

Disialogangliosides GD2 and GD3 Are Involved in the Attachment of Human Melanoma and Neuroblastoma Cells to Extracellular Matrix Proteins

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Abstract. Human melanoma cells express relatively large amounts of the disialogangliosides GD3 and GD2 on their surface whereas neuroblastoma cells express GD2 as a major ganglioside.¹ Monoclonal antibodies (Mabs) directed specifically to the carbohydrate moiety of GD3 and GD2 inhibit melanoma and neuroblastoma cell attachment to various substrate adhesive proteins, e.g. collagen, vitronectin, laminin, fibronectin, and a heptapeptide, glycyl-L-arginyl-glycyl-L-aspartyl-L-seryl-L-prolyl-L-cysteine, which constitutes the cell attachment site of fibronectin. Cells that are preattached to a fibronectin substrate can also be induced to detach and round up in the presence of purified anti-ganglioside Mab. Moreover, when melanoma cells that contain both GD2 and GD3 are incubated with Mabs directed to both of these molecules an additive inhibition is observed. The specificity of this inhibition is demonstrated since Mabs of various isotypes directed to either protein or carbohydrate epitopes on a number of other major melanoma or neuroblastoma cell surface antigens have no effect on cell attachment. A study of the kinetics involved in this inhibition indicates that significant effects occur dur-

ing the first 5 min of cell attachment, suggesting an important role for GD2 and GD3 in the initial events of cell-substrate interactions. The role of gangliosides in cell attachment apparently does not directly involve a strong interaction with fibronectin since we could not observe any binding of radiolabeled fibronectin or fragments of the molecule known to contain the cell attachment site to melanoma gangliosides separated on thin-layer chromatograms. An alternative explanation would be that gangliosides may play a role in the electrostatic requirements for cell-substrate interactions. In this regard, controlled periodate oxidation of terminal, unsubstituted sialic acid residues on the cell surface not only specifically destroys the antigenic epitopes on GD2 and GD3 recognized by specific Mabs but also inhibits melanoma cell and neuroblastoma cell attachment. In fact, the periodate-induced ganglioside oxidation and the inhibition of cell attachment are equally dose dependent. These data suggest that cell-substratum interactions may depend in part on the electrostatic environment provided by terminal sialic acid residues of cell surface gangliosides and possibly other anionic glycoconjugates.

SIALIC acid-bearing glycolipids, i.e., gangliosides, expressed on the cell surface have been implicated as possible receptors for hormones (2), toxins (12), viruses (24), and various growth factors (19, 20). It has also been suggested that gangliosides serve as functionally important molecules in cell-substratum interactions. The recent development of specific monoclonal antibodies directed against the carbohydrate moieties of gangliosides (3, 8, 20, 27, 31, 51) will aid in dissecting the biological functions of these membrane-embedded surface-exposed molecules. In this regard, we recently described monoclonal antibodies (Mabs)¹ directed against the disialogangliosides GD3 and GD2 (8).²

¹ Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; GRGDSPC, glycyl-L-arginyl-glycyl-L-aspartyl-L-seryl-L-prolyl-L-cysteine; Mabs, monoclonal antibodies.

² Gangliosides are termed according to the nomenclature of Svennerholm (43).

The epitopes recognized by these Mabs are known to involve the terminal sialic acid moiety since either neuraminidase treatment or mild periodate oxidation abolishes antibody reactivity (9). Using these Mabs we could localize both GD2 and GD3 in adhesion plaques and on the surface of human melanoma cells. Moreover, biochemical analysis of such substrate-attached adhesion plaques, left behind after EDTA treatment of metabolically labeled human melanoma cells, demonstrated that both GD2 and GD3 were indeed heavily concentrated at the interface of these cells and their substratum, suggesting that these molecules may play a role in melanoma cell attachment (8).

Initial evidence indicating ganglioside involvement in cell attachment came from a number of studies that demonstrated that the exogenous addition of gangliosides to cells in culture inhibited their attachment to the extracellular matrix protein

fibronectin (20, 31, 51). In these studies gangliosides with two or more sialic acids were more inhibitory than monosialogangliosides, and it was suggested that the sialic acid moiety was critically involved in this form of cell-substratum interaction. Recent reports by Yamada et al. (50) and Spiegel et al. (42) demonstrated that gangliosides added to cells embedded in their membrane and caused retention of fibronectin in cell surface-associated fibrillar networks. In addition, Dippold et al. demonstrated that Mab R24 directed to GD3 on human cells caused them to round up from tissue culture plastic (11).

The evidence presented by us and others suggesting that gangliosides may play a key role in cellular adhesion mechanism(s) served as a rationale for the experiments presented here. The results of these studies suggest that gangliosides may play a general, yet critical, role in cell-substratum interactions rather than acting as receptors for specific extracellular matrix adhesive proteins.

Materials and Methods

Mabs

The various sources of Mabs used in these studies are listed with their isotype denoted in parentheses. Mabs 126 (IgM), MB3.6 (IgG3), 11C64 (IgG3), 9.2.27 (IgG2a), KS1/4 (IgG2a), and KS1/9 (IgM) were produced in our laboratory. Mab 3F8 (IgG3) was provided by Dr. N. K. Cheung (Case Western Reserve School of Medicine, Cleveland, OH). Mab HNK-1 (IgM) and W6/32 (IgG2a) were generous gifts from Dr. R. Quarles (National Institutes of Health, Bethesda, MD) and Dr. P. Parham (Stanford University School of Medicine, Stanford, CA), respectively. Mab 390 (IgG3) was provided by Dr. R. Seeger (UCLA School of Medicine). Monoclonal antibodies were isolated from murine ascites fluid and purified by using a protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) immunoadsorbent procedure as described previously (30). The antibodies directed to GD2 and GD3 used in this study were shown not to react with carbohydrate determinants on glycoproteins. Specifically, these Mabs did not immunoprecipitate a protein from metabolically labeled melanoma cells, nor did they react by Western immunoblot analysis (data not shown). Furthermore, M21 human melanoma cells pretreated with mild trypsin actually reacted better with these Mabs yet failed to react with a Mab directed to the protein antigen recognized by Mab 9.2.27 (data not shown).

