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Altered Cell Surface Organization of Gangliosides and Sialylglycoproteins of Mouse Metastatic Melanoma Variant Lines Selected *in Vivo* for Enhanced Lung Implantation[1]

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ABSTRACT

Qualitative and quantitative analyses of cell surface sialylglycoproteins and glycosphingolipids of *in vivo* selected mouse melanoma variant lines that show either a high (F10) or low (F1) degree of lung implantation have been made in culture and in *in vivo*-grown tumors. The following observations have been made concerning the cell surface characteristics of the two cell lines: (*a*) although the total, protein-bound, and lipid-bound sialic acid is significantly decreased (20 to 35%), the cell surface-exposed sialylglycoproteins and gangliosides showed a moderate increase in F10 cells as compared to F1 cells; (*b*) surface glycoproteins of melanoma cells were studied with different labeling techniques followed by polyacrylamide gel electrophoresis and fluorography. The majority of surface glycoproteins were shown to be sialylfucosylgalactosyl and/or galactosaminyl glycoproteins; (*c*) the high-lung-implanting F10 cells reveal certain sialylglycoproteins with molecular weights of 66,000 (in culture) and 97,000, 84,000, 74,000, and 66,000 (*in vivo*), as detected by the galactose oxidase method, that are absent or weakly labeled in F1 cells; (*d*) metabolic labeling with glucosamine followed by neuraminidase hydrolysis reveals that F10 cells contain 80% more neuraminidase-accessible total cell surface sialic acid, the majority of which was contributed by AcNeu-α-(2→3)-Gal-β-(1→4)-GlcCer as compared to F1 cells; (*e*) F10 cells showed an enrichment of the quantity of ceramide dihexoside and a 5- to 6-fold higher cell surface exposure compared to F1 cells; (*b*) dramatic differences in ganglioside profile were seen between the *in vivo* and *in vitro* growth of F1 and F10 cells. The relationship of altered cell surface architecture of sialic acid-containing components to lung-specific implantation in experimental metastases is discussed.

INTRODUCTION

The changes of chemical composition and synthesis of GSL[3] and their organization have been studied by a number of investigators during the past few years. Simplification of the GSL profile, possibly due to blocked synthesis of complex gangliosides, neutral GSL, and fucolipids, has been the major change noticed in various types of transformed cells *in vitro* and *in vivo* (see Refs. 8, 29, and 30 for review). Some, but not all, GSL changes noticed in certain thermosensitive virus-transformed cells are suppressed at a nonpermissive temperature (5, 24), suggesting that GSL changes are related to loss of growth control seen in transformed cells.

Many transformed cells of hamster, mouse, and chicken fibroblasts are characterized by a considerable loss of large external transformation-sensitive protein compared to their untransformed counterpart (20, 32, 52, 61). Virus-transformed hamster and 3T3 fibroblasts show an increase of surface exposed sialylgalactosyl and/or galactosaminyl glycoproteins (20). The membranes of a wide variety of transformed cells showed an increase in high-molecular-weight glycopeptides over control cells (10, 53–55), which could be accounted for by increased levels of sialyltransferase in the malignant cell (53, 55).

The significance of cell surface GSL and glycoprotein changes seen in malignant transformation, as well as of the relationship to metastatic behavior of tumor cells, has not been studied in the past. Recently, Fidler (14, 17) has developed a model system for studying low- and high-lung-implanting properties using B16 mouse melanoma. With this model the cell surface glycoproteins of low- and high-lung-implanting F1 and F10 melanoma variant lines have been studied (57). A comparison of pronase-digestible sialylfucosylglycopeptides of these variant lines, with the use of chromatography on Sephadex G-50, revealed no significant difference in the molecular weights of the glycopeptides (57). However, a high electrophoretic mobility and an increase in NANase-accessible sialic acid was seen on the surface of the high-lung-implanting F10 melanoma line (6) as compared to the low-lung-implanting F1 line. In light of these contradictory findings, a further examination of the surface glycoproteins and GSL of low (F1)- and high (F10)-lung-implanting metastatic melanoma lines was carried out with various sensitive surface labeling and surface hydrolysis procedures. With these techniques marked differences in the architecture of surface glycoproteins and GSL and composition of GSL have been detected between the metastatic variant lines.

MATERIALS AND METHODS

Cell Culture. Two variant lines of B16 melanoma were used: (*a*) F1 cloned as a first passage from i.v.-introduced

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[3] The abbreviations used are: GSL, glycosphingolipid(s); NANase, neuraminidase; MEM, minimal essential medium; FCS, fetal calf serum; PBS, isotonic phosphate-buffered saline (0.8% NaCl in 0.01 M phosphate buffer containing 0.49 M $MgCl_2$ and 0.9 M $CaCl_2$); G_{M3}, AcNeu-α-(2→3)-Gal-β-(1→4)-GlcCer; CD, ceramide dihexoside; G_{D1a}, AcNeu-α-(2→3)-Gal-β-(1→3)-GalNAc-β-(1→4) [AcNeu-α-(2→3)]-Gal-β-(1→4)-GlcCer; G_{M2}, GalNAc-β-(1→4)-[AcNeu-α-(2→3)]-Gal-β-(1→4)-GlcCer; G_{M1}, monosialo+ganglioside; GalNAc, *N*-acetylgalactosamine; NAN, *N*-acetylneuraminic acid.

