

CLASS 1 (UNIQUE) TUMOR ANTIGENS OF HUMAN MELANOMA

Identification of a 90,000 Dalton Cell Surface Glycoprotein by Autologous Antibody

BY FRANCISCO X. REAL, M. JULES MATTES, ALAN N. HOUGHTON,
HERBERT F. OETTGEN, KENNETH O. LLOYD, AND LLOYD J. OLD

From the Memorial Sloan-Kettering Cancer Center, New York 10021

The central question of human cancer immunology continues to be whether malignant transformation is associated with antigenic changes that can be recognized as foreign by the host of origin. Our laboratory has been addressing this question through the analysis of the humoral immune reactions of patients to surface antigens of cancer cells. The approach we have evolved in these studies has been referred to as autologous typing and consists of identifying patients with antibodies to surface antigens of cultured autologous tumor cells and analyzing the specificity of these reactions by absorption tests with autologous and allogeneic normal and malignant cells (1). Three classes of cell surface antigens have been defined in this way through the study of patients with melanoma (2-6), astrocytoma (7), renal cancer (8), and acute leukemia (9, 10). Class 1 tumor antigens show an absolute restriction to the autologous tumor and cannot be detected on any other normal or malignant cell type. Class 2 tumor antigens are also found on a proportion of allogeneic tumors of related origin and, in certain instances, on a limited range of normal cell types. As shown by Watanabe et al. (11), some of these antigens have the characteristics of autoantigenic differentiation antigens. Class 3 tumor antigens have a broad distribution on normal and malignant cell types and do not follow any differentiation-related or tumor-restricted pattern.

In our study of 85 patients with malignant melanoma, autologous typing has identified 5 patients with antibodies to Class 1 tumor antigens (1-3, 5, 6). Four of these antibodies belonged to the IgG class and the fifth was an IgM antibody. Partial characterization of one of the Class 1 melanoma antigens (AU) was carried out using papain-solubilized antigen in conjunction with antibody inhibition assays (12). AU antigen appeared to be a glycoprotein (as indicated by affinity for lentil lectin), showed no relation to HLA or β_2 microglobulin (β_2m),¹ and had a molecular mass in the range of 25,000-40,000 daltons. Despite repeated

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¹ *Abbreviations used in this paper:* Con A, concanavalin A; DTT, dithiothreitol; EBV, Epstein-Barr virus; FBS, fetal bovine serum; GGF, gamma globulin-free; IA, immune adherence; ITS, insulin-transferrin-selenium; β_2m , β_2 microglobulin; MHA, mixed hemadsorption; PA, protein A; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; VSV, vesicular stomatitis virus.

attempts none of the antibodies to Class 1 melanoma antigens immunoprecipitated antigen from radiolabeled cell extracts.

In this report, we describe a new Class 1 melanoma antigen detected by antibody in the sera of patient FD. FD sera reacted exclusively with autologous FD melanoma cells and immunoprecipitated a 90,000 dalton glycoprotein from extracts of radiolabeled FD melanoma cells.

Materials and Methods

Tissue Culture. Tumor cell lines were established and propagated as previously described (2, 7, 8). B cell lines were established from peripheral blood lymphocytes of melanoma patients transformed by Epstein-Barr virus (EBV) released from the B-95-8 marmoset lymphoid cell line. Cultured cells were maintained in Eagle's minimum essential medium supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 7.5% fetal bovine serum (FBS) (complete medium). To remove heterologous FBS components, SK-MEL-131 (FD) melanoma cells were grown for at least four subcultures in 10% human sera selected from AB blood type donors. SK-MEL-131 cells were also adapted to growth in serum-free medium containing insulin (5 μ g/ml), transferrin (5 μ g/ml), and selenium (5 ng/ml) (ITS medium; Collaborative Research, Inc., Waltham, MA). A series of subcultures of SK-MEL-131 were obtained by limiting dilution in wells of Costar 3596 plates (Costar, Cambridge, MA). Cells were seeded at a concentration of 100, 10, or 1 cell per well. Cultures were checked for the presence of a single growing cluster of cells, and wells containing such clusters were subcultured and expanded. Cultures were regularly tested for mycoplasma, and contaminated cultures were discarded.

Serological Tests. The protein A (PA) hemadsorption and immune adherence (IA) assays were performed as previously described (2-4, 7). Indicator cells for the human mixed hemadsorption (MHA) assay were prepared using a human serum that contained natural hemagglutinating antibodies reactive with sheep erythrocytes, and goat anti-human Ig (Cappel Laboratories, Cochranville, PA) as the second antibody. Assays were performed in Falcon 3040 microtest II plates (Falcon Labware, Oxnard, CA). Target cells were plated 1-3 d before the assay and incubated with antibody for 1 h at room temperature. After washing with 5% gamma globulin-free FBS (GGF-FBS) (Gibco Laboratories, Grand Island, NY) in phosphate-buffered saline (PBS), indicator cells were added and incubated with the target cells for 1 h at room temperature. The plates were then washed three times with 5% GGF-FBS-PBS and reactions were evaluated under a light microscope. Titer refers to the highest serum dilution showing 10% of target cells with red cells attached to 50% or more of their perimeter. In tests with cells grown in human serum, 2% human serum albumin in PBS was substituted for the 5% GGF-FBS-PBS. Qualitative absorptions were performed by mixing 30 μ l of a dilution of serum (fourfold more concentrated than the serum dilution giving 50% rosetted target cells) with a 30 μ l pellet of packed cells for 30 min at room temperature and 30 min at 4°C. For quantitative absorptions, the cell pellet (ranging from 1 to 30 μ l) was suspended in 30 μ l of similarly diluted serum and incubated for 30 min at room temperature and 30 min at 4°C.

