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Immunology

Cell surface antigens of human malignant melanoma: Mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells*

(serology/human cancer/tissue culture)

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ABSTRACT **We studied how frequently patients with malignant melanoma have specific antibody to cell surface antigens of cultured autologous melanoma cells as demonstrated by mixed hemadsorption assays. Of 35 patients studied over periods ranging from 1 to 36 months with Stage II, III, and IV disease, two showed consistent and high titered reactivity against autologous melanoma cells, two showed less consistent and intermediate reactivity, seven showed sporatic, low titered reactivity, and the remainder were consistently negative. A detailed analysis was carried out with the sera of one patient with sufficiently high titer against autologous melanoma cells. By direct tests and by absorption analysis with a variety of melanoma and nonmelanoma cell lines which included autologous fibroblasts, the antigen could not be demonstrated on any cell type other than the autologous melanoma.**

Considerable attention has been focused on the question of whether melanoma cells are marked by distinctive surface antigens. An array of techniques measuring humoral (1–9) and cellular immunity (10, 11) has been applied to the antigens of melanoma. Both individually specific antigens unique for each melanoma (1, 4–6, 9) as well as common antigens shared by several melanomas have been reported (2, 3, 8). Interpretation of these findings is commonly limited by the handicaps confronting human cancer immunologists in their effort to establish the specificity and significance of *in vitro* reactions. In experimental systems, the availability of inbred strains permitted the transplantation studies that established the existence of distinctive surface antigens in tumors. The serological definition of these antigens in the mouse and rat has also depended on inbred populations to provide the necessary reagents—antisera prepared by hyperimmunization with tumor cells, reference tumor cell lines maintained by transplantation, and normal cells of known antigenic composition. In the absence of these advantages, the serologist studying human cancer is attempting to evolve techniques and approaches that come to grips with the issue of cancer specificity. The present report illustrates the way our laboratory is seeking evidence for melanoma-distinctive surface antigens. In these studies, we have stressed three features: (*i*) *autologous reactions*—to eliminate the contributions of antibodies to histocompatibility antigens; (*ii*) *cultured melanoma cells*—to provide target cells that can be repeatedly tested; and (*iii*) *absorption tests*—to establish specificity by determining the occurrence of antigen on a range of normal and malignant tissues.

MATERIALS AND METHODS

Tissue culture

Melanoma Cells. Sterile tumor specimens were dissected free of adherent normal tissue and were finely minced in Earle's balanced salt solution. The resulting cell suspensions were washed three times in Earle's balanced salt solution and resuspended in complete medium consisting of Eagle's minimum essential medium containing 1% nonessential amino acids, and supplemented with 2 mM glutamine, 100 international units/ml of penicillin, and 100 μg/ml of streptomycin. Fetal calf serum (FCS) was included at a final concentration of 20% for primary cultures and 10% for more advanced cultures. Cultures were passed when confluent, at 7- to 10-day intervals, by harvesting with 0.05% crystallized trypsin (Tryptar 250,000 units per vial, Armour Pharmaceutical Co.) for early cultures or with 0.01% trypsin (Bacto Trypsin, Difco Laboratories) for established cultures.

Details concerning the melanoma lines used in this study will be published elsewhere. Successful cultures have been initiated from approximately 30% of all melanoma specimens submitted for tissue culture. Cultures were examined repeatedly for mycoplasma, fungi, and bacteria; contaminated cultures were discarded.

Skin Fibroblasts. Normal fibroblasts were obtained from skin biopsies which were trimmed of subcutaneous fat and minced. The fragments were placed in culture flasks in complete medium with 10% FCS until sheets of fibroblasts were obtained. Fibroblasts were harvested with 0.01% trypsin, transferred to new flasks, and then passaged in a manner comparable to melanoma cell lines.

Other Cell Lines. The following cell lines were also used: Me Wo, melanoma (11); SK-Mel-1, melanoma-suspension culture (12); RPMI-7931, melanoma originated by G. Moore at Roswell Park Memorial Institute; J-82, bladder carcinoma (13); T-24, bladder carcinoma (14); SK-L-7, lymphoblast suspension culture (15); and SK-RC-1 and SK-RC-4, two lines derived from renal carcinoma by H. Shiku of this Institute.

Serological procedures

Mixed Hemadsorption Assay. Mixed hemadsorption (MHA) was performed by the method of Espmark and Fagreus (16) as modified by Metzgar and Oleinik (17). Antisera prepared in monkey and baboon against sheep red blood cells were generously provided by A. Espmark of the National Bacteriological Laboratory, Stockholm, Sweden and G. Giraldo of this Institute. Goat anti-human immunoglobulin serum was purchased from Meloy Laboratories. Polyvalent anti-HLA serum was a gift of C. Whitsett of this Institute.

