated sialic acid. Similar results were obtained with neuraminidase-released sialic acids.

DISCUSSION

Gangliosides are ubiquitous molecules present in the membranes of all eukaryotic cells. They have been implicated in a variety of cellular functions, including cell-cell adhesion and communication, as well as cell-substrate interactions (21–23). Gangliosides have been found to serve as putative cell membrane receptors for hormones (24, 25), toxins (26, 27), and extracellular matrix components (28, 29). Their role as developmental antigens, particularly in tissues of neuroectodermal origin, is currently being examined by numerous investigators. The application of monoclonal antibody production for the isolation and characterization of ganglioside antigens has aided significantly to delineate their roles in various biological processes. The ganglioside antigen defined by the monoclonal antibody D1.1 has been recently shown to be expressed on the surfaces of developing rat neuroectoderm (10). This anti-

[5] A. Varki and S. Diaz, submitted for publication.

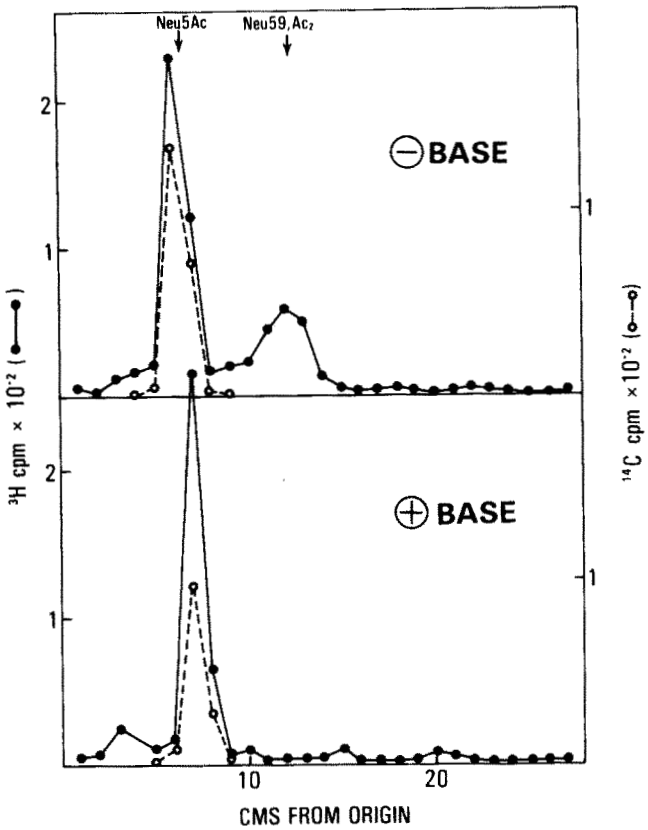

FIG. 6. **Paper chromatography of sialic acids from purified ganglioside.** The radiolabeled ganglioside recognized by the D1.1 antibody was purified by preparative TLC. The sialic acids were released from the ganglioside, purified, and subjected to paper chromatography as described with (*lower*) or without (*upper*) prior base treatment. The standards are *N*-acetylneuraminic acid (*Neu5Ac*) and 9-*O*-acetyl-*N*-acetylneuraminic acid (*Neu5,9Ac*$_2$).

gen was shown to be a differentiation marker since its expression disappears during neural differentiation.

We have presented evidence that in human tissue the antigen defined by monoclonal antibody D1.1 has a high degree of specificity for melanoma. Evidence for *in vivo* expression of this antigen has been documented by immunohistological staining patterns of tissue sections. In addition to its serological specificity, the D1.1 antibody is the first described which recognizes an alkali-labile ganglioside. Our evidence clearly indicates that the epitope recognized by monoclonal antibody D1.1 includes the *O*-acyl group. In contrast, the serological reactivity of three additional antibodies directed to gangliosides on melanoma cells did not show any loss in reactivity when tested on the same alkali-treated ganglioside preparations. Preliminary evidence presented here suggests that the ganglioside recognized by monoclonal antibody D1.1 may be an *O*-acylated version of the disialoganglioside G_{D3} since after de-*O*-acylation, it co-migrates with G_{D3}. In addition, the ganglioside elutes from a DEAE-Sepharose anion exchange column in the disialo fraction. However, further structural analysis will be necessary to confirm this hypothesis.