To detect shed antigens in spent culture medium, medium from SK-MEL-131 cells (or from normal human fibroblasts) was collected, ultracentrifuged at 100,000 g for 30 min, and tested for antibody inhibitory activity by serially diluting FD serum in the medium. After a 30 min incubation at room temperature, residual antibody activity was determined using SK-MEL-131 target cells. To test for the heat stability of FD antigen, SK-MEL-131 cells were incubated for 5 min in boiling water and then tested for FD antigen in absorption tests. Sensitivity to neuraminidase was determined by incubating attached SK-MEL-131 cells in Dulbecco's PBS (Gibco Laboratories) containing neuraminidase (Calbiochem-Behring Corp., La Jolla, CA) at a concentration of 50 U/ml for 1 h at 37°C before the serological assay. Trypsin sensitivity was determined by incubating SK-MEL-131 cells with trypsin (Gibco Laboratories) at a concentration of 0.5 mg/ml for 10 min at 37°C.

To investigate the reappearance of FD antibody, trypsinized SK-MEL-131 cells were washed and incubated for 0.5 and 6 h in complete medium containing (a) no additive, (b) cycloheximide (Sigma Chemical Co., St. Louis, MO) at a concentration of 10 $\mu\text{g}/\text{ml}$ or (c) monensin (Sigma Chemical Co.) at a concentration of 10 $\mu\text{g}/\text{ml}$. The IgG fraction of FD serum was obtained by DEAE-Sepharose chromatography (Pharmacia Fine Chemicals, Piscataway, NJ).

Immunoprecipitation. Metabolic incorporation of [^3H]glucosamine (13), [^3H]mannose (13), or [^{35}S]methionine (14) and solubilization of labeled cells (15) have been previously described. Membrane preparations were solubilized with Nonidet P-40 (Calbiochem-Behring Corp.) and [^{125}I]labeled with chloramine T (Eastman Kodak Co., Rochester, NY) (16). Culture medium from SK-MEL-131 cells grown in ITS medium was concentrated 10 times by pressure dialysis using an Amicon membrane (Amicon Corp., Danvers, MA) with a 10,000 dalton exclusion limit and then dialyzed for 72 h against PBS. Iodination was performed as described (16), except that the column buffer was 50 mM Tris, pH 7.5, 2% BSA, 10 mM NaI, 0.10 M NaCl (buffer A). Immunoprecipitation techniques have also been previously described (16). Immunoprecipitated molecules were extracted from pelleted *Staphylococcus aureus* (Enzyme Center, Boston, MA) with 60 μl 0.01 M Tris HCl, pH 7.2, 2% sodium dodecyl sulfate (SDS), 12 mg/ml dithiothreitol (DTT), 15% (wt/vol) sucrose, and 0.01% (wt/vol) pyronin Y by heating for 5 min at 100°C, and were analyzed by polyacrylamide gel electrophoresis (PAGE) (17) using 9% gels. For two-dimensional electrophoresis (isoelectric focusing followed by SDS-PAGE), immune precipitates were extracted and processed as described (13, 18). For unreduced samples, DTT was omitted and 14 mg/ml iodoacetamide was added to the sample. To analyze the specificity of the immunoprecipitating antibody in FD serum, absorption tests were performed. 30 μl of a 1:2 dilution of FD serum was absorbed with a 30 μl cell pellet of autologous or allogeneic cells for 30 min at room temperature and then for 30 min at 4°C. After removing the absorbing cells, FD serum was tested for residual immunoprecipitating activity.

Lectin Column Fractionation. Beads conjugated with concanavalin A (Con A), wheat germ agglutinin, Lentil lectin or *Helix pomatia* lectin were purchased from Pharmacia Fine Chemicals and beads conjugated with peanut agglutinin, soybean agglutinin, and lectins from *Ulex europaeus-I*, *Bandeira simplicifolia-I*, *Dolichos biflorus*, *Pisum sativum*, *Ricinus communis-I*, *Vicia villosa*, *Lotus tetragonolobus*, and *Limulus polyphemus* were purchased from E-Y Laboratories, San Mateo, CA. Columns (0.5 ml) were equilibrated with buffer A (see above) and [^{125}I]labeled antigen preparations were passed through the column. Material specifically bound to the column was eluted with the appropriate sugar at a 1 M concentration.

Results

Clinical History of Patient FD. Patient FD was a white male who presented in 1964 at age 50 with a primary melanoma of the right ear. He had a local excision and remained healthy until 1977 when a second primary melanoma was excised from his left upper back. Pathological examination of the lesion revealed a Clark's level III melanoma of 2 mm thickness. Due to regional recurrence, he underwent a left axillary dissection in August 1979, and 4 of 23 lymph nodes were found to contain metastatic melanoma. The SK-MEL-131 cell line was established from a left axillary metastasis excised in February 1980. A right lung metastasis was detected in April 1980 and the patient received leukocyte buffy-coat interferon. Because of disease progression, he was treated with four cycles of dacarbazine and achieved a partial response. He also received four subcutaneous injections of vesicular stomatitis virus (VSV) lysate of SK-MEL-131 cells during February and March, 1981. He was then treated with cis-platinum, vindesine, carmustine, and tamoxifen. His disease continued to progress and the patient died at another institution in November 1981. At autopsy, widely

metastatic melanoma was found. Patient FD also had a clinical history of meningioma and Paget's disease of bone.

Reactivity of FD Serum with Cultured Autologous Melanoma Cells (SK-MEL-131). Sera from patient FD were tested for reactivity with cell surface antigens of the autologous SK-MEL-131 cell line. Autologous reactions were detected with PA and human MHA assays, but not with the IA assay (Fig. 1). DEAE-Sepharose chromatography of FD sera showed that the reactivity was present in the IgG fraction. SK-MEL-131 cells cultured for four or more passages in medium containing normal human serum (rather than FBS) continued to be reactive with FD serum, indicating that heterologous serum components were not involved in the reactions observed. Fig. 2 summarizes the results of tests with 15 individual serum specimens obtained from patient FD over a 15-mo period. Serum specimens obtained between February and April, 1980 were not reactive.