Adhesive Substrates

Fibronectin and vitronectin were isolated from human serum as previously described (16a). Laminin and collagen IV were purchased from Bethesda Research Laboratories.

Ganglioside Standards

GD3 was generously provided by Dr. S. I. Hakomori (Fred Hutchinson Cancer Research Center, Seattle, WA). GD2 was a gift from Dr. R. K. Yu (Yale University, New Haven, CT). GM1, GD1a, and GT1 were purchased from Supelco, Inc. (Bellefonte, NY).

Cell Lines

The human M21 melanoma and UCLA-P3 lung adenocarcinoma cell lines were kindly provided by Dr. D. L. Morton (UCLA). The 983 B human melanoma cell line was provided by Dr. H. Koprowski (Wistar Institute, Philadelphia, PA), and the SK-NAS human neuroblastoma cell line was the gift of Dr. L. Helson (Sloan-Kettering Memorial Hospital, NY). The 983 B cells were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum. The M21 and SK-NAS cells were propagated in RPMI 1640 media, supplemented with 10% fetal calf serum. All cells were grown in tissue culture flasks (Corning Medical and Scientific, Medfield, MA) at 37°C in 7.5% CO₂/92.5% air and were shown to be free of mycoplasma by repeated testing during these studies.

Adhesion Assay

Cells were grown as monolayers in tissue culture flasks, labeled in leucine-free growth media, supplemented with [³H]leucine (50 μCi/ml) at 37°C for 48 h.

The cells were detached with an EDTA-containing buffer (11) as previously described. The cells were washed with growth medium containing 1% fetal calf serum and 10⁶ cells in a volume of 250 μl were incubated for 1 h at 4°C with the appropriate Mab at a concentration of 5 μg/ml. Cells were pelleted, washed twice to remove excess antibody, resuspended at 2 × 10⁵ cells/ml in growth medium containing 1% fetal calf serum, and seeded at 5 × 10³ cells/50 μl into individual 96-well polystyrene microtiter plates (Flow Laboratories, McLean, VA) that had been coated with adhesive substrates as previously described (16a, 36). All substrates were allowed to interact with microtiter wells overnight at room temperature in phosphate-buffered saline (PBS) at 5 μg/ml. Before addition of cells, wells were coated with PBS containing 1% BSA for 1 h. After plating, cells were allowed to adhere at 37°C in a humidified incubator containing 7.5% CO₂. At specified intervals, the plates were inverted to facilitate the removal of unbound cells, and all wells were washed with 150 μl PBS, pH 7.2. The remaining attached cells were removed by the addition of 100 μl/well of a solution of 0.5 mg/ml trypsin containing 0.2 mg/ml EDTA (Gibco, Grand Island, NY), followed by a 15-min incubation at 37°C and the harvesting of the cells onto glass fiber filters (Whatman Chemical Separation Inc., Clifton, NJ) using a mash harvester (Valcor Engineering Corp., Springfield, NJ). This was followed by the rinsing of all wells through the mash harvester with 150 μl PBS, followed by a wash with 5% trichloroacetic acid and finally methanol. The fiber filter disks were placed into vials containing 5 ml liquid scintillation cocktail (Liquiscint, National Diagnostics, Somerville, NJ), and the counts per minute were determined in a Liquid scintillation counter (Tracor Analytic, Inc., Elk Grove Village, IL). Wells were harvested containing cells that were not allowed to adhere in order to establish the total counts per minute to calculate the percentage of the total bound cell population. The labeling procedure described above routinely resulted in ~50 cpm/cell. Random fields were counted visually, and the results were correlated directly with those obtained by using the harvesting procedure described above (data not shown).

Fluorescence-activated Cell Sorting (FACS) Analysis

Quantitation of cell surface antigen expression was achieved by FACS analysis as previously described (49), using nonfixed viable cells stained at 4°C. The hybridoma fusion partner, i.e., mouse myeloma protein P3x63, served as the negative control for primary antibody binding. An additional negative control included monoclonal antibody KS1/4 produced in our laboratory and shown to react with a 40-kD glycoprotein on the surface of human lung adenocarcinoma cells. This antibody does not react with either human melanoma or neuroblastoma cells (48).

Enzyme-linked Immunosorbent Assay (ELISA)

The relative expression of each antigen was also measured with an ELISA assay on dried cell targets as previously described (16).

Periodate Oxidation

Cells were treated with sodium meta periodate in serum-free growth media at concentrations ranging from 0.5–4 mM for 30 min at 4°C in the dark. The cells were immediately washed twice with ice-cold growth medium and used in the adhesion assay or tested for antigen reactivity by ELISA.

Results

Monoclonal Anti-ganglioside Antibodies Inhibit Cell-Substrate Interactions

To determine the relative effects of Mabs directed to ganglioside antigens on the cell attachment process, human melanoma or neuroblastoma cells were allowed to attach to various adhesive substrates, immobilized on microtiter wells after being preincubated with one of several monoclonal antibodies. As shown in Fig. 1 and Table I, Mabs directed to either GD3 or GD2 inhibited attachment of human melanoma cells 983 B to fibronectin, laminin, vitronectin, and collagen type IV, whereas no such effect was observed when these same cells were tested in the absence of Mab pretreatment or after preincubation with Mab 9.2.27 directed to a 250-kD melanoma-associated chondroitin sulfate core glycoprotein (16). In several experiments we observed essentially identical cell attachment when 983B human melanoma cells were pre-

treated with Mab 9.2.27, W6/32 (anti-HLA), KS1/4 (non-binding antibody), or PBS; thus, any one of these reagents could represent control binding.