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lung tumor; and (*b*) F10 cloned by repeated i.v. passage of tumor cells from culture. Both these variant lines were originally isolated by Fidler (14, 17) and obtained from Dr. G. Nicolson. Fidler (14) showed that, by successive i.v. administration of B16 melanoma cells in syngeneic animals and removal of lung tumor foci followed by culture *in vitro*, a 3- to 4-fold increase in lung implantation (17, 18) and a moderate increase (66%) in spontaneous postsurgical metastasis (17) were seen in F10 cells as compared to F1 cells. Dr. Nicolson, who provided the initial stock cultures for these studies, tested the lung implantation characteristics and found that low and high lung implantation characteristics by "i.v. assay" (quantitation of lung foci formation followed by i.v. administration of tumor cells) reproduced (19) those described by Fidler (17, 18).

The cultures were maintained in Falcon tissue culture dishes in Eagle's MEM, supplemented with 10% FCS (Microbiological Associates, Bethesda, Md.), nonessential amino acids, penicillin:streptomycin, and L-glutamine (Grand Island Biological Co., Grand Island, N. Y.) as described by Fidler (14, 17). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°. Cells were harvested for subcultures with the use of 2 mM EDTA: PBS. The high and low lung implantation and metastatic characteristics of F1 and F10 cells have been reported to be stable during long-term culture *in vitro* (14).

Animals and Propagation of *In Vivo* Tumors. Male C57BL/6J mice, 8 to 10 weeks old, purchased from The Jackson Laboratory, Bar Harbor, Maine, were utilized throughout these studies. Tumors were produced s.c. by the injection of 2×10^5 viable cells in a volume of 0.2 ml serum-free medium under the loose skin of the mid-back region of the animals. Tumors were produced i.p. by injecting 2×10^5 viable cells i.p. Both i.p. and s.c. tumors were removed when the animals were sacrificed 12 to 15 days later.

Preparation of Tumor Cells for Assays. The tumor tissue removed from animals was placed in serum-free MEM, cut into small pieces with scissors, placed over an 80 mesh stainless steel sieve, and teased with a Teflon policeman. The tumor cell suspension was centrifuged at $500 \times g$ and resuspended in 20 ml of a RBC-lysing solution (40) (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA, and 10% FCS) and kept on ice for 5 min. Then the suspension was centrifuged to obtain a RBC-free tumor cell suspension. Approximately 40 to 65% trypan blue-excluding tumor cells were obtained by this method. For lipid work the RBC-free tumor cell suspension was directly used. When surface labeling of short-term cultures of *in vivo* tumors was performed, this procedure was carried out under sterile conditions. For such surface labeling experiments, approximately 1×10^6 trypan blue-excluding tumor cells were plated in 15-cm tissue culture dishes in MEM containing 1% FCS and incubated overnight. Floating dead cells were rinsed off the plates the following day, leaving macrophages and viable tumor cells attached to the dishes. Viable tumor cells were then freed of macrophages by removing tumor cells from culture dishes with 2 mM EDTA:PBS for 5 to 7 min. The viability of the tumor cells obtained ranged between 90 and 95%. The tumor cells were then used for surface labeling experiments as described in the next section.

Direct and Indirect Galactose Oxidase Surface Labeling. Approximately 1×10^6 cells were cultured in triplicate 10-cm tissue culture dishes. After 72 hr of growth, the surface labeling was performed as previously described (21, 22), with the following modifications. Enzyme treatments were performed *in situ* with 2 ml of the appropriate buffer solutions. Cells on 1 dish were treated for 45 min at room temperature with 25 units of affinity-purified (62) *Vibrio cholerae* NANase (Behring Diagnostics, Inc., Woodbury, N. Y.) in 0.1 M phosphate buffer containing 5 mM $CaCl_2$ and 0.85% NaCl, pH 6.0. NANase-treated and untreated dishes of cells were then incubated at room temperature with 10 units of galactose oxidase (AB Kabi, Stockholm, Sweden) in 0.1 M phosphate buffer containing 5 mM $CuSO_4$, pH 7.0, on a rocker platform for 2 to 2.5 hr. One dish of cells was kept as a control with pH 7.0 buffer and remained untreated. The final yield of cells was >95%, and >90% were alive as shown by the trypan blue exclusion test. The enzyme-treated cells were thoroughly washed with PBS and tritiated with 0.5 mCi NaB^3H_4, pH 7.4. After tritiation of oxidized cells and control cells (21), further reduction with nonradioactive $NaBH_4$ (2.5 mg/ml of 0.01 N NaOH) was carried out. Excess radioactivity was removed by washing with PBS, and 0.1 ml of a 1-ml final cell suspension was added to a preweighed Millipore membrane. The Millipore membranes were dried, desiccated, and reweighed to find the dry cell residue weight. Incorporated radioactivity was measured after the cell residue was solubilized in Protosol:water (9:1) overnight. One-tenth of the total labeled cells (approximately 8×10^5 cells) in 100 μl was mixed with 100 μl of sample buffer and prepared for gel electrophoresis.

Glucosamine Incorporation and NANase Treatment. Each cell line was set up in duplicate 10-cm plates containing 1×10^6 cells/plate. After 20 hr of growth, cells were labeled for 8 hr in 3 ml of MEM with one-tenth the normal amount of dextrose and 20 μCi D-[1-^{3}H]glucosamine. Incorporation was continued for an additional 40 hr with further addition of 6 ml of regular medium. Radioactive medium was removed, the cells were thoroughly washed, and the monolayer of cells was covered with 2 ml 0.1 M phosphate buffer pH 6.0, containing 5 mM $CaCl_2$ and 0.85% NaCl. One dish was kept as control, and the other dish was treated with 25 units of affinity-purified NANase (62). The dishes were incubated at 37° in a warm room on a rocker for 1 hr. Radioactivity released in the cell-free supernatant was measured for both plates. The NANase-releasable sialic acid was calculated by subtracting the radioactivity in the control supernatant. The control and NANase-treated cells were prepared for gel electrophoresis as described above.