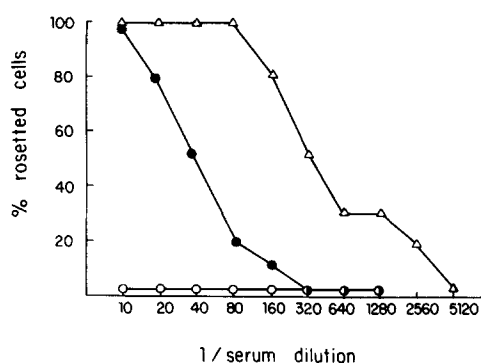


FIGURE 1. Reactivity of serum from patient FD with autologous SK-MEL-131 cells as determined by three serological assays: (○) immune adherence; (●) protein A; (△) mixed hemadsorption.

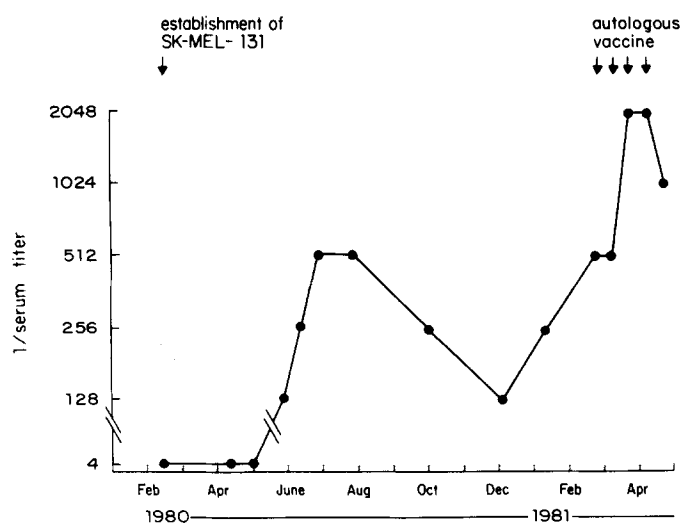


FIGURE 2. Reactivity of sequential serum samples from patient FD with autologous SK-MEL-131 melanoma cells. Serological test: protein A assay.

Reactivity was found with serum obtained in June 1980 (titer 1:128), and serum titers ranged from 1:128 to 1:512 over the next 8 mo. After four vaccinations with a VSV-lysate of SK-MEL-131 cells during February and March, 1981, serum titers rose to 1:2048. No serum specimens were obtained after April 1981.

Absorption Analysis of FD Serum Reactivity. The specificity of FD autologous reactivity was analyzed by absorption tests. An individual absorption test is illustrated in Fig. 3 and the results of the complete analysis are summarized in Table I. Of the 34 melanoma cell lines tested, only SK-MEL-131 cells absorbed autologous reactivity. SK-MEL-131 cells adapted to growth in human serum or in serum-free medium absorbed autologous reactivity completely, further demonstrating that heterologous serum components contributed by FBS were not involved in FD serum reactivity. A variety of other cell types were used in absorption tests; these included 32 cell lines derived from other tumor types, EBV-transformed lymphocytes of eight individuals including patient FD, and normal kidney epithelial cells and normal skin fibroblasts. None of these cells had absorbing activity. These absorption tests indicate that FD sera detect a Class I or unique antigen on FD (SK-MEL-131) melanoma cells and that the FD antigen is distinct from the five other Class I antigens previously defined on melanoma cells: AU (SK-MEL-28), BD (SK-MEL-13), BI (MeWo), DX (SK-MEL-93), and FT (SK-MEL-147).

Allogeneic Typing with Serum from Patient FD. A serum specimen obtained in February 1981 with a titer of 1:1280 for autologous SK-MEL-131 cells was tested on a panel of 63 allogeneic cell lines and one culture of normal allogeneic melanocytes (Table II). No reactivity was detected with any of these allogeneic cells. To determine whether FD reactivity could be transferred to allogeneic melanoma cells, three melanoma cell lines (SK-MEL-26, -43, and -127) were incubated for 5 d with supernatants from SK-MEL-131 cultures and then cultured in fresh medium for 3 wk. No FD reactivity was induced in these

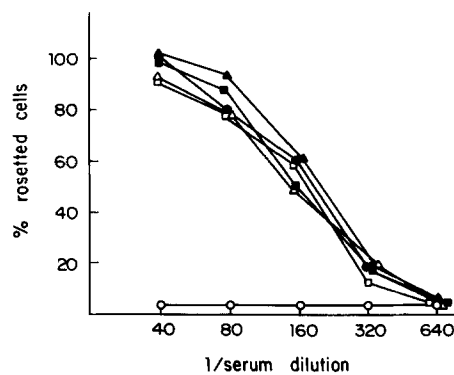


FIGURE 3. Absorption analysis of the protein A reactivity of FD serum (March 1981) for autologous SK-MEL-131 melanoma cells. SK-MEL-131 cells absorbed FD serum reactivity completely. SK-MEL-28, SK-MEL-37, SK-MEL-93 allogeneic melanoma cells and EBV-transformed B cells from patient FD did not absorb FD serum reactivity. (●) Unabsorbed. Absorbed with (○) SK-MEL-131; (△) SK-MEL-28; (□) SK-MEL-37; (▲) SK-MEL-93; (■) FD EBV-transformed B cells. Serological test: protein A assay.