Cultured cells were harvested, washed, and distributed to the wells (1000 cells per well) of 3040 microtest II plates (Falcon Plastics, Oxnard, Calif.) and the plates were incubated at 37° in a CO_2 incubator. Multiple plates were prepared for each test and examined in MHA assays at several different time intervals

Abbreviations: FCS, fetal calf serum; MHA, mixed hemadsorption; P_i/NaCl, phosphate-buffered saline.

* This is paper no. I in a series.

Table 1. Summary of serological results on autologous melanoma cell lines

	Cell Lines		Sera		Results of MHA tests	
Patient	Designation	Passage generations studied	No. of serum samples	Months between first & last serum samples	No. positive/ total tests	Range of serum titers†
AA	SK-Mel-5	11–24	6	5	0/8	
AB	SK-Mel-6	9–21	8	36	1/11	1/16–1/32
AC	SK-Mel-7	1–12	2	4	0/14	
AD	SK-Mel-8*	1–51	13	23	0/24	
AE	SK-Mel-9	1–16	4	5	0/11	
AF	SK-Mel-11	7–16	1	1	0/7	
AG	SK-Mel-12	3–29	2	2	0/7	
AH	SK-Mel-13*	13–50	8	15	3/16	1/4
AI	SK-Mel-14*	3–36	4	9	0/12	
AJ	SK-Mel-17	5–30	2	1	0/6	
AK	SK-Mel-18	2–18	2	3	0/9	
AL	SK-Mel-19	3–13	9	21	0/9	
AM	SK-Mel-20*	1–17	2	8	0/7	
AN	SK-Mel-21	2–23	5	7	2/12	1/4–1/16
AO	SK-Mel-22	2–6	6	9	3/8	1/4
AP	SK-Mel-23	4–11	12	20	0/5	
AQ	SK-Mel-24*	1–12	9	5	0/11	
AR	SK-Mel-25*	1–17	2	3	0/9	
AS	SK-Mel-26*	2–14	20	25	0/11	
AT	SK-Mel-27*	1–24	5	6	8/22	1/4–1/64
AU	SK-Mel-28*	1–22	30	28	21/23	1/16–1/512
AV	SK-Mel-29*	1–12	13	10	0/7	
AW	SK-Mel-30	4–	1	1	0/1	
AX	SK-Mel-31	3–10	11	5	1/15	1/4
AY	SK-Mel-32	3–	1	1	0/4	
AZ	SK-Mel-33	2–24	10	21	0/10	
BA	SK-Mel-34	1–24	4	2	2/10	1/4–1/16
BB	SK-Mel-35	4–18	1	1	0/7	
BC	SK-Mel-36	1–18	22	16	1/9	1/16–1/64
BD	SK-Mel-37*	1–25	8	7	9/22	1/16–1/64
BE	SK-Mel-38	5–8	7	20	0/8	
BF	SK-Mel-39	1–9	17	6	0/8	
BG	SK-Mel-40	2–	2	3	0/4	
BH	SK-Mel-41*	1–3	13	25	0/2	
BI	MeWo*	18–29	7	10	17/20	1/64–1/128

* Companion lines of autologous skin fibroblasts were also cultured.
† Titers refer to the highest serum dilution showing 5% positive target cells.

after cell passage to insure detection of surface antigens with variable expression.

Serum dilutions were prepared in phosphate-buffered saline (P_i/NaCl) containing 5% FCS. The medium was decanted from the test plates and 0.05 ml of each serum dilution was added to replicate wells. The plates were then incubated at room temperature for 45 min and washed three times with P_i/NaCl-FCS. Indicator sheep red blood cells were suspended in P_i/NaCl-FCS (0.2% vol/vol) and 0.1 ml aliquots were added to each well. The plates were again incubated at room temperature for 45 min, agitated gently, washed three times in P_i/NaCl-FCS, and examined under a light microscope. Each well was scored for percent positive target cells, and for the intensity of the reaction. A cell was considered positive when ¼ or more of its perimeter was covered by indicator cells. For a well to be scored positive, 5% or more positive cells needed to be present. (This represents a stringent criteria as 1% positive cells in a well could usually be detected without question.)