Fucose Incorporation. The variant cultured melanoma cells were labeled with the use of 2×10^6 cells in 10-cm tissue culture dishes with 10 μCi L-[6-^{3}H]fucose per 10 ml culture medium for 48 hr. After incorporation the cells were prepared for gel electrophoresis.

The isotopes used for various labeling procedures were obtained from Amersham/Searle Corporation, Arlington Heights, Ill.; these were L-[6-^{3}H]fucose, 18.5 Ci/mmol; D-[1-^{3}H]glucosamine, 3.2 Ci/mmol; and NaB^3H_4, 6.0 Ci/mmol.

Aliquots of the surface-labeled cell suspension absorbed on Millipore membranes solubilized by Protosol:water (9:1),

and GSL zones on thin-layer chromatograms were counted in toluene-based scintillation fluid on a Beckman scintillation counter. NANase supernatant hydrolysates, sialic acid, and hexosamine eluates from glucosamine-labeled cells were counted with Aquasol as scintillation fluid.

Polyacrylamide Slab Gel Electrophoresis, Fluorography, and Carbohydrate Staining. Electrophoresis was performed according to the method of Laemmli (33) in 7% polyacrylamide gel with marker proteins, tritium labeled by the reductive alkylation method (44), in peripheral slots. The molecular weights of marker polypeptides were: β-galactosidase, 130,000; bovine serum albumin, 68,000; egg albumin, 43,000. The gels were fixed overnight in 50% trichloroacetic acid and fluorographed according to the procedures of Bonner and Laskey (5, 35). The gels were exposed to Kodak RP-X-Omat X-ray film at −70° (5) for 1 to 2 months and then developed. The apparent molecular weights of polypeptides on the gels were determined according to the method of Weber and Osborn (58). Two identical gels were stained for carbohydrates with periodic acid-Schiff (47) and Stains-All (25); sialylglycoproteins were detected by comparing these 2 gels.

Isolation and Estimation of Protein-bound Sialic Acid. Protein-bound sialic acid was estimated from the nonlipid residue obtained after lipid extraction by the procedure of Aminoff (1). Typically, 5 to 10 mg of the nonlipid residue were taken into a test tube and digested at 80° for 2 hr with 2 ml of 0.1 M H_2SO_4. The clear hydrolysate was added to a 3-cm Pasteur pipet column of Dowex 1X-acetate. After adsorption of sialic acid, the column was washed with 2 ml of water. The sialic acid was eluted with 5 ml of 1 M formic acid, a 2.5-ml aliquot was evaporated in a Buchler evaporator, and a calorimetric assay was performed (1).

Radioactivity bound to sialic acid of the glycoproteins was estimated on the glucosamine-labeled melanoma variant cell lines (before and after NANase treatment) by the above-mentioned technique.

Total sialic acid was determined by the method described above, directly after acid hydrolysis of cell preparations.

Isolation of Gangliosides and Neutral GSL and Quantitation of Gangliosides. Gangliosides were extracted from frozen cell pellets according to the procedure of Suzuki (50). Lipid-bound sialic acid in the ganglioside fraction was estimated by the procedure of Svennerholm (51), as modified by Miettinen and Takki-Luukkainen (37), which measures the total ganglioside content of cells. The neutral GSL present in the lower phase of Folch extracts obtained during the procedure for ganglioside isolation were separated from the rest of the phospholipids and neutral lipids according to the procedure of Saito and Hakomori (45).

Separation of Neutral GSL and Gangliosides. Aliquots of purified neutral GSL fraction equivalent to 5 mg nonlipid residue from each preparation of cells were spotted on a thin-layer plate (0.25 mm thick; Aneltech, Inc., Newark, Del.). The plates were developed in chloroform:methanol:water (66:30:8, by volume; resulting lower phase) and sprayed with orcinol reagent (34) to detect the GSL and ceramide. The ganglioside fraction corresponding to 5 mg of nonlipid residue from each preparation of cells was spotted into each channel in a thin-layer plate. The plates were sprayed with resorcinol reagent (51) to detect the sialic acid-positive spots.

Characterization of Gangliosides and Neutral GSL. Chemical characterization of the gangliosides of s.c. grown F1 and F10 cells was accomplished with the use of pooled ganglioside fractions from these cells. Individual ganglioside homologs were prepared by column chromatography on Anasil-S (Analabs, New Haven, Conn.), according to the procedure of Penick *et al.* (41). The stoichiometric analysis of the carbohydrate moieties of the individual gangliosides was performed as trimethylsilyl derivatives of the *O*-methyl-glycosides by gas-liquid chromatography (11, 13).

RESULTS

Sialic Acid Content of B16 Melanoma Cells *in Vivo* and *in Vitro*. The results of quantitative determination of the total, lipid-bound, and protein-bound sialic acids of the variant melanoma lines *in vitro* and *in vivo* are presented in Table 1. As shown in the table, the results are similar in the cells grown both *in vivo* and *in vitro*. Approximately 49 to 54% of the total sialic acid is seen in the glycoproteins, and the remaining was found in the lipid fraction of the cells. A considerable decrease in total, lipid-bound, and protein-bound sialic acids is seen in F10 cells compared to F1 cells.