TABLE I
*Absorption Analysis of FD Serum Reactivity for Autologous SK-MEL-131 Melanoma Cells:
 Summary of Results*

Positive absorption	Negative absorption	
Melanomas	Melanomas	Renal carcinomas
SK-MEL-131 (FBS)	SK-MEL-13, 19, 23(I), 26, 28, 29, 33,	SK-RC-1, 2, 4, 6, 7, 9, 11, 16,
SK-MEL-131 (NHS)	37, 40, 42, 44, 57, 61, 67, 72, 75,	18, 28
SK-MEL-131 (ITS)	90, 93(I), 93(II), 93(III), 94, 110,	Other carcinomas
	111, 119, 122, 147, 158, 161, 162,	MCF-7 (breast), ME-180
	165, 169, MeWo, WM77	(cervix), HT-29 (colon),
	Astrocytomas	SW-620 (colon), GCC-SV(C)
	SK-MG-3, 5, 6, 9, 12, U251 MG, MS	(choriocarcinoma)
	Bladder carcinomas	B lymphocyte lines
	Scaber, RT4, T24, 486-P, 639-V,	(EBV-transformed)
	647-V, 253-J	WM51, FD(autologous), AZ,
	Lung carcinomas	AV, BD, FG, DX, EI
	SK-LC-6, 8, Calu-1	Normal cells
		Skin fibroblasts (DX, EZ)
		Fetal fibroblasts (Flow 5000)
		Kidney epithelium (KM, KU,
		KY,NO)
		Type O erythrocytes
		Xenogeneic cells
		Sheep erythrocytes
		VERO (monkey kidney)
		CHO (hamster ovary)
		B16 (mouse melanoma)

allogeneic melanomas, suggesting that a transmissible agent, such as a virus, was not involved in FD antigen expression.

Characteristics of FD Antigen. Absorption tests with SK-MEL-131 cells heated for 5 min at 100°C showed that FD antigen was heat labile. Treatment with trypsin also destroyed FD antigen expression. No antigen could be detected at 0.5 h after trypsinization, but FD expression was partially restored at 12 h and reached maximal levels at 48–72 h after trypsinization. Reexpression of FD antigen was inhibited completely by cycloheximide and partially by monensin (Fig. 4). Neuraminidase treatment of adherent cells did not affect the expression of FD antigen but abolished the reactivity of R₂₄ antibody, which reacts with G_{D3} disialoganglioside (Fig. 5). Absorption tests indicated that FD antigen could be detected in conditioned medium from SK-MEL-131 cells and that centrifugation at 100,000 g for 2 h did not sediment the antigen.

A progressive decline in the expression of FD antigen was found during successive passaging of SK-MEL-131 cells (Fig. 6). The titer of FD serum (March 1981) tested on SK-MEL-131 cells at passage 7 (10 wk after culture initiated) was 1:1280. Eight weeks later, at passage 14, the titer was 1:320. After 8 wk of further culture, the titer decreased to 1:80, and after 10 mo of culture the titer was <1:10. This decline in antigen expression was related to time in culture and not to passage number; this was established by testing cells that had been

TABLE II
Allogeneic Typing with FD Serum: Lack of Reactivity of FD Serum with Cell Surface Antigens of 63 Cultured Cell Types

Negative cell lines	
Melanomas	Lung carcinomas
SK-MEL-13, 19, 21, 23(I), 26, 27, 28, 33, 37, 41, 75, 93(I), 113, 119, 127, 143, 147, 153, 158, 161	SK-LC-6, 13
Astrocytomas	Ovarian carcinomas
U251 MG, AN, AS	SK-OV-3, 2774
Neuroblastomas	Pancreatic carcinomas
SK-N-MC, SH-EP, LA-N-I,	ASPC-1, Capan-1
Renal carcinomas	Other carcinomas
SK-RC-6, 7, 9, 29, 31, 37, 39, 42, 44	MCF-7 (breast); GCC-SV(C) (choriocarcinoma); SK-UT-1 (uterine)
Colon carcinomas	B Lymphocyte lines (EBV-transformed)
HT29, SW48, SW676, SW1083, SW1417, SK-CO-1, 10, 11	FD, AU, AV
Bladder carcinomas	Normal cells
T24, 253-J, Scaber	Kidney epithelium
Teratocarcinomas	Fetal brain cells
Tera-1, Tera-2	Fetal fibroblasts
	Melanocytes

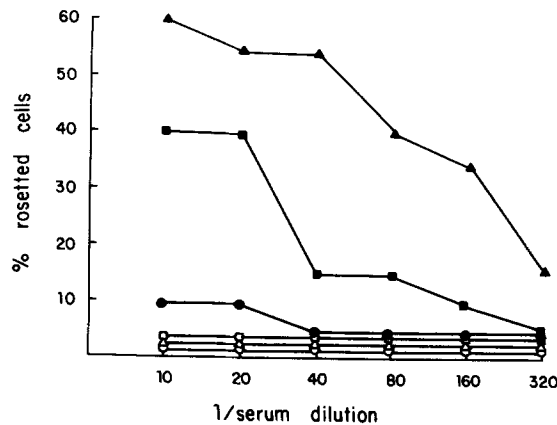


FIGURE 4. Reactivity of FD serum with SK-MEL-131 cells after trypsin treatment. Reexpression of FD antigen is completely inhibited by cycloheximide and partially inhibited by monensin. (○, △, □) Protein A assay performed 0.5 h after trypsinization; (●, ▲, ■) protein A assay performed 6 h after trypsinization. (△, ▲) untreated; (□, ■) monensin treatment; (○, ●) cycloheximide treatment. Serological test: protein A assay.

subcultured 14 vs. 4 times over a period of 6 mo. Reactivity with eight mouse monoclonal antibodies to unrelated cell surface antigens of SK-MEL-131 did not vary significantly during prolonged culture. Absorption tests indicated that FD antigen continued to be expressed in SK-MEL-131 cells even after a year in