Alkali-labile gangliosides have only recently been recognized (9, 30, 32). Older methods for the preparation of gangliosides (saponification of contaminating acidic lipids) undoubtedly resulted in destruction of the alkali-labile *O*-acyl esters. Recently, however, several *O*-acylated gangliosides have been reported. The presence of 9-*O*-acetylated sialic acids in total brain gangliosides from several vertebrate species was first reported by Haverkamp *et al.* (33). The structure of one *O*-acetylated ganglioside from mouse brain is definitely known (30). Several other *O*-acetylated gangliosides have recently been found in mouse thymus gangliosides. In this case, it was assumed that the alkali-labile nature of the gangliosides was due to the presence of *O*-acetylated sialic acids (32).

In the present study, the two-dimensional TLC with intermediate alkali hydrolysis as described by Sonnino *et al.* (15) has been a useful tool for the analysis of these substituted molecules. In our hands, this technique has allowed us to demonstrate that the TLC migration of the ganglioside defined by monoclonal antibody D1.1 behaves similarly to those previously described as having an *O*-acylated sialic acid residue. Since the ganglioside in question was such a minor component of the total gangliosides, we chose to use a radiolabeling method to demonstrate that it contained an *O*-acylated sialic acid. Total labeled gangliosides from a melanoma cell line contained about 10% *O*-acylated sialic acid. When radiolabeled D1.1 antigen was purified from this mixture and subjected to similar analysis, it was found to contain ~30% base-labile sialic acids. For reasons discussed in detail elsewhere (20), the 30% value is likely to be an underestimate. Therefore, these data are most compatible with one of two sialic acids in the ganglioside being *O*-acylated. Additional studies will be required to define this further.

Since the base-labile sialic acid co-migrated with the 9-*O*-acetyl-*N*-acetylneuraminic acid, it is likely to be an *O*-acetylated sialic acid. However, the present data do not allow us to exclude other base-labile *O*-acyl groups, such as *O*-lactyl esters, that have been reported in the sialic acids (9). Previous experiments have shown that prolonged treatment with *Vibrio cholerae* neuraminidase is required to completely destroy the antigenicity of the ganglioside, both in rat brain and in the B49 cells.[6] This partial resistance to the *Vibrio* neuraminidase is in keeping with the fact that side chain (7/8/9) mono-*O*-acetylated sialic acids are relatively more resistant to release by this enzyme (9).

There are other known causes for base-labile groups in glycolipids. Inner lactone esters can occur in the sialic acids, either naturally (34), or as an artifact induced during purification. This appears unlikely because similar peaks were not seen in identical analyses of radiolabeled sialic acids from many other metabolically labeled tumor cell lines.[5] Furthermore, the sialic acids of the ganglioside in question are susceptible to release by *Arthrobacter ureafaciens* neuraminidase, which will release *O*-acetylated sialic acids (18) but would not be expected to release sialic acid lactones. *O*-Acyl groups have also been reported in the simple neutral glycolipid galactosylceramide (35). The ganglioside described here is considerably larger. However, we cannot absolutely rule out the possibility of such substitutions occurring *in addition* to the substitution of the sialic acids.

Although the epitope defined by monoclonal antibody D1.1 involves an *O*-acylated sialic acid, several facts suggest it is a co-determinant and that this sialic acid residue is not the entire epitope responsible for antibody recognition. First, this antibody was raised by immunization of BALB/c mice, which are known to have extensive *O*-acetylation of the sialic acids of their red blood cell membranes (31). In addition, murine erythroleukemia cells, which are known to have *O*-acetylated sialic acids on their surface,[5] do not show any reaction with this antibody (data not shown). Finally, the antibody fails to react with another alkali-labile ganglioside present on the

[6] J. Levine, unpublished observations.

same melanoma cells (see Fig. 2).