Glycoproteins of B16 Melanoma Cells. The glycoproteins of variant B16 melanoma cell lines were studied by a variety of techniques as shown in Figs. 1 to 3. The chemical staining of total cellular glycoproteins of the variant melanoma cells with periodic acid-Schiff and parallel staining of similar samples with carbocyanin dye (Stains-All) did not reveal any differences in glycoprotein profile between the variant lines. This method permits the identification of 21 major glycoproteins of which 9 are sialylated as could be detected as blue bands with carbocyanin dye. Fucose

Table 1

Sialic acid content of B16 melanoma variant lines in culture and in vivo

Cell lines	Sialic acid (nmol/mg nonlipid residue)		
	Total	Protein bound	Lipid bound
Cultured cells			
F1	13.5 ± 1.6[a] (3)[b]	6.7 ± 0.4 (4)	6.8 ± 0.3 (3)
F10	9.2 ± 1.4 (3)	4.8 ± 0.3 (3)	4.4 ± 0.5 (8)
% decrease[c]	31.8	28.4	35.3
Purified i.p. tumor cells			
F1	14.8 ± 1.2 (3)	7.7 ± 1.4 (3)	6.8 ± 0.5 (5)
F10	10.7 ± 0.5 (3)	5.8 ± 0.5 (3)	4.7 ± 0.3 (4)
% decrease	27.8	24.7	30.9
Purified s.c. tumor cells			
F1	12.9 ± 2.0 (3)	6.6 ± 1.9 (3)	6.3 ± 0.5 (3)
F10	9.9 ± 0.3 (3)	5.2 ± 0.2 (3)	4.7 ± 0.6 (4)
% decrease	23.2	21.2	25.4

[a] Mean ± S.D.

[b] Numbers in parentheses, number of batches of cells used in these analyses.

[c] $\%\ \text{decrease} = \frac{F1 - F10}{F1} \times 100$

incorporation studies help in the detection of fucose-containing glycoproteins. Nearly 22 glycoproteins could be detected by this method. No qualitative difference could be detected between the different variant lines (Fig. 2).

Glucosamine Labeling and NANase Hydrolysis. Preliminary experiments indicated that 28.5 and 29.5% of the radioactivity of glucosamine could be incorporated into sialic acid moieties of glycoproteins and gangliosides of F1 and F10 cells, respectively. Hence glucosamine-labeled cells can be treated with NANase to detect NANase-accessible sialyl components (glycoproteins and gangliosides). Exposure of sialyl components to NANase was studied by this approach; an example of such quantitative analysis is presented in Table 2. F10 cells contained 80% more NANase-accessible total sialic acid as detected in the supernatant after hydrolysis. Only a small percentage of total sialylglycoproteins was hydrolyzed by NANase during 1 hr incubation, indicating that 8.15% was exposed in F1 cells and 9.5% was exposed in F10 cells. Thus F10 cells showed a 16.5%

$$\frac{F10 - F1}{F1} \times 100$$

higher amount of surface-exposed sialylglycoproteins in culture. Qualitative analysis of the glucosamine-labeled glycoproteins before and after NANase hydrolysis was done by gels as shown in Fig. 3. The fluorograms showed about 29 radioactive glycoprotein bands of which only 7 were altered in intensity after NANase treatment indicating they are exposed on the cell surface. These sialylglycoproteins had apparent molecular weights of 154,000, 143,000, 127,000, 110,000, 97,000, 77,000, and 73,000. No qualitative difference in NANase-accessible sialylglycoproteins could be detected between F1 and F10 cells. These experiments were performed twice with reproducible results.

Analysis of the ganglioside fraction of glucosamine-labeled cells before and after NANase treatment suggests that F10 cell surface ganglioside released 87% more sialic acid than did F1 cells. Although 15 to 17% of the glucosamine radioactivity was seen in the ganglioside fraction, a large proportion of it was accessible to NANase during 1 hr incubation, indicating that 21.5% was exposed in F1 cells and 40.1% was exposed in F10 cells. Thus the glucosamine-NANase method shows that F10 cells contain 80% more NANase-accessible total cell surface sialic acid, the majority of which is contributed by G_{M3} ganglioside, as compared to F1 cells.

Sialylgalactosyl and/or Galactosaminyl Glycoproteins *in Vitro* and *in Vivo*. Quantitative analysis of direct and indirect galactose oxidase labeling of cultured melanoma variant lines is presented in Table 3. Qualitative analyses of surface glycoproteins of variant melanoma lines maintained in culture and of short-term-cultured s.c. tumor cells as detected by the galactose oxidase method are presented in Fig. 1. All experiments were performed 3 times with reproducible results. Most of the glycoproteins could be detected by the indirect method, *i.e.*, after NANase and galactose oxidase labeling (Fig. 1, *A* and *B*, *Channels 2* and *4*).

Indirect galactose oxidase labeling aids in the detection of 9 to 10 surface glycoproteins in cultured and short-term-cultured *in vivo* cells. A glycoprotein with an apparent molecular weight of 66,000 is seen in F10 cells maintained in culture (Fig. 1*A*, *Channel 2*) but is not seen in F1 cells. Similarly, short-term-cultured F10 s.c. tumor cells show intensely labeled glycoprotein bands with apparent molecular weights of 97,000, 84,000, 74,000, and 66,000 (Fig. 1*B*, *Channel 4*). These bands are not heavily labeled in F1 cells (Fig. 1*B*, *Channel 2*). Analysis of short-term-cultured F10 i.p. tumor cells also shows the presence of the above-mentioned bands (M.W. 97,000, 84,000, 74,000, and 66,000) as compared to F1 cells (results not shown).