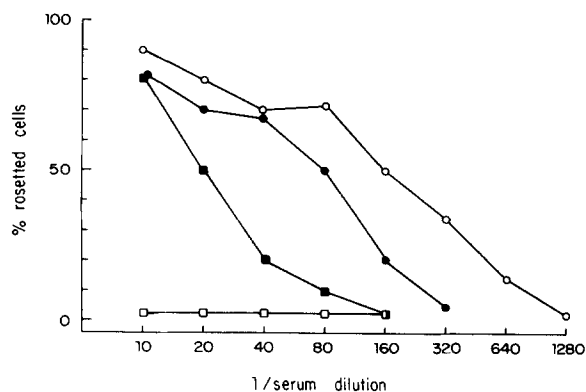


FIGURE 5. Reactivity of FD serum with SK-MEL-131 melanoma cells after neuraminidase treatment. Neuraminidase does not affect FD antigen expression, but abolishes reactivity with R₂₄ monoclonal antibody—detecting G_{D3} disialoganglioside. (●, ■) Untreated; (○, □) neuraminidase treatment. (○, ●) FD serum; (□, ■) R₂₄ monoclonal antibody. Serological test: protein A assay.

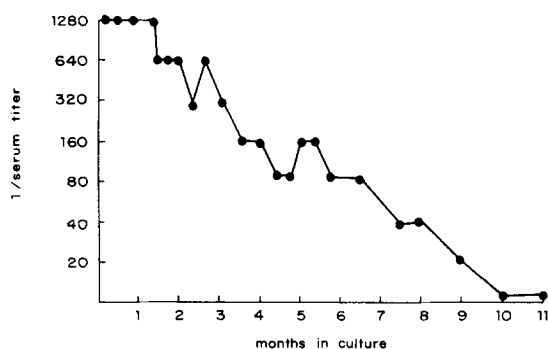


FIGURE 6. Reactivity of FD serum (March 1981) of patient FD with autologous SK-MEL-131 melanoma cells kept in continuous culture for 12 mo. Serological test: protein A assay.

continuous passage, despite the fact that no FD antigen could be detected in direct tests. SK-MEL-131 cells stored at -70°C after varying periods of culture retained their characteristic prestorage reactivity with FD sera.

Subcultures obtained by limiting dilution of early passages of SK-MEL-131 yielded lines with stable high or low antigen expression (Fig. 7). One line designated 1.36 has been cultured for 26 mo and maintains reactivity with FD serum at a titer of 1:320. 12 sublines of 1.36 have been isolated by limiting dilution, and the majority of these maintain high FD antigen expression (Fig. 7), although there is a variation in the amount of antigen expressed by individual sublines as indicated by direct tests and quantitative absorption analysis (Fig. 8).

A variety of agents were tested for their ability to influence FD antigen expression. SK-MEL-131 cells were incubated for 48 h in the presence of cholera toxin, dibutyryl cAMP, theophylline, phorbol-12-myristate-13-acetate, 5-azacytidine, retinoic acid, nerve growth factor, α -interferon, β -interferon, γ -interferon, phytohemagglutinin, or Con A. No change was found in the reactivity of

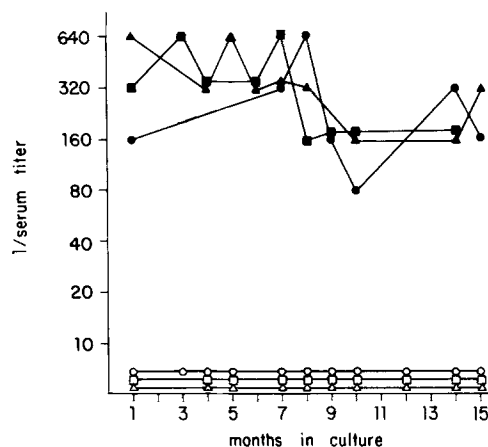


FIGURE 7. Reactivity of FD serum with subcultures of SK-MEL-131 melanoma cells. Lines 2.42 (○), 3.44 (□), 3.45 (△), and 1.36 were derived by limiting dilution from parental SK-MEL-131 after 2 mo in culture (passage 9). Sublines 1.36-1-5 (●), 1.36-1-9 (■), and 1.36-1-13 (▲) were derived by limiting dilution of the 1.36 line. Serological test: protein A assay.

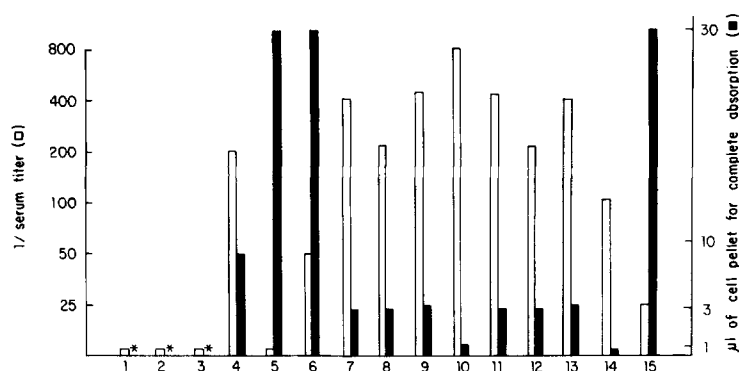


FIGURE 8. Expression of FD antigen by SK-MEL-131 melanoma cells: comparison of direct protein A assays and quantitative absorption analysis. Lines 2.42 (column 1), 3.44 (column 2), 3.45 (column 3), and 1.36 were derived from parental SK-MEL-131 cells and individual sublines (columns 4–15) were derived from the 1.36 line (see Fig. 7). (*) No absorption of FD reactivity with a 30 μ l cell pellet.