Absorption Procedure (18). On the day of the absorption test, the serum to be examined was titrated against target cells by MHA and the dilution yielding 25% positive cells was determined. A dilution of serum two doubling dilutions below this end-point was prepared. One aliquot remained unabsorbed, while other aliquots were each mixed with an equal volume of packed cells. (In order to avoid possible enzymatic destruction of surface antigens, cultured cells used for absorption were harvested by mechanical scraping.) Absorptions were carried out, with frequent mixing, first at room temperature for 45 min, and then on ice for an equal period. The absorbing cells were then removed by centrifugation, and the absorbed and unabsorbed sera were serially diluted and tested against the target cells.

RESULTS

Mixed hemadsorption tests on autologous melanoma cells

Results of MHA tests on the serum and melanoma cell lines of 35 patients are summarized in Table 1. On the basis of serum

Table 2. Details of studies on serum reactivity to autologous melanoma cell lines by MHA assays

	MHA titer*												
	1972	1973	1974				1975						1976
Patient AU	Apr	Oct	Jan	Apr	Aug	Dec	Jan	Apr	Aug		Dec		Jan
Initial diagnosis: Nov 1971													
Recurrences	↑	↑						↑					
Tissue culture cell line								Δ SK-Mel-28					
Serum specimens		1	2	3	4	5	6	7	8	9	10	11 12	13
MHA results*: SK-Mel-28													
P-1			++	++	++	+	++	++	–				
P-3			+	+	++	++	–	–		++			
P-6				++	++		++	+	–	+			
P-9		++	++	++				++	–				
P-15			++			+	+	+		+	+	++ ++	++
P-18			++	++	++	+	++	++	–	+	+	++ +	++

	1973		1974				1975				1976
Patient AD	Sep	Dec	Jan	Apr	Aug	Dec	Jan	Apr	Aug	Dec	Jan
Initial diagnosis: 1959											
Recurrences	↑	↑		↑					↑		
Tissue culture cell line		Δ SK-Mel-8									
Serum specimens		1	2	3 4 5	6	7	8	9	10 11	12	
MHA results*: SK-Mel-8											
P-2		–	–	– –	–	–					
P-9			–	–	–	–	–	–	–		
P-14						–	–	–	– –	–	
P-31		–	–	– – –	–	–					
P-51		–	–	– – –	–	–	–				

* MHA titer: ++ = ≥1/64; + = 1/4 – 1/32; – = <1/4.

reactivity, four groups of patients can be defined. The majority, 24 out of 35 patients, showed no detectable antibody to autologous melanoma cells. Seven patients showed weak, sporatic reactivity, usually at low serum dilution (patients AB, AH, AN, AO, AX, BA, and BC). Two patients, AT and BD, showed intermediate reactions with titers in the range 1/16–1/64. Patients AU and BI belonged to the highly reactive group; their sera were consistently positive with titers as high as 1/128 to 1/512.

Details of studies on two patients are illustrated in Table 2.

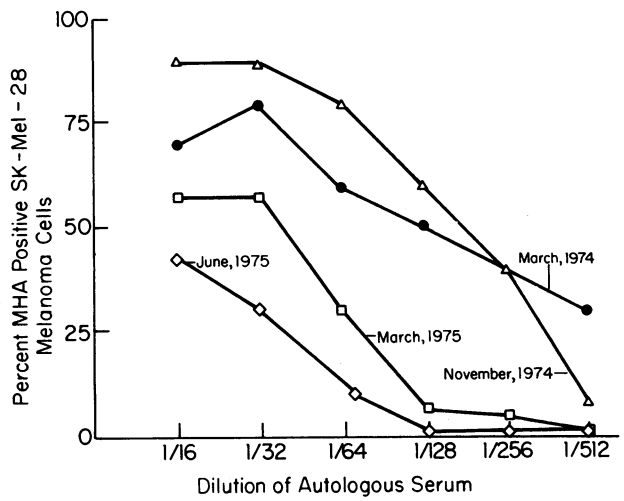

FIG. 1. Titrations of four serum samples from patient AU tested by MHA against a single passage of autologous SK-Mel-28 cells (passage 11). Sera were collected over a period of 15 months (see Table 3).

Mixed hemadsorption tests on autologous fibroblasts

Cultures of normal skin fibroblasts have been obtained from 13 of the melanoma patients, including five patients whose serum showed reactivity with autologous melanoma cells (Table 1). In no instance did we observe positive reactions with autologous fibroblasts.

Analysis of sera from patient AU

Serum Titration on Autologous Melanoma Cells. Fig. 1 illustrates titrations of four serum specimens taken over a period of 15 months and tested simultaneously on a single passage of autologous SK-Mel-28 cells. Tests on subsequent as well as earlier passages of autologous melanoma cells have shown the same relative levels of reactivity of these sera.