In all cells studied, some incorporation was seen after

Table 2

Exposure of sialyl components on the surface of cultured B16 melanoma variant lines

Results are a representative sample of 1 of the 3 "glucosamine:NANase" experiments performed. In 2 other experiments, similar (F10:F1) ratios were seen. No significant difference in the metabolism of glucosamine was seen between the variant cell lines. Nearly 6 to 7% of the total glucosamine radioactivity added to culture plates incorporated in variant melanoma cells in 48 hr of incubation. Of the total incorporated radioactivity (proteins and lipids), nearly 83 to 85% was seen in the glycoprotein (nonlipid residue) and 15 to 17% was seen in the ganglioside fraction in the different variant lines. Approximately 92 to 95% of the ganglioside-bound radioactivity was bound to sialic acid moieties in these cells. The amount of radioactivity incorporated into sialic acid was approximately 28.5 and 29.5% in the F1 and F10 cells, respectively.

	cpm/mg cell residue			% exposure[a]	
Cells analyzed	Supernatant NANase releasable (T − C)[b]	Sialylglycoproteins NANase releasable (C − T)	Gangliosides NANase releasable (C − T)	Sialylglycoproteins	Gangliosides
F1	10,385	6,565 (80,640 − 74,075)	3,105 (14,455 − 11,350)	8.15	21.50
F10	18,645	7,050 (74,035 − 66,985)	5,810 (14,480 − 8,670)	9.50	40.10
F10:F1	1.80	1.07	1.87	1.16	1.86

[a] Percentage of the incorporated radioactivity that is NANase accessible

$$\frac{C - T}{C} \times 100$$

For example, percentage exposure of sialylglycoproteins of F1 cells is calculated as follows: 80,640 − 74,075/80,640 × 100.

[b] T, enzyme treated; C, control (untreated).

Table 3
Direct and indirect surface labeling of B16 melanoma variant lines by galactose oxidase:NaB^3H_4 method in tissue culture

Cells and treatment	Incorporation of ^3H (cpm/mg dry cell residue)[a]		
	Total	Galactose and/or GalNAc on surface glycoproteins[b]	Sialyl galactose and/or GalNAc on surface glycoproteins[c]
F1			
Control	20,000		
GaO[d]	36,500	16,500	
N, GaO	87,700		51,200
F10			
Control	20,800		
GaO	44,700	23,900	
N, Gao	111,700		67,000

[a] Average of duplicate analyses of 2 batches of delipidated cell residue that were solubilized and subjected to radioactivity determination.
[b] Direct galactose oxidase: GaO − control.
[c] Indirect galactose oxidase method: N, GaO − GaO.
[d] GaO, galactose oxidase treatment; N, GaO, NANase followed by galactose oxidase treatment.

treatment with NaB^3H_4 only. Gel electrophoresis of these control samples revealed the presence of 4 protein bands (M.W. 52,000, 47,000, 45,000, and 41,000).

Ganglioside and Neutral GSL Profile of Cultured and *in Vivo* Melanoma Variant Lines. Qualitative data on the patterns of neutral GSL and gangliosides of melanoma variant cell lines are shown in Fig. 4. A relatively simple neutral GSL pattern is seen in cultured and *in vivo*-grown melanoma cells. These correspond in chromatographic migration to mono- and diglycosylceramide. The neutral GSL of s.c. tumors of F1 melanoma cells were characterized by gas chromatography to contain glucosylceramide and CD (glucose:galactose, 1.00:0.96). As seen in culture and in s.c. tumors, F10 cells contain more CD than do F1 cells, which contain only traces (see Fig. 4*A*, *Channels 1* and *5 versus Channels 2* and *6*). On the other hand, i.p. grown tumors of F1 and F10 cells did not exhibit any difference in neutral GSL (Fig. 4*A*, *Channels 3* and *4*).

Cultured melanoma variant cell lines exhibited a simple ganglioside pattern consisting solely of G_{M3} and traces of G_{Dla}, whereas *in vivo*-grown tumors of F1 and F10 cells contain G_{M3}, G_{M2}, G_{M1}, and G_{Dla}. There was very little qualitative difference between the variant lines (F1 *versus* F10) whether grown in culture or *in vivo*. However, a small decrease in quantities of G_{M3} was seen in tissue culture, s.c. and i.p. grown tumors, which is consistent with the decrease in total lipid-bound sialic acid (Table 1). A preliminary characterization of the gangliosides of s.c.-grown F1 tumors was attempted. Gas chromatographic analysis of the carbohydrate moieties of the principal gangliosides of F1 cells *in vivo* revealed a carbohydrate stoichiometry generally similar to the values expected from their corresponding chromatographic mobilities [Band 1 (G_{M3}): glucose:galactose:NAN, 1.00:0.84:0.98; Band 2 (G_{M2}): glucose: galactose:GalNAc:NAN, 1.00:0.82:0.88:0.95; Band 3 (G_{M1}): glucose:galactose:GalNAc:NAN, 1.00:1.85:0.92:0.90; Band 4 (G_{Dla}): glucose:galactose:GalNAc:NAN, 1.00:1.72:0.82:1.78].

Cell Surface Exposure of Gangliosides (G_{M3}) and CD. The cell surface exposure of ganglioside (G_{M3}) found in cultured cells was studied with the glucosamine:NANase method and indirect galactose oxidase labeling followed by lipid extraction and thin-layer chromatography as shown in Table 4. With these procedures the G_{M3} molecule showed 45 to 56% greater exposure to NANase in F10 than in F1 cells. Glucosamine-derived radioactivity was found only in the G_{M3} zone in the thin-layer plates.