FD serum with SK-MEL-131 cell lines or sublines expressing low or high levels of FD antigen.

Biochemical Characterization of the FD Antigen. FD serum immunoprecipitated a 90,000 dalton component from 125 I-labeled membrane preparations of SK-MEL-131 cells (Fig. 9). This component showed an isoelectric point (pI) of 5.5 in two-dimensional gel electrophoresis. The 90,000 dalton component was not immunoprecipitated from lysates of SK-MEL-131 cells metabolically labeled with [35 S]methionine or [3 H]glucosamine, but it was detected with [3 H]mannose-labeled samples (Fig. 9). Similar to the restricted reactivity of FD sera for SK-MEL-131 cells in direct tests and absorption tests (see above), the immunoprecipitating activity of FD serum for labeled membrane preparations of SK-MEL-131 cells could be absorbed only by SK-MEL-131 cells, but not by any of the 14

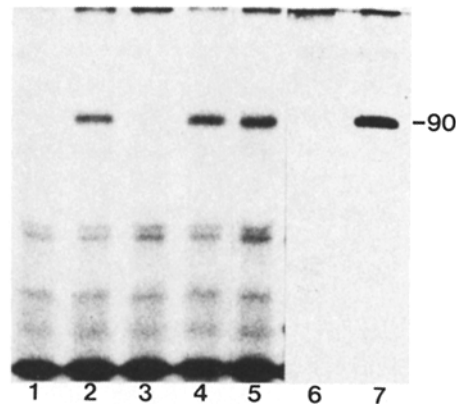


FIGURE 9. Immunoprecipitates of ^{125}I -labeled SK-MEL-131 cell membrane preparations (1-5) or [^3H]mannose-labeled SK-MEL-131 cell lysates (6-7) with normal human serum (1, 6), FD serum (March 1981) (2, 7), or FD serum absorbed with SK-MEL-131 cells (3), allogeneic melanoma SK-MEL-28 (4), or allogeneic bladder carcinoma 253-J (5). A component of 90,000 daltons is precipitated from ^{125}I - or [^3H]mannose-labeled SK-MEL-131 by FD serum. The reactivity of FD serum is specifically absorbed by SK-MEL-131 cells, but not by the two allogeneic tumor cell lines.

other cell lines tested, including four melanomas, three astrocytomas, four carcinomas, two cultures of normal fibroblasts, or by autologous EBV-transformed lymphocytes (Fig. 9). Further evidence indicating that the cell surface reactivity and immunoprecipitating activity of FD serum are directed against the same determinant(s) came from cotyping a series of 15 serum specimens from patient FD for both activities and finding a direct correspondence between titers in serological reactions and immunoprecipitating activity.

A 90,000 dalton component was also immunoprecipitated from spent medium obtained from SK-MEL-131 cultures grown in serum-free medium, which had been labeled with ^{125}I after ultracentrifugation and 10-fold concentration by pressure dialysis. This antigen preparation was fractionated on 14 different lectin columns (see Materials and Methods), and the effluent and eluate from each column were tested by immunoprecipitation. The 90,000 dalton antigen was found in the effluent in each case, and, with the exception of the Con A column, no antigen was detected in the eluate. With the Con A column, about one-third of the recovered antigen was in the eluate fraction. When a ^{125}I -labeled SK-MEL-131 cell membrane preparation was similarly fractionated on a Con A column, the antigen was not present in the effluent but was detected in the eluate, indicating a difference between the antigen present in the medium and the antigen present in the membrane.

Discussion

The description of the FD Class 1 melanoma antigen brings the number of such antigens that have been serologically defined in melanoma to six. Whether they belong to a single family of related molecules or represent products that are structurally unrelated remains to be determined. As previous attempts to characterize Class 1 tumor antigens by immunoprecipitation were unsuccessful,

the fact that FD antigen could be precipitated by autologous sera and identified as a 90,000 dalton glycoprotein is an important step in the analysis of these antigens. The generation of mouse monoclonal antibodies to the different epitopes on the 90,000 dalton glycoprotein may provide insight into some of the key questions about Class 1 tumor antigens: Are there structurally related products on all tumors? Do normal cells express such molecules? What is the relation of Class 1 tumor antigens to other polymorphic systems, such as major histocompatibility complex antigens and Ig products?

A class of tumor antigens known from work with chemically induced tumors of inbred rodents shows the same restriction to individual tumors as the Class 1 tumor antigens of humans. These individually specific antigens of mouse and rat tumors were initially detected by transplantation techniques involving graft rejection in preimmunized animals (19). Although much effort has gone into developing serological reagents to define these antigens, this has generally not been successful. However, studies with one tumor (BALB Meth A) have resulted in the production of a mouse antibody to a restricted antigen that parallels the distribution of the transplantation antigen on this tumor (20) and that is coded for by genes associated with the IgH region of chromosome 12 (21). Biochemical characterization of the Meth A transplantation antigen has been facilitated by the finding that the cytosol is a rich source of the Meth A antigen (22) and recent evidence indicates that molecules of approximately 75,000–86,000 daltons are responsible for transplantation rejection (23–25). Another example of an individually distinct transplantation antigen that has been serologically defined was recently reported by Srivastava and Das in studies of a chemically induced rat hepatoma (26). This antigen was found to have a molecular mass of 95,000–100,000 daltons. Whether the individually distinct antigens of experimental tumors and the Class 1 antigens of human tumors are related also remains to be determined.