MHA Tests of AU Sera on Allogeneic Melanoma Cells.

Table 3. MHA reactivity of sera from patient AU against melanoma and nonmelanoma cultured cell lines

	Negative	
Positive	Melanomas	Nonmelanomas
SK-Mel-28 (AU)	SK-Mel-5,6,7,8,9,10, 11,13,14,15,16,19, 20,25,27,33,35,36, 37; MeWo; RPMI 7931	SK-RC-1, RC-4 SK-OV-3 T-24 J-82

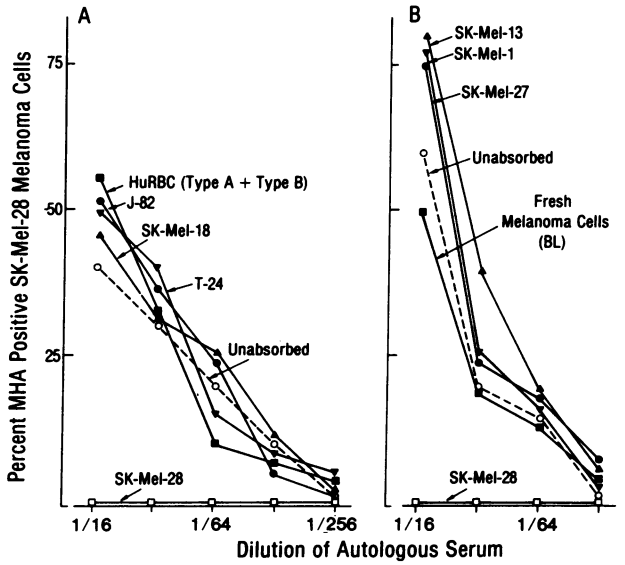

FIG. 2. Absorption of AU serum; results of two experiments. For each experiment, AU serum was diluted to 1:16 and divided into aliquots (see *Materials and Methods*). One aliquot was left unabsorbed (O- - -O) and the others were absorbed with equal volumes of packed autologous SK-Mel-28 cells (□——□), or various allogeneic cells (●——●, ▲——▲, ■——■, ▼——▼). After absorption, each aliquot of absorbed and unabsorbed serum was serially diluted and tested for residual MHA reactivity against SK-Mel-28 cells. Only autologous SK-Mel-28 absorbed MHA reactivity.

High titered (1/512) AU sera were repeatedly tested on a variety of cells, including 22 melanoma cell lines (Table 3). No cells other than autologous SK-Mel-28 showed positive reactions. In addition, AU sera were negative in cytotoxic tests against a panel of lymphocytes from 30 normal donors (C. Whitsett, personal communication).

Absorption Analysis of AU Sera. Fig. 2 illustrates two absorption tests with AU sera. Autologous SK-Mel-28 cells removed all MHA reactivity from AU sera; no absorption was seen with any other cell types. Table 4 summarizes the extensive absorption analysis with autologous, allogeneic, and xenogeneic cell types. Of the range of cells examined, only autologous SK-Mel-28 cells absored specific reactivity from AU sera.

Fluctuation in expression of AU antigen and alloantigens on SK-Mel-28 cells

Throughout this study, we have been impressed by the marked variation in surface antigen expression that SK-Mel-28 and other melanoma cell lines show in different passage generations, as well as during the same passage generation. This fluctuation is seen with both melanoma antigens and alloantigens (Fig. 3). At present, we know of no way to control this rapid and unpredictable variation. Its practical implications are clear. All cell lines must be tested repeatedly in serological tests before any confidence can be attached to negative findings.