The exposure of CD was studied with the use of direct galactose oxidase labeling followed by thin-layer chromatography of the neutral GSL extracts. With these procedures a 5- to 6-fold greater exposure of CD to the enzyme galactose oxidase was seen in F10 cells than in F1 cells (Table 4).

DISCUSSION

Increases in total sialic content *in vivo* have been observed for human tumors of lung (9), colon, stomach, breast (3), pancreas, liver, skin, and lymph nodes (36) compared with normal tissue. Similar increases have been observed in the plasma membranes of rat hepatomas (4, 12). In contrast there are several reports that animal cells in culture show a decrease of total sialic acid after viral transformation (26, 27, 63), although the accessibility of sialic acid to NANase is increased compared to control cells (7). These studies thus suggest an association between the amount of sialic acid exposed on the cell surface and the malignant state. In this study we sought to determine whether or not a correlation would also be observed between the cell surface exposure of sialic acid and its ability to implant in lung after i.v. administration. Our findings indicate that total, protein-bound, and lipid-bound sialic acids of high-lung-implanting melanoma cells (F10) are decreased in both solid tumors and cultured cells when compared to low-lung-implanting F1 cells. However, the amount of enzyme-accessible cell surface sialic acid was increased in both the sialylglycoprotein and ganglioside fractions in F10 cells, as determined by the galactose

Table 4
Exposure of GM_3 and CD on the surface of metastatic variant melanoma lines

Component studied and method	cpm/mg cell residue[a]		
	F1	F10	F10:F1
Ganglioside (G_{M_3}) exposure			
$GluNH_2$-NANase[b]	4,585	7,145	1.56
Indirect galactose oxidase treatment[c]	5,500	8,000	1.45
CD exposure			
Direct galactose oxidase labeling[d]	6,500	36,000	5.53

[a] Results represent the actual radioactivity present in the GSL zone of a thin-layer chromatography plate of a single experiment. A repeat experiment produced similar results.
[b] Radioactivity in the G_{M3} zone of thin-layer chromatography plate from glucosamine-labeled cells: control minus NANase treated = NANase-releasable counts.
[c] Radioactivity in the lipid extract of the galactose oxidase-labeled cells after initial NANase treatment minus radioactivity in the extract of untreated galactose oxidase-labeled cells.
[d] Radioactivity in lipid extracts of galactose oxidase-labeled cells minus radioactivity in the extracts of control cells that were tritiated without prior enzyme treatment.

oxidase and glucosamine:NANase methods. A similar decrease in total sialic acid content accompanied by an increased cell surface exposure of sialic acid has also been observed in cells obtained from spontaneous lung metastases derived from F1 s.c. tumors, as compared to the original F1 cells (G. Yogeeswaran and B. S. Stein, unpublished observation). Thus there does appear to be a correlation between cell surface sialic acid and the ability to implant and grow in lung tissue in the B16 melanoma system.

In the studies reported here, we have examined the GSL of metastatic variant melanoma cells grown both *in vivo* and *in vitro*. The results of these studies demonstrate a small decrease in quantity of G_{M3} in F10 cells grown *in vivo* and *in vitro* compared to similarly grown F1 cells which is accompanied by an accumulation of the G_{M3} precursor, CD.

Differences were observed in the ganglioside profiles of the cell lines depending on whether the cells were grown *in vitro* or *in vivo*. The changes seen include a total absence of G_{M2} and G_{M1} in cultured cells, whereas these molecules were found in *in vivo*-grown tumors (both i.p. and s.c.). The reason for such changes is not known; it may be due to increased biosynthesis of these molecules *in vivo* or to the uptake of these gangliosides from serum by tumor cells or due to the presence of contaminating host-derived cells. Presently, we are testing these 3 possibilities. Differences in gangliosides and neutral GSL profile have also been found in *in vivo*- and *in vitro*-grown mouse adrenocortical tumor cells (39) and hamster sarcoma cells (46).

GSL analysis following direct galactose oxidase labeling aids in measuring the cell surface exposure of neutral GSL. The higher-lung-implanting F10 cells showed a 5- to 6-fold higher CD exposure which is partly reflected by a rise in quantity of CD relative to F1 cells. In several other tumors (42, 43), an increase in quantity of a tumor antigen characterized as CD has been reported.

An increase in sialic acid in glycopeptides in the membrane glycoproteins of transformed cells in tissue culture (53, 56) and human leukemic cells (54) is well documented. Bosmann *et al.* (6) have shown that F10 cells have a 2-fold higher NANase-accessible sialic acid content relative to F1 cells, but the nature of the sialic acid-bearing moieties was not investigated. The present investigation, in which radioactive glucosamine as precursor for sialic acid was used, enabled a detailed analysis of NANase-accessible sialic acid-containing molecules in these cells. This approach, in confirmation of the findings of Bosmann *et al.* (7), has shown a 1.8-fold increase in NANase-accessible sialic acid on F10 cells as compared to F1 cells. The accessibility of NANase to glycoprotein sialic acid on F10 cells was only moderately higher than that for F1 cells (7%). Analysis of the gangliosides of NANase-treated cells showed a significantly higher NANase-accessible G_{M3} (56%), although the content of G_{M3} decreased 35% in F10 cells compared to F1 cells. This suggests that gangliosides contributed to the NANase-releasable sialic acid to a greater degree than glycoproteins in F10 cells compared to F1 cells. Increased cell surface exposure of G_{M3} has been seen in other transformed fibroblasts compared to their normal counterparts (31).