Antibodies to Class 1 antigens have been identified with low frequency in patients with melanoma (1). Although this could indicate that such antigens are present on only a small proportion of melanomas, there are a number of other reasons why antibody is found so infrequently, including poor immunogenicity of Class 1 antigens, loss of antigen expression after *in vitro* culture, and the possibility that recognition of Class 1 antigens is primarily in the province of cellular immunity and not humoral immunity. Another fact that may contribute to the low frequency of detection of Class 1 reactivity relates to heterogeneity in the levels of antigen expressed by cultured tumor cells. This was emphasized in the study of the DX Class 1 melanoma antigen (6). Cell lines were established from six metastatic deposits occurring in patient DX. Only one of these lines expressed sufficient antigen to permit detection of autologous antibody, even though four of the five other DX lines expressed the same antigen at lower levels (as shown in absorption tests). The analysis of the AU Class 1 melanoma antigen illustrated another problem with regard to target cells in the study of these antigens: an unpredictable cyclic variation in the expression of AU antigen (2). AU melanoma cells that showed strong expression of AU antigen on one day (titer 1:128–1:512) became nonreactive the next day and then strongly reactive on a subsequent test. A number of variables that might account for this variation

in AU antigen expression were analyzed, including culture conditions, cell cycle, and passage generation, but no explanation emerged from these studies. The present analysis of the FD antigen also reveals a marked variation in antigen expression by FD melanoma cells, but, in this case, antigen loss was clearly related to time of in vitro culture. Early cultures of FD melanoma had strong Class 1 antigen expression, whereas later cultures showed progressive diminution in antigenic expression. Limiting dilution experiments with early FD cultures were carried out to determine whether subsequent antigen loss affected the population as a whole or was due to the emergence of low expression variants. Sublines of FD melanoma have been isolated that have maintained stable expression of FD antigen over a period of up to 26 mo, favoring the idea that the loss of antigen expression by FD cultures represents the outgrowth of low expression variants. To deal with this problem of antigen variability of melanoma cells in the search for Class 1 antigens, we are now deriving a panel of cloned lines from each melanoma specimen as the source of target cells for initial serological tests, rather than depending on a single culture from each specimen, as we have in the past.

The failure to immunoprecipitate serologically defined Class 1 melanoma antigens in previous work may have several explanations, including insufficient antibody levels, antibodies that do not immunoprecipitate effectively, poor radiolabeling of Class 1 tumor antigens, or the relative paucity of such molecules on melanoma cells. Although FD antigen could be labeled by ^{125}I and by [^3H]-mannose, it could not be immunoprecipitated from cells labeled with [^3H]-glucosamine or [^{35}S]methionine, and this could be due to a peculiar carbohydrate or amino acid composition or to a slow rate of synthesis, among other explanations. The latter possibility is suggested by the slow rate of reappearance of FD antigen after trypsinization of target cells. The finding of FD antigen in spent medium provides an additional source of antigen for purification and characterization. The antigen in spent medium differs from the membrane-bound form in binding less strongly to Con A. Whether FD is a true secreted protein or its presence in the culture medium is due to passive shedding from the cell membrane needs to be determined. Further biochemical and genetic characterization of the FD antigen and additional examples of similarly defined Class 1 melanoma antigens will be necessary before the significance of these antigens in terms of immune responses to tumors and relation to malignant transformation can be assessed.

Summary

Analysis of the humoral immune response of patients with melanoma has identified a small group of individuals with antibody to cell surface antigens that are restricted to autologous melanoma cells. These antigens, referred to as Class 1 or unique tumor antigens, are demonstrated by reactions between serum and cultured melanoma cells from the same patient and absorption tests with autologous and allogeneic normal and malignant cells to determine antibody specificity. Five Class 1 melanoma antigens have been defined to date, but insight into the nature of these antigens has been limited because antibodies identifying these antigens lacked detectable immunoprecipitating activity. We have now defined a Class 1 melanoma antigen (designated FD) that is immunoprecipitated by

autologous antibody. FD antigen is identified by an IgG antibody present in the sera of patient FD, and peak titers of this antibody in tests with cultured autologous melanoma cells are in the range of 1:2048. By absorption tests, FD antigen could not be detected on any other cell type, including 33 allogeneic melanomas. Prolonged culture of FD melanoma cells resulted in decreased expression of FD antigen, but sublines could be obtained with stable antigen expression. FD antigen is trypsin and heat sensitive, neuraminidase resistant, and is shed in the culture medium. Immunoprecipitation of ^{125}I -labeled cell membrane preparations revealed a 90,000 dalton component of pI 5.5. Serum immunoprecipitating activity could be absorbed by autologous melanoma cells but not by autologous B cells or allogeneic cell lines. A component of the same molecular mass could be precipitated from lysates of cells metabolically labeled with [^3H]mannose. The membrane form of the FD antigen binds strongly to Con A-Sepharose and can be eluted with methyl- α -D-mannoside. The identification of a precipitating Class I antigenic system of melanoma facilitates efforts to generate monoclonal antibodies to this tumor antigen and to clone its coding sequence.

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References

1. Old, L. J. 1981. Cancer immunology: the search for specificity. G. H. A. Clowes Memorial Lecture. *Cancer Res.* 41:361.
2. Carey, T. E., T. Takahashi, L. A. Resnick, H. F. Oettgen, and L. J. Old. 1976. Cell surface antigens of human malignant melanoma. I. Mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proc. Natl. Acad. Sci. USA.* 73:3278.
3. Shiku, H., T. Takahashi, H. F. Oettgen, and L. J. Old. 1976. Cell surface antigens of human malignant melanoma. II. Serological typing with immune adherence assays and definition of two new surface antigens. *J. Exp. Med.* 144:873.
4. Shiku, H., T. Takahashi, L. A. Resnick, H. F. Oettgen, and L. J. Old. 1977. Cell surface antigens of human malignant melanoma. III. Recognition of autoantibodies with unusual characteristics. *J. Exp. Med.* 145:784.
5. Takeyama, H., H. Shiku, L. A. Resnick, A. N. Houghton, A. P. Albino, H. F. Oettgen, and L. J. Old. 1981. Detection of a unique antigen and a differentiation antigen of malignant melanoma by autologous and allogeneic typing. *Proc. Am. Assoc. Cancer Res.* 21:300. (Abstr.)
6. Albino, A. P., K. O. Lloyd, A. N. Houghton, H. F. Oettgen, and L. J. Old. 1981. Heterogeneity in surface antigen expression and glycoprotein expression of cell lines derived from different metastases of the same patient: implications for the study of tumor antigens. *J. Exp. Med.* 154:1764.
7. Pfreundschuh, M., H. Shiku, T. Takahashi, R. Ueda, J. Ransohoff, H. F. Oettgen, and L. J. Old. 1978. Serological analysis of cell surface antigens of malignant human brain tumors. *Proc. Natl. Acad. Sci. USA.* 74:5122.
8. Ueda, R., H. Shiku, M. Pfreundschuh, T. Takahashi, L. T. C. Li, W. F. Whitmore,