Although sialic acids more often serve to mask underlying antigens, there are several examples in which they can be antigenic themselves. The human M and N blood groups are partly determined by sialic acids (36). There are naturally occurring human monoclonal IgMs from patients with Waldenström's macroglobulinemia that react with antigens containing sialic acid as a determinant (37). Polyclonal antibodies to sialyloligosaccharides coupled to proteins have been successfully raised (38). Sialic acid is an integral component of the antigenic epitope recognized by several monoclonal antibodies raised against tumor gangliosides from colon carcinoma (1), neuroblastoma (5), and melanoma (2). In none of these cases was there any indication of *O*-acylation of sialic acids. As far as we are aware, this is the first example of a monoclonal antibody directed against an alkali-labile ganglioside. Furthermore, the epitope recognized includes the *O*-acyl group itself. Further studies are currently underway to define the complete structure of this ganglioside.

O-Acylation of sialic acids appears to be a tissue- and species-specific characteristic (9). However, it is currently unclear whether *O*-acylation in a given cell type is a general characteristic of all sialylated compounds produced by that cell or whether it is restricted to specific sialic acid residues. In a study of sialic acids in mouse red cells, different sialoglycoproteins were found to have similar extents of *O*-acetylation (31). This suggested that *O*-acetylation may not be very rigorously regulated with regard to its distribution on different sialic acid residues. However, in this study we found that of several gangliosides produced by a monoclonal population of tumor cells in culture, only one or two appeared to be alkali-labile. There is no evidence in any biological system for different sialyltransferases inserting *O*-acylated and non-*O*-acylated sialic acids. Therefore, it appears likely that in melanoma cells, the *O*-acylation reaction is a carefully regulated event that occurs after the sialylation of gangliosides, resulting in selective *O*-acylation of only certain sialic acid residues. Consequently, quantitative or qualitative differences in specific *O*-acyltransferases may cause the appearance of new antigens during the process of malignant transformation. Such antigens provide potentially useful molecular markers for the study of these human tumor cells.

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SUPPLEMENTARY MATERIAL

TO

A MONOCLONAL ANTIBODY RECOGNIZES AN O-ACYLATED SIALIC ACID IN A HUMAN MELANOMA-ASSOCIATED GANGLIOSIDE

David A. Cheresh, Ajit P. Varki, Nissi M. Varki,

William B. Stallcup, Joel Levine, and Ralph A. Reisfeld

EXPERIMENTAL PROCEDURES

Materials

Cells Lines: Human melanoma cell lines M14 and M21 originally provided by Dr. D. L. Horton, UCLA, have been in long term culture in our laboratory and the melanoma cell lines FM3, FM5, FM7, FM8, and FM9 were recently established by us. HS-1 was kindly provided by Dr. Mitchell at USC and FDSS was obtained from Dr. Giovanella, Houston, Texas. The melanoma cell line Melur was provided by Dr. Ursula Koldovsky, Dusseldorf, Germany.
The B-lymphoblastoid cells, L1O and L14 originally obtained from Dr. D. L. Horton at UCLA, are in culture in our laboratory as are the B-lymphoblastoid cells 4098 and Daudi, as well as the T-lymphoblastoid cell Molt-4 originally obtained from the American Type Culture Collection (ATCC), Rockville, MD. The B-lymphoblastoid cell LG-2, originally provided by Dr. Gatti, UCLA, has been in long term culture in our laboratory.
The B-49 cell was obtained from Dr. D. Schubert, Salk Institute, La Jolla, CA. The neuroblastoma cells, LAN1, LAN2, and LAN5 were kindly provided by Dr. R. Seeger, UCLA, and have been in culture in our laboratory. The bronchogenic cell lines CALU1, CALU6, A427 as well as the T-cell leukemia cell HPBALl were obtained from the American Type Culture Collection, Rockville, MD. The oat cell lung carcinoma cells, NCI-H69 and T293, were kindly provided by Dr. J. Minna, NCI, and by Dr. N. O. Kaplan, UCSD, respectively. The PANC-1 cell line derived from an adenocarcinoma of the pancreas originally obtained from Dr. Nelson-Reese at Navel Research Lab., Oakland, CA, and the UCLA-P3 lung adenocarcinoma cell line originally provided by Dr. D. L. Horton, UCLA, have been in long-term culture in our laboratory. The neuroblastoma cell lines, Sk-N-MC and Sk-N-SH as well as the glioblastoma cell lines U-87, U-373 MG and U138 MG were obtained from the ATCC.