Qualitative analysis of the glycoproteins before and after NANase treatment by gel electrophoresis detected 7 surface sialylglycoproteins in both variant melanoma lines. This method could detect 4 other NANase-accessible surface glycoproteins that could not be detected by the galactose oxidase method. In contrast to the galactose oxidase gels (see below), no difference in the glycoprotein with a molecular weight of 66,000 was detected between the variant lines on the gels prepared from glucosamine:NANase-treated cells, but this may be due to a high background of hexosamine-bound radioactivity in the glycoprotein band.

The indirect galactose oxidase technique combined with fluorography of gels permits the visualization of surface-exposed glycoproteins with good resolution. Without NANase treatment the melanoma variant lines were poorly labeled, suggesting that the glycoprotein chains of B16 melanoma variants are highly sialylated. The high-lung-implanting F10 cells contain a surface sialylglycoprotein with a molecular weight of 66,000 that is absent in F1 cells in tissue culture-maintained cells. The surface glycoproteins of F1 and F10 cells were studied with the use of short-term-cultured *in vivo* tumor cells to see whether the difference in glycoprotein (M.W. 66,000) seen between these variant lines in culture is seen *in vivo*. Such studies showed a quantitative increase of at least 4 major surface sialylglycoproteins (including the glycoprotein with a molecular weight of 66,000) in F10 cells (s.c. tumors) relative to F1 cells.

B16 melanoma variant lines both *in vivo* and *in vitro* show 4 nonspecifically labeling proteins. Nonspecifically labeled proteins were detected in other lymphoid (23) and human leukemic lymphocytes (2). The nature of the reducible group(s) responsible for this nonspecific labeling reaction is not known, but it could be due to the reduction of Schiff's base formed between pyridoxal phosphate and an amino group of the neighboring enzyme protein. Enzymes of this nature, such as ornithine decarboxylase, are known to be activated in proliferating (malignant) cells (38).

The differences in the galactose oxidase-labeling glycoprotein profile between F1 and F10 cells indicate that there are architectural or oligosaccharide sequence differences between these variant lines. This conclusion is strengthened by the fact that there was no detectable difference between these cell lines in glycoproteins detected by metabolic labeling with fucose or glucosamine or chemical staining. Studies done by Warren *et al.* (57) suggest that there is no difference between F1 and F10 cells in the size of glycopeptides isolated from pronase digests. One may ask the question of whether the differences in the cell surface-exposed sialylglycoproteins may account for the observed increase of lung implantation (17–19) in F10 cells as seen by Fidler.

In this investigation the well-documented high-lung-implanting F10 cells (15–17) show increased NANase and galactose oxidase-accessible neutral GSL, gangliosides, and glycoproteins when compared with low-lung-implanting F1 cells. Also, the studies described here present evidence that highly tumorigenic and metastatic mouse melanoma cells are enriched with highly sialylated glycoproteins. These results are consistent with the findings of Sinha and Goldenberg (48) and Weiss *et al.* (60), who

showed that NANase treatment of tumor cells decreases lung implantation following i.v. administration of treated cells. An indirect experiment by Hagmer (28), in contrast with the previously mentioned findings (48, 60), suggests that decreasing the exposure of cell surface sialic acid and thereby decreasing the negative charge on the cell may enhance experimental pulmonary metastases. Therefore the cell surface sialyl components may enhance or retard pulmonary metastases in different systems. The reasons for these differences must be further explored.

Therefore, the study gives additional support to the previously proposed role of sialic acid in lung implantation (48, 60) and tumorigenicity (59). Further it is possible that the increased surface sialic acid-containing components seen in higher-lung-implanting melanoma F10 cells may decrease tumor cell immunogenicity (59) and hence decrease susceptibility to killing by macrophages (16) as well as intervene in the phenomenon of clumping with sensitized lymphocytes (16, 19).

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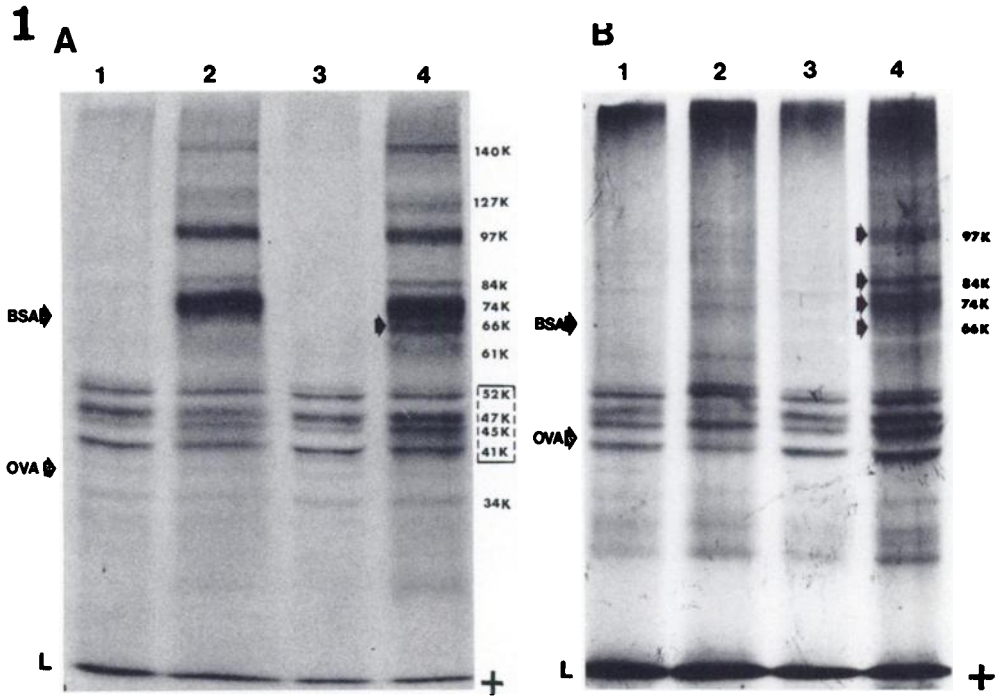