As expected, an additive inhibition of cell attachment was observed in each case when anti-GD2 and anti-GD3 were used in combination. This finding suggests that both gangliosides are involved in the attachment process of these cells and may interact independently, although possibly by a similar mechanism. The M21 melanoma cells were also examined

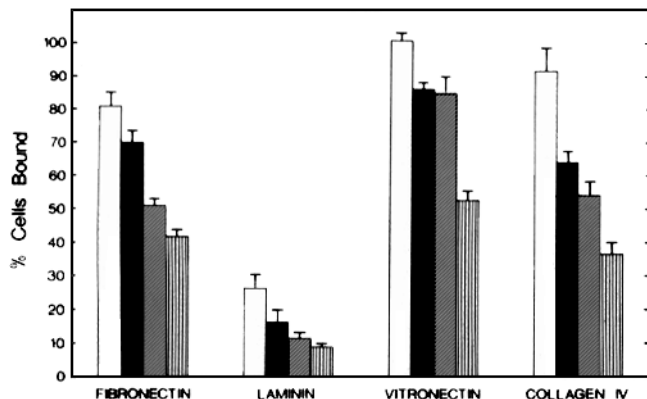


Figure 1. The effects of monoclonal anti-ganglioside antibodies on binding of human melanoma cells to various substrates. Metabolically labeled 983B human melanoma cells (5×10^3) were allowed to attach to microtiter wells which had been coated overnight with $5 \mu\text{g/ml}$ in PBS of either fibronectin, laminin, vitronectin, or collagen IV, as described in Materials and Methods. Before addition to coated wells, the cells were allowed to react with either Mab 9.2.27 (anti-250-kD chondroitin sulfate core glycoprotein, □), 11C64 (anti-GD3, ■), 3F8 (anti GD2, ▨), or the combination of Mabs 11C64 and 3F8 (▤) for 1 h at 4°C and washed free of excess antibody. The data are expressed as the percentage of the total cell population attached after 30 min at 37°C . Each bar represents the mean \pm SD of five replicates. In control wells not treated with substrate we never observed more than 5% of the cells attached over the course of the assay.

for their ability to attach specifically to the heptapeptide glycyl-L-arginyl-glycyl-L-aspartyl-L-seryl-L-prolyl-L-cysteine (GRGDSPC), which represents the cell attachment site of the fibronectin molecule (32, 33, 16a, 36). As shown in Fig. 2 both monoclonal anti-ganglioside antibodies inhibit this interaction. In control experiments in which these cells were treated with anti-HLA or no antibody at all, 54% of the cells were bound in 30 min. Prior treatment with either anti-GD2 or anti-GD3 reduced this by $\sim 50\%$. The control peptide GRGESPC (33), which was synthesized by substituting a glutamic acid (E) for an aspartic acid residue (D), did not mediate significant cell attachment, never promoting more than 5% cell attachment (data not shown). As with the other substrates tested, Mabs directed to GD2 and GD3, when used together, produced an additive inhibition of cell attachment of M21 cells (data not shown).

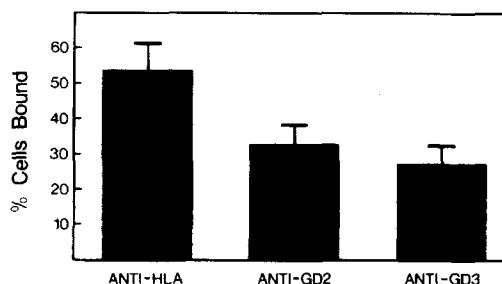


Figure 2. Monoclonal anti-ganglioside antibodies inhibit binding of human melanoma cells to the synthetic peptide GRGDSPC. Metabolically labeled M21 human melanoma cells (5×10^3) were allowed to attach to microtiter wells which had been precoated with the synthetic peptide GRGDSPC as described (33). Before addition to coated wells, the cells were allowed to react with Mabs W6/32 (anti-HLA), MB3.6 (anti-GD3), or 126 (anti-GD2) for 1 h at 4°C and washed free of excess antibody. The data are expressed as the percentage of the total cell population attached after 30 min at 37°C . Each bar represents the mean \pm SD of five replicates.

Table I. Reactivity of Mabs and Their Effect on Adhesion of Human Melanoma and Neuroblastoma Cells to Various Substrates*

MAb (isotype)	Antigen	M21		FACs analysis [‡] 983B		SK-NAS		ELISA reactivity (OD492)			Inhibition of cell-substratum interaction [§]		
		MIF	%+	MIF	%+	MIF	%+	M21	983B	SK-NAS	M21	983B	SK-NAS
MB3.6 (IgG ₃)	Carbohydrate on GD ₃ (8)	69	64	65	88	0	0	0.929	0.492	0.039	+	+	-
11C64 (IgG ₃)	Carbohydrate on GD ₃ (8)	75	70	68	86	0	0	0.585	0.631	0.025	+	+	-
126 (IgM)	Carbohydrate on GD ₂ (8)	186	100	137	99	118	86	1.112	0.664	1.61	+	+	+
3F8 (IgG ₃)	Carbohydrate on GD ₂ (39)	127	100	110	86	87	75	1.933	NT	0.688	+	+	+
9.2.27 (IgG _{2a})	Chondroitin sulfate proteoglycan (16)	137	99	61	89	0	0	1.150	0.816	0	-	-	-
KS1/9 (IgM)	Carbohydrate neutral glycolipid (48)	42	5	39	4	0	0	0.209	NT	0	-	-	-
W6/32 (IgG _{2a})	Histocompatibility class I protein (29)	56	99	92	96	60	81	1.616	0.625	0.943	-	-	-
390 (IgG _{2a})	26-kD Thy-1 protein (41)	0	0	0	0	50	86	0	0	0.608	-	-	-
KS1/4 (IgG _{2a})	Adenocarcinoma-associated 40-kD glycoprotein (48)	0	0	0	0	0	0	0	0	0	-	-	-
P3X62 (IgG ₁)	None	0	0	0	0	0	0	0	0	0	-	-	-

* Adhesion of cells to various immobilized extracellular matrix proteins was performed as described in Materials and Methods.