Monoclonal Antibodies: MAb D1.1 was produced as originally described by Levine et al. and was shown to recognize a ganglioside present on developing embryonic rat neuroectoderm (10). MAb 9.2.27 which recognizes a melanoma associated chondroitin sulfate proteoglycan (11-12), and mAb R_{24} (generously provided by Dr. K. Lloyd, Memorial Sloan-Kettering) which reacts with the melanoma associated ganglioside GD3 (2) were used for control purposes. MAb W6/32 directed to all HLAL-A,B,C antigens was provided by Dr. P. Parham (Stanford Univ., Palo Alto, CA).

Tissues: Portions of fresh, normal and malignant tissue were obtained from the Surgical Pathology Department of the Ida M. Green Hospital of Scripps Clinic. Specimens were embedded in Tissue Tek Medium (Scientific Products) and frozen in blocks in isopentane at liquid nitrogen temperature. They were then stored at -70°C.

Materials for Purification and Analysis of Sialic Acids: Dowex 3x4A (100-200 mesh, chloride form) and Dowex 50 AG 1x2 (100-200 mesh, hydrogen form) were from BioRad. The Dowex 3x4A resin was converted to the formate form, using the method recommended by the manufacturer. [^{14}C]N-acetylneuraminic acid was from New England Nuclear. Chemically synthesized N-acetylneuraminic Acid (Neu5Ac) (99% purity) was from Kantoishi Pharmaceutical Co., Tokyo, Japan. N-glycolyl-neuraminic acid (Neu5Gc) was from Sigma. Authentic N-acetyl-9-O-acetyl-neuraminic acid (Neu5, $9Ac_2$) was kindly provided by Professor Roland Schauer, Kiel, Federal Republic of Germany.

The micropartition units (Amicon) were used as recommended by the manufacturer. All evaporations of aqueous samples were performed on a Buchler Shaker-Evaporator with the water bath set at 35°C or a Savant Centrifuge evaporator at 35-40°C.

Preparation and Purification of Gangliosides: Biopsy material or cultured tumor cells, equivalent to 1-2 ml of packed cells, were homogenized in 20 volumes of chloroform-methanol, 2:1, for 5 minutes with mild sonication and vortexing. The homogenate was filtered through a sintered glass filter, the residue re-extracted as above with 10 volumes of chloroform-methanol, 1:1, refiltered, and the filtrates were combined, evaporated and dissolved in 10 ml of chloroform-methanol, 2:1. Gangliosides were partitioned into an aqueous phase as described by Ledeen and Yu (13). This material was dialyzed exhaustively against cold distilled water and lyophilized, and called "crude gangliosides".

The lyophilized crude ganglioside preparation was further purified as follows: dissolved in 50 ml of methanol-chloroform-water, 60:30:8, and applied slowly to a column (1 cm x 15 cm) of DEAE-Sepharose CL-6B (Pharamacia Fine Chemicals, Piscataway, New Jersey). The column was washed extensively with the above solvent and gangliosides were eluted with methanol:chloroform:0.8 M aqueous sodium acetate, (60:30:8.) Fractions were collected and assayed for the presence of gangliosides by TLC using a variety of ganglioside standards. Fractions containing gangliosides were evaporated, and dissolved in distilled H_2O to be dialyzed and lyophilized. The freeze-dried material was dissolved in chloroform-methanol (1:1) and applied to a column of Iatrobeads as described by Ledeen and Yu (13). The material eluted from this column was considered relatively free from contaminants.