Fig. 1. Fluorography pattern of sodium dodecyl sulfate:polyacrylamide gel electrophoresis of ^{3}H-labeled tissue culture-maintained and short-term-cultured cells derived from s.c. tumors of B16 melanoma variant lines after galactose oxidase treatment. *A*, tissue culture-maintained cells: *Channel 1*, F1 cells treated with galactose oxidase only; *Channel 2*, F1 cells after NANase and galactose oxidase treatment; *Channel 3*, F10 cells with galactose oxidase only; *Channel 4*, F10 cells after NANase and galactose oxidase treatment. *B*, short-term-cultured s.c. tumor cells: *Channel 1*, F1 cells with galactose oxidase only; *Channel 2*, F1 cells after NANase and galactose oxidase treatment; *Channel 3*, F10 cells with galactose oxidase only; *Channel 4*, F10 cells after NANase and galactose oxidase treatment. Apparent molecular weights of major bands are shown in thousands. Apparent molecular weights indicated inside the box are nonspecifically labeled (see "Discussion"). *L*, lipid band; *arrow*, components relatively increased in F10 cells; *BSA*, bovine serum albumin; *OVA*, ovalbumin.

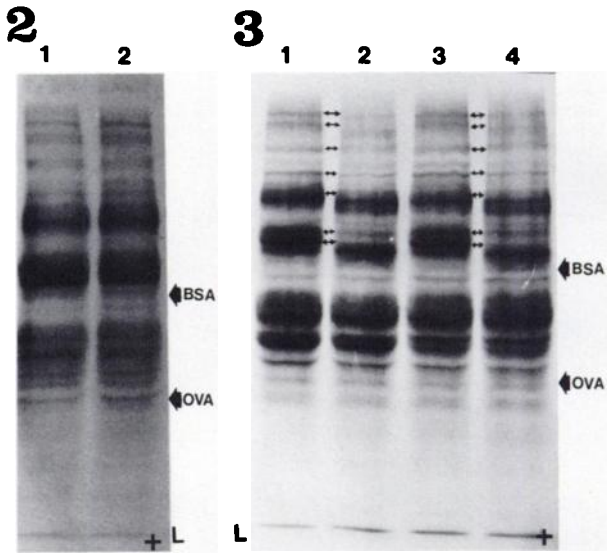

Fig. 2. Fluorography pattern of sodium dodecyl sulfate:polyacrylamide gel electrophoresis of [^{3}H]fucose-labeled cultured B16 melanoma variant lines. *Channel 1*, F1 cells; *Channel 2*, F10 cells. *BSA*, bovine serum albumin; *OVA*, ovalbumin; *L*, lipid band.

Fig. 3. Fluorography pattern of sodium dodecyl sulfate:polyacrylamide gel electrophoresis of [^{3}H]glucosamine-labeled cultured melanoma variant lines with and without NANase treatment. *Channel 1*, F1 cell control; *Channel 2*, F1 cell after NANase treatment; *Channel 3*, F10 cell control; *Channel 4*, F10 cell after NANase treatment; *double-headed arrow*, bands that change in intensity after NANase treatment; *BSA*, bovine serum albumin; *OVA*, ovalbumin.

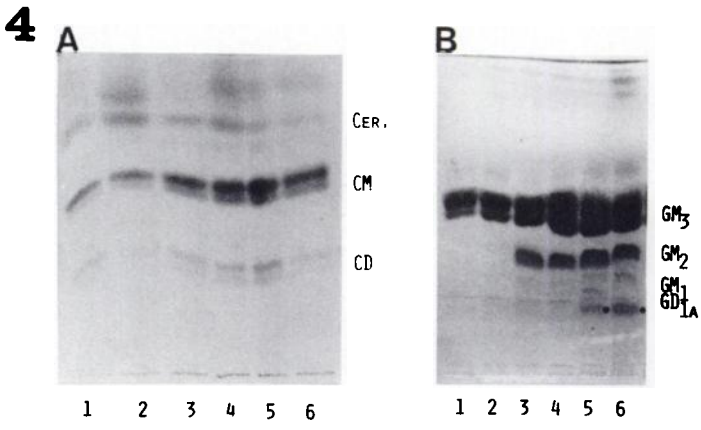

Fig. 4. *A*, Thin-layer chromatogram of the neutral GSL of F10 and F1 melanoma variant lines in culture and *in vivo*. Neutral GSL of (*Channel 1*) cultured F10 cells, (*Channel 2*) cultured F1 cells, (*Channel 3*) i.p.-grown F10 tumor cells, (*Channel 4*) i.p.-grown F1 tumor cells, (*Channel 5*) s.c.-grown F10 tumor cells, (*Channel 6*) s.c.-grown F1 tumor cells. *B*, thin-layer chromatogram of the gangliosides of F10 and F1 melanoma variant lines in culture and *in vivo*. Gangliosides of (*Channel 1*) cultured F10 cells, (*Channel 2*) cultured F1 cells, (*Channel 3*) s.c.-grown F10 cells, (*Channel 4*) s.c.-grown F1 cells, (*Channel 5*) i.p.-grown F10 cells, (*Channel 6*) i.p.-grown F1 cells. The system of nomenclature is that of Svennerholm (34). *Bands* shown by *dots*, non-resorcinol-positive yellow spots that are not gangliosides.