- Jr., H. F. Oettgen, and L. J. Old. 1979. Cell surface antigens of human renal cancers defined by autologous typing. *J. Exp. Med.* 150:564.
9. Garrett, T. J., T. Takahashi, B. D. Clarkson, and L. J. Old. 1977. Detection of antibody to autologous human leukemia cells by immune adherence assays. *Proc. Natl. Acad. Sci. USA.* 74:5487.
 10. Naito, K., H. Yamaguchi, K. Horibe, H. Shiku, T. Takahashi, S. Suzuki, and K. Yamada. 1983. Autologous and allogeneic typing of human leukemia cells: definition of surface antigens restricted to lymphocytic leukemia cells. *Proc. Natl. Acad. Sci. USA.* 80:2341.
 11. Watanabe, T., C. S. Pukel, H. Takeyama, K. O. Lloyd, H. Shiku, L. T. C. Li, L. R. Travassos, H. F. Oettgen, and L. J. Old. 1982. Human melanoma antigen AH is an autoantigenic ganglioside related to G_{D2}. *J. Exp. Med.* 156:1884.
 12. Carey, T. E., K. O. Lloyd, T. Takahashi, L. Travassos, and L. J. Old. 1979. Solubilization and partial characterization of the AU cell surface antigen of human malignant melanoma. *Proc. Natl. Acad. Sci. USA.* 76:2898.
 13. Ogata, S., R. Ueda, and K. O. Lloyd. 1981. Comparison of ³H-glucosamine-labeled glycoproteins from human renal cancers and normal kidney epithelial cultures by the two-dimensional polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci. USA.* 78:770.
 14. Dippold, W. G., K. O. Lloyd, L. T. C. Li, H. Ikeda, H. F. Oettgen, and L. J. Old. 1980. Cell surface antigens of human malignant melanoma: definition of six new antigenic systems with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 77:6114.
 15. Lloyd, K. O., J. Ng, and W. G. Dippold. 1981. Analysis of the biosynthesis of HLA-DR glycoproteins in human malignant melanoma cell lines. *J. Immunol.* 126:2408.
 16. Cairncross, J. G., M. J. Mattes, H. R. Beresford, A. P. Albino, A. N. Houghton, K. O. Lloyd, and L. J. Old. 1982. Cell surface antigens of human astrocytoma defined by mouse monoclonal antibodies: identification of astrocytoma subsets. *Proc. Natl. Acad. Sci. USA.* 79:5641.
 17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
 18. O'Farrell, D. M., and P. Z. O'Farrell. 1977. Two-dimensional polyacrylamide gel electrophoresis fractionation. *Methods Cell Biol.* 16:407.
 19. Prehn, R. T., and J. M. Main. 1957. Immunity to methylcholanthrene-induced sarcomas. *J. Natl. Cancer Inst.* 18:769.
 20. DeLeo, A. B., H. Shiku, T. Takahashi, M. John, and L. J. Old. 1977. Cell surface antigens of chemically induced sarcomas of the mouse. I. Murine leukemia virus-related antigens and alloantigens on cultured fibroblasts and sarcoma cells: description of a unique antigen on BALB/c Meth A Sarcoma. *J. Exp. Med.* 146:720.
 21. Pravtcheva, D. D., A. B. DeLeo, F. H. Ruddle, and L. J. Old. 1981. Chromosome assignment of the tumor-specific antigen of a 3-methylcholanthrene-induced mouse sarcoma. *J. Exp. Med.* 154:964.
 22. DuBois, G. C., E. Appella, L. W. Law, A. B. DeLeo, and L. J. Old. 1980. Immunogenic properties of soluble cytosol fractions of Meth A Sarcoma cells. *Cancer Res.* 40:4204.
 23. DuBois, G. C., E. Appella, L. W. Law, A. B. DeLeo, and L. J. Old. 1981. Soluble antigens of BALB/c sarcoma Meth A: relationship between the serologically defined tumor-specific surface antigen (TSSA) and the tumor-associated transplantation antigen (TATA). *Transplant. Proc.* 13:1765.
 24. DuBois, G. C., L. W. Law, and E. Appella. 1983. Purification and biochemical properties of tumor-associated transplantation antigens from methylcholanthrene-induced murine sarcomas. *Proc. Natl. Acad. Sci. USA.* 79:7669.
 25. DuBois, G. C., E. Appella, and L. W. Law. 1984. Further characterization of purified

- tumor-associated transplantation antigens (TATA) from methylcholanthrene-induced sarcomas and an SV-40-induced sarcoma. *Proc. Am. Assoc. Cancer Res.* 25:279. (Abstr.)
26. Srivastava, P. K., and M. R. Das. 1984. The serologically unique cell surface antigen of Zajdela ascitic hepatoma is also its tumor-associated transplantation antigen. *Int. J. Cancer.* 33:407.