[‡] FACs analysis was performed on nonfixed viable cells using indirect immunofluorescence as described in Materials and Methods. The antigenic expressions was quantitated in terms of mean intensity of fluorescence and percentage of cells in the population demonstrating positive staining above background, which was determined using P3x63 mouse myeloma protein as nonbinding control.

[§] A+ indicates the ability of each Mab to reduce cell attachment to various substrates by at least 2 SD of the mean control binding as described in Materials and Methods.

M21 and 983B human melanoma cells were also tested for their ability to attach to tissue culture plastic. In this case, we observed little or no inhibition with anti-ganglioside Mabs within the first 30 min of cell attachment (data not shown). However, optimal binding of these cells requires ~2 h at 37°C, when cell-derived and serum-derived cell adhesive proteins would probably play a role.

To examine the kinetics of inhibition of cell attachment by anti-ganglioside Mabs, M21 melanoma cells were allowed to attach to either fibronectin (Fig. 3, top) or laminin (Fig. 3, bottom) for various times after being pretreated with either anti-GD3, anti-GD2, or control Mab, i.e., anti-HLA (W6/32). It is apparent that both monoclonal anti-ganglioside antibodies significantly inhibit the attachment of these cells to both substrates. Furthermore, this inhibition of cell attachment obtained with each antibody is detectable within the first 5 min of binding time, suggesting that GD2 and GD3 may be involved in the initial events of M21 cell attachment. Specifically, anti-GD3 inhibited M21 cell binding to both substrates by ~50% whereas anti-GD2 inhibited 90% of cell binding to fibronectin and 60% to laminin after 15 min. To determine whether ganglioside involvement in cell attachment was restricted to human melanoma cells, a human neuroblastoma cell line (SK-NAS) was investigated that expressed high levels of GD2 but contained no detectable GD3. As shown in Table II, Mab 126 directed to GD2 significantly inhibited these cells' capacity to attach to fibronectin or

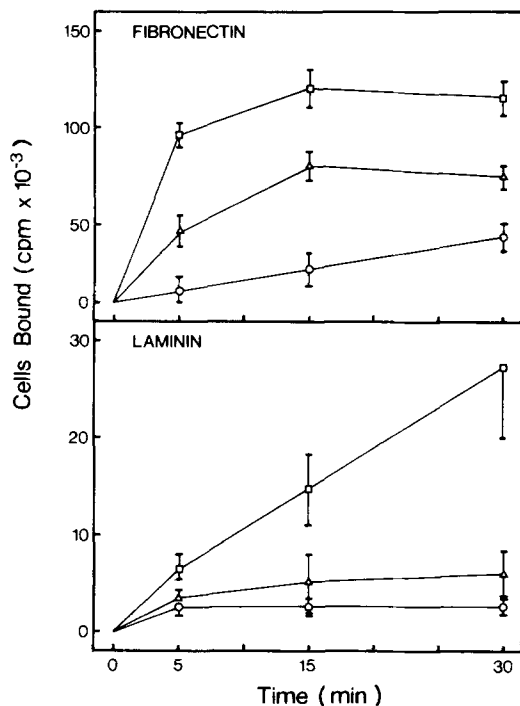


Figure 3. Effects of monoclonal antiganglioside antibodies on the kinetics of M21 human melanoma cells attachment to fibronectin or laminin. Metabolically labeled M21 human melanoma cells (5×10^3) were allowed to attach for various times to microtiter wells coated with either fibronectin or laminin. Before addition to substrate-coated wells, the cells were allowed to react with Mabs W6/32 (anti-HLA, \square), 11C64 (anti-GD3, Δ), or 3F8 (anti-GD2, \circ) for 1 h at 4°C and washed free of excess antibody. The data are expressed as the total number of cells (counts per minute bound) at the designated times. Each point represents the mean \pm SD of five replicates.

Table II. Effects of Anti-GD₂ Mab on the Attachment of SK-NAS Human Neuroblastoma Cells to Fibronectin and Laminin

Mab	Cell binding* (% of control)	
	Fibronectin	Laminin
KS1/4	100	100
390	88	—
W6/32	97	100
126	47	52

* SK-NAS cells were preincubated with various Mabs and allowed to attach to substrate-coated microtiter wells for 10 min as described in Materials and Methods. Cell attachment is expressed as the percentage of control in which cells were preincubated with Mab SK1/4, which does not bind to these cells.