DISCUSSION

A number of laboratories have reported that sera from patients with melanoma react with surface antigens of cultured melanoma cells. Many of these studies dealt with allogeneic reactions—i.e., reactions between serum and target cells from different individuals. We too, have observed a high frequency of positive reactions in allogeneic combinations, but on further analysis most of these were found to be due to alloantibodies. As a consequence of the difficulty, if not impossibility, at this stage in the development of human cancer serology to prove the specificity of an allogeneic reaction, we turned to tests of serum and melanoma cells from the same patient (autologous reactions). Of the 35 patients studied, only 11 showed reactivity to their cultured melanoma cells. Is this low frequency of autologous reactions a true reflection of the incidence of antibody in melanoma patients? We think not. The population we drew from was predominantly patients with Stages II and III melanoma, and it is possible that patients with more restricted disease would have shown higher reactivity. However, the technical problems involved in initiating cultures from primary biopsies—usually minute and required in their entirety for pathology examination, make direct proof of this point difficult. Another factor that might account for the low incidence of autologous reactivity is inadequate sensitivity of the serological assay. Although we have convinced ourselves that the MHA technique is a highly sensitive method for analysis of cell surface antigens and one that lends itself easily to tests for specificity, other techniques might detect antibody that MHA does not. Finally, the fact that cultured cells rather than cells directly from the surgical specimen were used should be considered in interpreting this study. What is the evidence that the cell lines were actually derived from melanoma cells? Synthesis of melanin, presence of premelanosomes, and characteristic epithelioid morphology are criteria we use. Growth in immunologically deficient mice is a more convincing characteristic and is being determined for each of our melanoma lines. Another possibility is that cultured tumor cells undergo a decrease in the expression of tumor antigens. However, results in animal systems point to the opposite conclusion—cultured cells express more tumor antigen, not less (19, 20). The striking fluctuation in the expression of cell surface antigens on cultured melanoma cells which range from strongly reactive to practically nonreactive, is another pitfall for the serologist. Positive sera can

Table 4. Summary of absorption tests on sera from patient AU: Results of absorption with autologous, allogeneic, and xenogeneic cells

Positive absorption	Negative absorption	
SK-Mel-28(AU)	**Normal autologous cells:** lymphocytes, platelets, red blood cells	**Microorganisms:** BCG
	Normal autologous cultured cells: AU fibroblasts	**Fresh melanoma cells:** Patients BJ, BK, BL, BM, BN
	Normal allogeneic cells: pooled buffy coat lymphocytes, pooled tonsil cells, A, B, or AB red blood cells	**Cultured melanoma cells:** SK-Mel-1, 11,13,18,20,21,23,26; MeWo
	Xenogeneic cells and serum: sheep red blood cells, FCS	**Cultured nonmelanoma cells:** T-24 (bladder cancer), J-82 (bladder cancer), SK-L-7 (B-cell line)

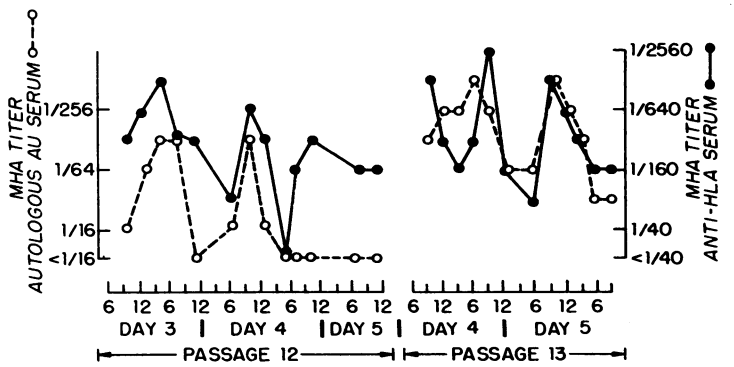

FIG. 3. Fluctuation in the expression of AU melanoma antigen and alloantigens. SK-Mel-28 cells were from passage 12 (left-side of figure) and passage 13 (right-side of figure). The cells were tested by MHA for expression of AU melanoma and HLA antigens at 3-hr intervals. In this figure, titer refers to the serum dilution giving ≥50% positive SK-Mel-28 target cells.

be missed if tests are performed at a time of low antigen expression. For this reason, repeated testing is essential before negative results can be considered valid.

Now that consideration has been given to the low frequency of positive reactions in this study, we must ask what significance can be attached to the reactions that were observed. With regard to specificity, the AU reaction is the most comprehensive analysis of a melanoma antigen to date. Direct MHA tests and extensive absorption analysis indicate that the AU antigen is restricted to autologous melanoma cells. No other melanoma or autologous, allogeneic, or xenogeneic tissue tested expresses AU melanoma antigen. However, two facts limit further speculation concerning the AU reaction: (*i*) the absence of the corresponding normal counterpart to melanoma cells, the vexing and perennial problem confronting the serologist studying melanoma. (*ii*) Our present ignorance with regard to the correlation between clinical course and the presence and titer of antibody. However, the present study was not meant to answer questions about the clinical significance of specific humoral immunity to melanoma cells—it was directed toward establishing whether such antibodies actually exist. Once analysis has advanced to the point of developing a serological classification of melanoma on the basis of its cell surface antigens, questions related to clinical relevance and application can be more profitably addressed.

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