One-dimensional thin-layer chromatography (TLC): Silica gel plates (plastic backed, E.M. Merck, Darmstadt, Germany) were activated by heating at 110°C for 1 hour. Chloroform-methanol-0.2% aqueous $CaCl_2$, 60:45:10, was used for the development of the chromatograms. Samples were spotted 1.5 cm from the bottom of the TLC plate which were then placed in a developing tank, presaturated with 100 ml of the above solvent. Chromatography development was for 2 hours, after which the plate was allowed to dry. Appropriate lanes of the chromatogram were cut and sprayed with resorcinol reagent (14) to visualize gangliosides. The standards used included GM_3, GM_2, kindly supplied by Dr. J. Sundsmo (Scripps Clinic, La Jolla, California) and GM_1, GD_{1a}, and GT_1 were purchased from Supelco For all experiments 1-2 ug of each standard were spotted in appropriate lanes. For resorcinol visualized M14 gangliosides, 5 ug of ganglioside-bound sialic acid (14) was spotted per lane.

Two-Dimensional TLC for detection of alkali-labile gangliosides: Alkali-labile melanoma gangliosides were detected by a modification of the 2D-TLC procedure originally described by Sonnino et al. (15). M14 gangliosides (10 ug of ganglioside bound sialic acid) were spotted at the origin. The solvent used was 100 ml of chloroform-methanol, 0.2% aqueous $CaCl_2$ (60:45:10), and the chromatograms were developed in each dimension for 2 hours with an intermediate overnight incubation at room temperature in an air tight chamber saturated with ammonia.

Immunoperoxidase Staining on Frozen Tissues: Sections of frozen tissue blocks, 4-6 micron thick, were cut on the microtome/cryostat. The sections, mounted on glass slides were briefly air-dried, and either stained immediately or stored at -70°C in airtight boxes. An indirect immunoperoxidase assay, essentially as described by Taylor (16), was used to stain these slides. Briefly, after washing in phosphate buffer saline (PBS) pH 7.1, the sections were pre-incubated for 15 minutes at room temperature in PBS containing 10% goat serum and 0.1% bovine serum albumin. Excess serum was then removed and monoclonal antibody diluted 1:2 overlayed onto the sections. The sections were allowed to incubate in a humid chamber for one hour and then briefly washed in PBS and overlayed with a 1:50 dilution of peroxidase-conjugated goat anti-mouse antibody (IgG + IgM; Tago Chemicals). This step was followed by a 1-hour incubation at room temperature, followed by a wash in PBS. The color reaction was developed with 1 mg/ml of diaminobenzidine and 0.03% H_2O_2. After a brief counterstain with 1% methylene blue, the slides were washed in water and dehydrated in isopropyl alcohol. They were then cleared in xylene, mounted in parmount, coverslipped, and examined using an American Optical microstar Series 20 microscope.

ELISA Binding Assay: Reaction patterns of D1.1 activity were analyzed by serological means using a solid-phase enzyme-linked immunosorbent assay (ELISA). Target cells were dried onto 96-well flat bottom polyvinyl microtiter plates (Dynatech) and the ELISA binding assay was performed as described previously by Harper et al. (12). MAb was diluted 1:2 in PBS containing 0.1% BSA and 0.02% Tween 20.

Detection of D1.1 reactivity after separation of gangliosides by TLC:
The reactivity of mAb D1.1 with glycolipids (0.5-1 ug of ganglioside-bound sialic acid (14) separated by TLC was directly determined by using a modification of the method originally described by Magnani et al. (17). We used an ELISA assay to demonstrate D1.1 activity directly on the TLC plate using the secondary antibody goat anti-mouse IgG coupled to horseradish peroxidase (BioRad) diluted 1:1000. This was followed by the addition of 10 ml of a substrate solution which consisted of 400 ug/ml O-phenylenediamine (Sigma) in 80 mM citrate-phosphate buffer, pH 5.0 containing 0.12% H_2O_2 (Eastman). Color development of the reactive band was stopped after a 15-minute exposure to the substrate solution following which the TLC plate was dried at 56°C for 30 min.