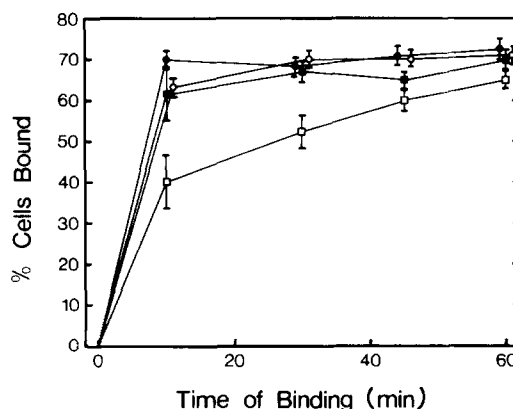


Figure 4. Effects of anti-GD₂ Mab on the kinetics of SK-NAS human neuroblastoma cell attachment to fibronectin. Metabolically labeled SK-NAS human neuroblastoma cells were allowed to attach for various times to microtiter wells which had been coated with fibronectin. Before addition to substrate-coated wells, the cells were allowed to react with Mabs KS1/4 (nonbinding control, \circ), W6/32 (anti-HLA, \blacksquare), 390 (anti-Thy1, \bullet), or 126 (anti-GD₂, \square) for 1 h at 4°C and washed free of excess antibody. The data are expressed as the percent of cells attached at each designated time. Each point represents the mean \pm SD of five replicates.

laminin as compared with two other antibodies that bind to the neuroblastoma cell surface (Mabs 390 and W6/32) or to a Mab that does not bind to these cells (Mab KS1/4). Moreover, Mab 3F8 (IgG3) also directed to GD2 showed a similar capacity to inhibit SK-NAS cell attachment to fibronectin or laminin (data not shown). As expected, the anti-GD3 antibodies had no effect on the attachment of this cell line (Table I) since neuroblastoma cells do not express GD3. In two independent experiments, Mab MB3.6 was shown to inhibit <5–10% of SK-NAS cell attachment, which readily compares with either binding or nonbinding control antibodies as documented in Table II. A kinetic analysis of the inhibition induced by anti-GD₂ antibody indicated that the binding of SK-NAS cells to either fibronectin (Fig. 4) or laminin (data not shown) can also be inhibited by 40–50% within 10 min of cell attachment. These data suggest that GD₂ expressed on neuroblastoma cells probably plays a similar role in cell-substratum interactions, as do both GD₃ and GD₂ that are expressed on the melanoma cells examined in this study. At later times (30–40 min) of cell attachment, the inhibition of neuroblastoma cell attachment decreased somewhat, ranging from 20 to 30%.

Addition of Anti-GD3 to M21 Human Melanoma Cells Preattached to Immobilized Fibronectin Causes Cell Rounding and Detachment

To determine whether cells preattached to fibronectin could be displaced by monoclonal anti-GD3 antibody, M21 melanoma cells were allowed to bind to fibronectin for 1 h, after which they were overlaid with either growth medium alone, or with growth medium containing purified Mabs MB3.6 (anti-GD3) or 9.2.27 (anti-250-kD glycoprotein). As shown in Fig. 5, the addition of MB3.6 to these cells caused a significant cell rounding and detachment within 4 h. The observed effect increased proportionally with increasing amounts of antibody added, with maximum effects being observed when melanoma cells were incubated with 0.5–1.0 mg/ml antibody. Cell viability is not affected by this treatment, since trypan blue exclusion demonstrated >95% viability among the MB3.6-treated cells even after 24 h. Identical concentrations of Mab 9.2.27 used as a control produced no effect on either cell morphology or adherence to fibronectin at any concentration tested (Fig. 5, *B* and *C*). In a similar experiment Mab 126 (anti-GD2), which reacts with 100% of the M21 cells, could cause >95% cell rounding, whereas, as expected, Mab W6/32 (anti-HLA) had virtually no effect (data not shown).

Relative Expression of Ganglioside Antigens on the Surface of Human Melanoma and Neuroblastoma Cells

The relative surface expression of various antigens defined by the Mabs used in this study is presented in Table I. It is apparent that the two anti-GD3 Mabs, MB3.6 and 11C64, that inhibit melanoma cell attachment, bind to both melanoma cell lines in an equivalent manner to that seen with Mabs W6/32 and 9.2.27 that have no effect on the cell attachment process. This can be observed by either ELISA reactivity on whole cells or by applying FACS analysis to determine the relative antigen density by measuring mean intensity of fluorescence and the percentage of cells in the population that express the antigen. The specificity of the anti-GD3 and GD2 antibodies to inhibit melanoma or neuroblastoma cell attachment to extracellular matrix proteins is further underlined by the observation that a variety of Mabs directed to either protein or carbohydrate determinants on a number of other cell surface molecules did not cause any significant inhibition of cell attachment (Table I). In addition, similar results were obtained with both anti-GD2 Mabs, 126 (IgM) and 3F8 (IgG3), demonstrating that the inhibition of cell attachment observed is independent of antibody isotype. Further evidence for the specificity of this reaction comes

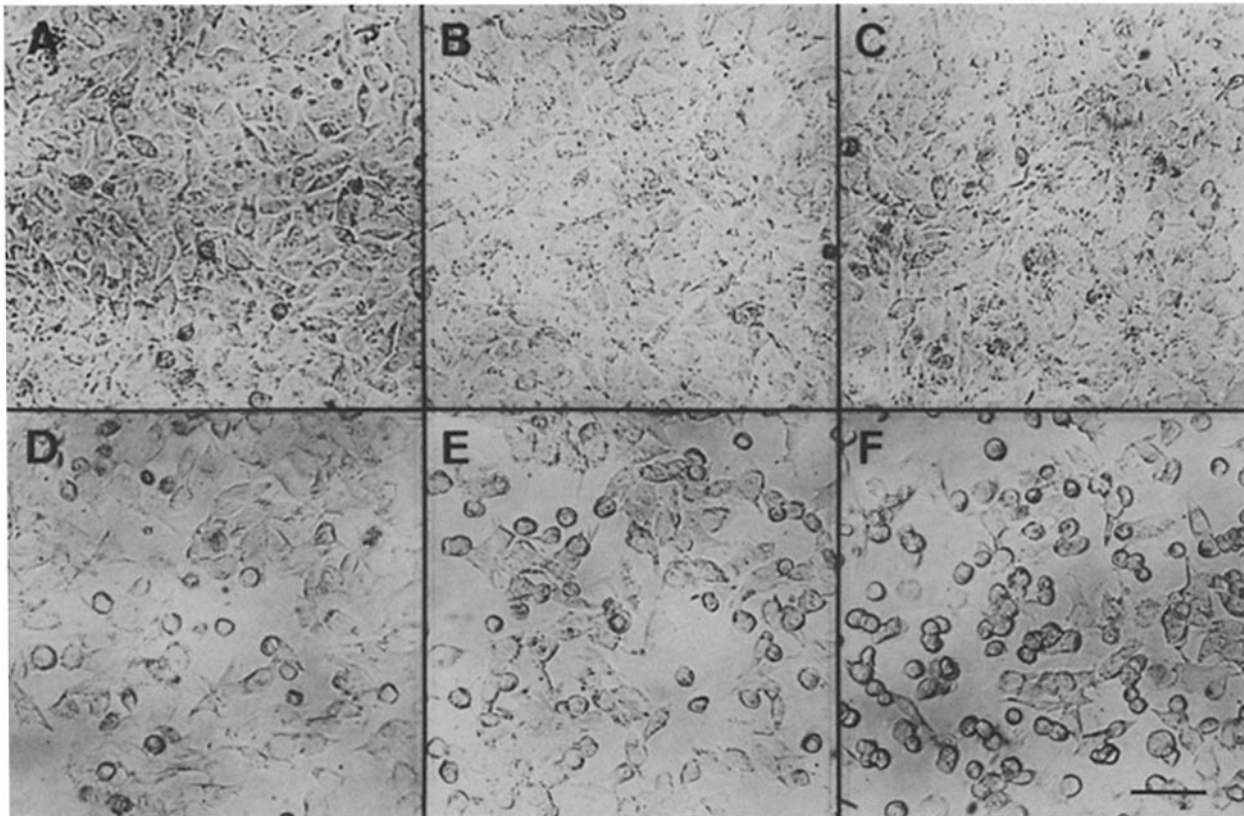


Figure 5. Effects of anti-GD3 Mab on M21 human melanoma cells preattached to a fibronectin substrate. M21 cells were allowed to attach to fibronectin (5 μ g/ml)-coated plastic for 1 h at 37°C. These attached cells were then overlaid either with growth media alone (*A*), growth media containing purified Mab 9.2.27 (anti-250-kD chondroitin sulfate proteoglycan core protein) at either 0.5 or 1.0 mg/ml (*B* and *C*), or purified Mab MB3.6 (anti-GD3) at 0.1, 0.5, or 1.0 mg/ml (*D–F*, respectively). After incubation was carried out at 37°C for 4 h, the cells were photographed using a Leitz inverted microscope at 23 \times . Bar, 100 μ m.

from the finding that the SK-NAS neuroblastoma cell line that expresses GD2 but not GD3 can only be inhibited in its ability to attach to various substrates by anti-GD2 Mabs. This cell line, similar to the melanoma cell lines studied, expresses other cell surface antigens that apparently have no involvement in the cell attachment process.

Periodate Oxidation of Cell Surface Gangliosides Inhibits Attachment of Human Melanoma and Neuroblastoma Cells to Fibronectin and Laminin

To assess the structural components of GD2 and GD3 involved in cell attachment, human melanoma cells 983B and M21, SK-NAS neuroblastoma, or UCLA-P3 lung adenocarcinoma cell lines were treated with growth medium containing from 0.5 to 4 mM sodium-meta-periodate. This reaction on ice and in the dark for 30 min preferentially oxidizes the exocyclic arm of terminal, unsubstituted sialic acids (47). Cells treated in this manner were then tested for their ability to adhere to a fibronectin and/or laminin substrate to determine whether surface exposed terminal sialic acid residues are involved in their cell attachment processes. As shown in Table III, all cell lines treated in this manner show a dose-dependent decrease in cell attachment. These same conditions of periodate oxidation also destroyed the epitope on the GD3 and GD2 antigens recognized by Mab 11C64 and 126, respectively (Fig. 6). In contrast, Mabs 9.2.27 and W6/32, which react with protein epitopes on these same cells, were unaffected at all concentrations of periodate used. In this regard, we have previously shown that periodate oxidation of melanoma gangliosides under these same conditions results in a specific attack on the exocyclic arm of sialic acid, causing a loss of binding of Mabs directed to either GD3 or GD2 (19). However, treatment of cells with periodate in this manner affected neither cell viability, as measured by trypan blue exclusion, nor protein synthesis, as determined by [³H]leucine incorporation into total cell protein after 24 h of labeling (data not shown). These data suggest that the cells are not compromised metabolically by this type of mild periodate oxidation of the cell surface.

The UCLA-P3 lung adenocarcinoma cell line was used as a control since it expresses GM3 as its major ganglioside yet contains neither GD2 nor GD3 (data not shown). As shown in Table III, periodate oxidation inhibits UCLA-P3 cells from binding to fibronectin, suggesting that in this case, the cell attachment process may involve oligosaccharides other than GD2 or GD3.

Table III. Effect of Periodate on Cell Attachment*

Periodate mM	M21		938B		SK-NAS	UCLA-P3
	Fibronectin	Laminin	Fibronectin	Laminin	Fibronectin	Fibronectin
0	77 ± 3 [‡]	35 ± 5	84 ± 8	27 ± 3	67 ± 3	15 ± 3
0.5	71 ± 4	23 ± 2	74 ± 4	25 ± 2	80 ± 3	11 ± 3
1.0	68 ± 4	18 ± 1	72 ± 7	24 ± 2	62 ± 2	9 ± 3
2.0	62 ± 2	13 ± 1	62 ± 4	20 ± 3	58 ± 2	5 ± 2
4.0	49 ± 1	12 ± 3	57 ± 6	12 ± 3	26 ± 1	4 ± 1

* Adhesion of periodate treated cells to immobilized fibronectin or laminin was performed as described in Materials and Methods.