Aqueous alkali-treatment of gangliosides: Gangliosides obtained from the melanoma cell line M14, representing 30 ug of TLC'd bound sialic acid were dried down in an Eppendorf tube, resuspended in 0.25 ml of 2.5 N aqueous ammonium hydroxide and allowed to incubate for 1 hour at 37°C. The ammonium hydroxide was rapidly evaporated in a speed-vac concentrator (Savant). A control was prepared by incubating gangliosides with distilled water in place of ammonium hydroxide.

Inhibition of Antibody Binding by Gangliosides: Antibody was pre-incubated for 1 hour at 25 C with purified M14 gangliosides (30 ug sialic acid) previously dried in glass tubes. The tubes were centrifuged at 500 x g for 10 minutes and the supernatants were diluted serially. These samples were then transferred to ELISA plates containing cultured M14 cells as targets. Antibody reactivity was measured by ELISA as described above. Unabsorbed antibody served as a positive control.

Metabolic Labeling of Melanoma Gangliosides: Melur cells (2.5 x 10^6) were seeded in (75 cm^2) tissue culture flasks (Corning) each containing 2 mCi D- 6 ^{3}H-glucosamine hydrochloride and 0.2 mCi D- 6 ^{3}H-galactose (New England Nuclear, Boston, MA) in 30 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) and allowed to grow for 72 hours at 37C in a humidified incubator supplied with a constant amount of 5% CO_2 in air. The cells were harvested by scraping and were washed three times in phosphate buffered saline pH 7.2. Gangliosides were extracted from the cell pellet as described above.

Purification and Analysis of Sialic Acids: Total purified gangliosides from 6-[^{3}H] glucosamine labeled cells were dissolved in water with sonication, adjusted to 2M acetic acid (final volume, 1 ml) and heated at 80°C for three hours to release the glycosidically-bound sialic acids. The reaction mixture was then chilled on ice, evaporated on a Buchler Shaker-Evaporator to remove the acid, reconstituted in 1 ml of water, and subjected to ultrafiltration through an Amicon Centrifree Micropartition system at 4°C. All subsequent steps were carried out at room temperature. The ultrafiltrate was passed over a 1 ml column of Dowex-50 (hydrogen form). The column effluent and 3 ml of water washings were collected in a tube containing 40 ul of 1M formic acid and then taken to dryness. The sample was dissolved in 1 ml of 10 mM sodium formate, pH 5.5 and passed over a 1 ml column of Dowex 3x4A (formate form) equilibrated in the same buffer. This column was washed with 7 ml of 10 mM formic acid, and the washings discarded. The sialic acids were eluted with 10 ml of 1 N formic acid, and the acid removed by evaporation. These conditions for release and purification were specifically chosen to maximize recovery of sialic acids with minimal loss of O-acyl groups (18). Recoveries of radioactivity were monitored at all steps. [^{14}C]N-acetyl-neuraminic acid was added to the formic acid eluate as an internal standard in amounts equal to approximately 1/4 of the cpm of ^{3}H-labeled sialic acids. After evaporation, the sample was split into two equal portions. One half was applied directly to Whatman 3MM paper while the other was treated with 2 ml of 2M NH_4OH at 60C for 60 min in order to de-O-acylate sialic acids, evaporated to remove the ammonia, and then spotted on the paper. The chromatogram was developed for 15 hours in n-Butanol:acetic acid:water (4:1:5, upper phase), dried, and cut into 1 cm strips that were monitored for [^{3}H]- and [^{14}C]-radioactivity. This method allows the detection of sialic acids containing O-acyl groups (e.g. Neu5,$9Ac_2$), or N-glycolyl groups (Neu5Gc), because such molecules have a migration different from that of the [^{14}C]Neu5Ac Standard.