[‡] Percentage of cells attached in 15 min (mean ± SE of four replicates).

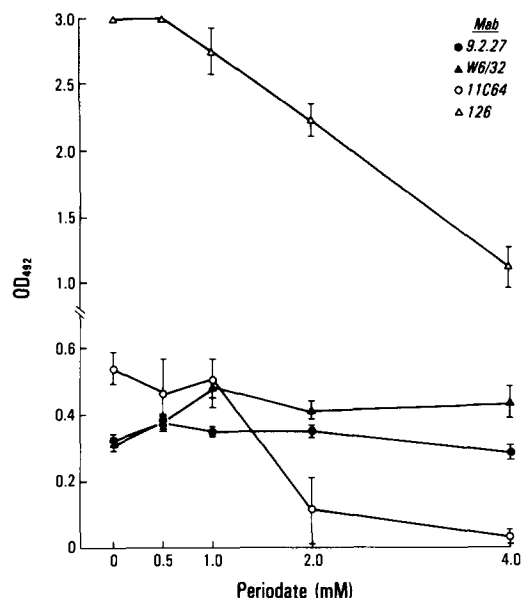


Figure 6. Periodate oxidation on cells of M21 human melanoma cells depletes their expression of GD2 and GD3 gangliosides. M21 Human melanoma cells were treated with growth media containing 0.5–4.0 mM sodium-meta-periodate on ice and in the dark for 30 min. After they were washed, the cells were dried onto microtiter wells for antigen detection by ELISA as described in Materials and Methods. Mabs 9.2.27 (anti-250-kD chondroitin sulfate core glycoprotein), W6/32 (anti-HLA), 11C64 (anti-GD3), and 126 (anti-GD2) were used as primary antibodies. Each point represents the mean ± SE of four replicates.

Discussion

Specific Mabs directed to well-defined epitopes expressed on the surface of human melanoma and neuroblastoma cells were used as molecular probes to provide evidence that the carbohydrate moieties of the disialogangliosides GD3 and GD2 are involved in the attachment of these cells to a variety of substrate adhesive proteins. Furthermore, these studies demonstrate that Mabs directed to either GD2 or GD3 also inhibit melanoma cell attachment to the GRGDSPC peptide that is known to specify the cell attachment site of fibronectin (33). Moreover, after separating GD3 and GD2 on thin-layer chromatography plates, we could not observe any specific interaction with ¹²⁵I-labeled fibronectin or a 120-kD cell attachment domain-containing fragment (34) with either of these gangliosides; however Mabs directed to GD2 and GD3 reacted specifically with their respective antigens on these chromatograms (data not shown). It is also important to note that none of the Mabs directed to either GD2 or GD3 could induce 100% inhibition of cell attachment. This observation is consistent with the concept that gangliosides expressed on these cells do not independently promote cell binding and thus fail to act as specific receptors for a substrate. Taken together, these observations suggest that the role of these gangliosides in the cell attachment process of human melanoma cells may involve secondary events on the surface that result in the promotion of optimal cell-substratum interactions.

A kinetic analysis of the inhibition of neuroectoderm-derived tumor cell attachment to extracellular matrix protein by anti-GD2 and GD3 antibodies suggests that these disialo-

gangliosides may be involved in some of the initial events of this process since a significant inhibition of cell attachment was observed in the presence of these antibodies within the first 5–10 min of binding time. Mabs directed to gangliosides on these tumor cells failed to induce a significant redistribution of this antigen at 37°C up to 30 min, suggesting that the observed effects were not due to antibody-mediated capping on the surface of these cells (Cheresh, D. A., and J. Claypool, unpublished observations). However, one cannot rule out that gangliosides may play a role in cell attachment at later times as well since inhibition was still observed after 30 min. In this regard, it is not inconceivable that anti-ganglioside Mabs in some way induce an as yet undefined membrane perturbation resulting in an altered cell metabolism and reduced cell attachment. This hypothesis is supported by other reports indicating that anti-ganglioside antibodies can alter cell growth and metabolism that may explain in part how these antibodies inhibit cell attachment (21, 22). However, this contention does not explain our results since we observed no decrease in cell viability when M21 melanoma cells were allowed to bind as much as 1 mg/ml of purified Mab MB3.6 (anti-GD3) during a period of 24 h. Indeed, if the anti-ganglioside Mabs could perturb the cell membrane one would expect an increased inhibitory effect on cell attachment with binding time; however, this is not the case. The fact that Mabs of various isotypes directed to a number of other antigen epitopes on melanoma and neuroblastoma cells fail to inhibit cell attachment suggests that disialogangliosides GD2 and GD3 indeed play a role in the attachment processes of these cells to extracellular matrix proteins. This contention is strengthened and extended by the finding that the addition of purified MB3.6 (anti-GD3) to M21 melanoma cells that were allowed to preattach to immobilized fibronectin caused significant cell rounding and detachment from the substrate. In fact, after 4 h of antibody exposure most (~62%) of the cell population had lost its spread appearance. Since FACS analysis demonstrated that Mab MB3.6 reacted with 64% of the M21 melanoma cells, one would not expect inhibition of attachment to exceed this percentage. This effect was dependent on both antibody concentration and time and could not simply be attributed to loss of cell viability, which was found to be >95% by trypan blue exclusion. As expected, Mab 126 that reacts with 100% of the M21 cell population by FACS analysis (Table I) caused cell detachment and rounding of >95% of the cell in the population. In a control experiment, purified Mab 9.2.27 or W6/32 at identical concentrations failed to change cell morphology or cell attachment, suggesting that mere binding of an antibody to an epitope that is expressed on the melanoma cell surface is insufficient to mediate this effect.

We previously reported the presence of GD2 and GD3 in focal adhesion plaques of M21 cells and suggested that these molecules are involved in the attachment of such cells at the cell-substrate interphase (8). Reports by others also indicated that cell surface-associated gangliosides are involved in cell adhesions processes (3, 20, 27, 31, 51). Thus, Perkins et al. demonstrated that exogenously added gangliosides could inhibit cell binding to fibronectin (31). These investigators proposed that the binding affinity of fibronectin immobilized in the extracellular matrix may be increased by gangliosides at the cell surface by some form of cooperative interaction.

Their results also support the findings of Rauvala et al. (38) that indicate that exogenously added gangliosides could inhibit cell attachment to immobilized lectin. Both of these findings are consistent with our results and strongly implicate gangliosides as playing a general role in cell attachment.

Kleinman et al. (20) and Yamada et al. (51) demonstrated that di- and trisialogangliosides added to the culture medium effectively inhibit cell binding to fibronectin. Furthermore, Kleinman et al. showed that prior treatment of these gangliosides with periodate eliminated their ability to inhibit cell attachment (20). These observations are in accord with our results, which clearly demonstrate their similar conditions of periodate oxidation could not only destroy the antigenic epitope on the cell surface recognized by the anti-ganglioside Mabs but also cause a significant reduction in cell attachment, indicating that the antibody binding site on these gangliosides involves a defined structure that can be specifically oxidized by periodate. This contention is strengthened further by our previous observation that whenever GD3 contains a relatively rare 9-O-acetyl ester on the exocyclic arm of its terminal sialic acid residue, periodate oxidation is prevented, thus protecting this antigen epitope against periodate-induced oxidation (9). These data suggest that an intact, unsubstituted terminal sialic acid residue is critical for ganglioside involvement in cell attachment. The fact that attachment of UCLA-P3 cells, which do not contain GD2 or GD3, can be inhibited by periodate oxidation suggests that sialic acid present on other gangliosides may be involved, i.e., possibly GM3 which represents the major ganglioside expressed by these cells (data not shown). The role of sialic acid in melanoma and neuroblastoma cell attachment was further established using neuraminidase. Thus, pretreatment of 983B and M21 melanoma cells or SK-NAS neuroblastoma cells with *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, MO) at 100 mU/ml for 1 h at 37°C resulted in >80% loss of GD2 and GD3 antigen expression and an ~40% reduction in cell attachment to immobilized fibronectin (Cheresh, D. A., and K. Mujoo, unpublished data). Removal of sialic acid in this manner did not decrease cell viability or protein synthesis and failed to alter the protein antigens recognized by Mab 9.2.27 or W6/32.

One possible functional role suggested for the terminal sialic acid moiety on di- and trisialogangliosides has been that of Ca⁺⁺ binding (1, 14), an event known to require not only the carboxyl group on the 1 position of sialic acid but also an intact exocyclic arm (7, 8, and 9 positions) (18). In fact, recent studies have demonstrated that complexes between polysialogangliosides and Ca⁺⁺ induce a specific protein phosphorylation pattern within a membrane fraction isolated from rat brain (14). A possible involvement of Ca⁺⁺ with GD2 and GD3 is suggested by our preliminary observations that ⁴⁵Ca⁺⁺ interacts with either of these two purified gangliosides immobilized on microtiter wells. The specificity of this interaction was indicated by competition with excess cold Ca⁺⁺.

In view of recent reports indicating the presence of specific cell surface glycoprotein receptors for fibronectin (5, 13, 36) and laminin (37), it is important to determine whether gangliosides that are somehow involved in cell adhesion processes are either dependent or independent of a protein receptor(s). Recent reports that demonstrated the presence of a trypsin-resistant glycoprotein receptor for fibronectin (13, 45, 46)

suggest that this receptor is only resistant to proteolytic cleavage in the presence of Ca⁺⁺ since chelation of this cation sensitized it to trypsin degradation, causing subsequent loss of cell attachment (7, 28). Thus, it is possible that the electrostatic environment created by a ganglioside-Ca⁺⁺ complex may indeed stabilize the interaction of a given cell surface glycoprotein receptor with a given adhesive protein substrate. This hypothesis is strengthened by a number of reports indicating that gangliosides can cooperatively interact with glycoprotein receptors at the cell surface (2, 4). In this regard, it has been shown that the receptor for thyroid-stimulating hormone may involve a glycoprotein/ganglioside complex (2). Also, in a recent report, Okada et al. demonstrated a close functional association between gangliotriaosylceramide (GM3) and the transferrin receptor on murine lymphoma cells which, in turn, may regulate the internalization of transferrin (26). Thus, depending on its particular oligosaccharide moiety, a ganglioside may serve to induce a preferred orientation or create an electrostatic environment suitable for optimal receptor-ligand interaction on the cell surface.

The fact that GD2 and GD3 represent the major gangliosides on the surface of both human melanoma and neuroblastoma cells and apparently play a role in their attachment to extracellular matrix proteins suggests that structurally similar gangliosides on other cell types may have similar functional capacities. In fact, it was recently demonstrated that GM3, a major ganglioside on the surface of baby hamster kidney cells, could be localized in the detergent-insoluble substrate attachment matrix of these cells. Furthermore, when GM3 was added to the culture media of these cells, attachment and spreading were inhibited (27).

The model system described in the present report was used for two reasons; first, we had well characterized Mabs directed to GD2 and GD3, and second, these are the major gangliosides associated with these particular cell types. Whenever additional Mabs become available, it will be important to determine whether gangliosides on other cell types play a similar role in cell-substrate attachment as that described for GD3 and GD2 on human melanoma and neuroblastoma cells to ascertain whether this is a common biological event.

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