CELL SURFACE ANTIGENS OF HUMAN MALIGNANT MELANOMA

II. Serological Typing with Immune Adherence Assays and Definition of Two New Surface Antigens*

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Study of malignant melanoma has dominated much of the activity in human cancer immunology (1–9). This reflects the widespread belief that melanoma represents an immunogenic tumor in man. Although many indications exist that melanoma patients respond to their tumor by developing humoral and cellular immunity, critical evidence that these immune reactions are directed to melanoma-specific antigens is lacking. Thus, for instance, interpreting results of tests between serum or lymphoid cells of one patient and melanoma cells of another is frequently difficult, if not at times impossible, because of the unknown participation of alloantigens in the reactions observed. Also, results restricted to melanoma biopsy material or primary cultures (in contrast to sequentially cultured melanoma cells) commonly pose questions related to specificity and reproducibility, since the analysis is necessarily limited by the number and type of tests that can be performed.

For this reason, we have directed our serological studies of cell surface antigens of melanoma to analyzing autologous reactions, i.e., serum and melanoma cells from the same patient. The need for repeated tests in substantiating the specificity and reliability of positive or negative reactions requires the ready and continuing availability of target cells, and this has been met by establishing melanoma and accompanying autologous skin fibroblasts in cell culture.

We have recently reported that 11 of 35 patients showed reactivity to autologous melanoma by mixed hemadsorption assays (10). Autologous fibroblasts from these same patients were negative. One patient (AU) showed a sufficiently high autologous reactivity to conduct an extensive absorption analysis of his serum. Tests on a range of malignant cell lines, melanoma and nonmelanoma, and normal cells, autologous and allogeneic, revealed that this melanoma surface antigen was restricted to AU melanoma cells.

The present report summarizes our experience with immune adherence (IA)[1] assays for the detection of specific antibody to cell surface antigens of melanoma.

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[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; IA, immune adherence; VBM, Veronal buffer medium.

Materials and Methods

Tissue Culture. For derivation of melanoma and other cell lines see Carey et al. (10) and Fogh and Trempe (11). Cells were cultured in Eagle's minimum essential medium supplemented with 2 mM glutamine, 1% nonessential amino acids, penicillin 100 IU/ml, streptomycin 100 μg/ml, and 15% fetal bovine serum (FBS). All cultures were checked repeatedly for mycoplasma, fungi, and bacteria; contaminated cultures were discarded.

Serology. IA assays were performed according to the method of Tachibana and Klein modified for monolayer target cells (12). Cultured cells were harvested with 0.25% trypsin, washed, and distributed to wells of Microtest II plates (no. 3040, Falcon Plastics, Oxnard, Calif.) so that each well received 500–1,500 cells. Plates were then incubated in a humidified 5% CO_2 incubator until use.

Serum titrations were prepared in Veronal buffer medium (VBM) with 5% FBS (VBM-FBS) on ice. Medium was decanted from the test plates, the wells washed three times with VBM-FBS, and 0.05 ml of each serum dilution was added per well. The plates were then incubated at the prescribed temperature (4, 24, or 37°C) for 1 h, washed three times with VBM-FBS, and then washed with VBM. Indicator cells (0.2% [vol/vol] human O-type erythrocytes, and 1/60 diluted guinea pig serum as a source of complement in VBM) were added in 0.05-ml aliquots to each well. The plates were further incubated at 37°C in a humidified 5% CO_2 incubator for 30 min. After incubation, the plates were agitated gently, washed once with VBM-FBS, and evaluated under a light microscope.

Individual target cells were scored as positive when 50% or more of the cell perimeter was surrounded by indicator cells. A well was considered positive only if at least 10% of the target cells met this criterion. In each experiment, control wells incubated with VBM-FBS alone or with human serum from normal pretested donors were included. The assay was considered evaluable only if these control tests showed less than 1% positive cells. Titers refer to the highest serum dilution showing 10% positive target cells.

Absorption tests were performed as previously described (10, 13, 14). Serum was absorbed at two doubling dilutions below its end point (titer). One aliquot of diluted serum remained unabsorbed, while other aliquots were mixed with cells to be tested. After preliminary experiments to determine optimal conditions, absorption was carried out on ice for 1 h with occasional shaking. For qualitative absorption, 0.15 ml diluted serum was absorbed with 0.1 ml packed cells. In quantitative absorption, diluted serum was absorbed with a range of counted numbers of viable cells. Cell cultures were harvested for absorption tests with 0.05% EDTA or 0.25% trypsin, as tests showed that the melanoma antigens detected in IA assays were not destroyed by EDTA or trypsin at these concentrations. After absorption, the cells were removed by centrifugation, and the absorbed and unabsorbed sera were retested on the appropriate target cell.

Results

IA Assays on Autologous Melanoma Cells

FREQUENCY OF DEMONSTRABLE IA ANTIBODY. Table I summarizes results of tests with sera from 18 melanoma patients on autologous melamona cell lines. To detect antibodies with differing affinity, incubation was carried out at 4, 24, or 37°C. Considering all tests together, autologous serum reactivity was demonstrable in 10 patients, with serum titers ranging from 1/4 to 1/160.

INFLUENCE OF TEMPERATURE ON IA REACTIVITY. On the basis of frequency and strength of reaction (Table I), three categories can be defined: (*a*) optimal reactivity at 24 or 37°C: sera from patients AL, AO, AR, AU, AV and BD; (*b*) optimal reactivity at 4°C: sera from patients AH and AX, and (*c*) no temperature preference: sera from patients AP and AT.

Fig. 1 illustrates the IA reactivity of sera from patients BD and AH at different incubation temperatures. BD sera reacted with autologous SK-MEL-37 cells at 24 and 37°C, but not at 4°C. In contrast, AH sera were far more reactive with autologous melanoma SK-MEL-13 at 4°C than at higher temperatures.

TABLE I

Immune Adherence Assays for Autologous Serum Reactivity to Surface Antigens of Cultured Melanoma Cells

Patient	Age	Sex	Designation of melanoma cell line	No. of sera tested (months between collection of first and last serum specimens)	Frequency of IA reaction and titer of antibody					
					4°C*		24°C		37°C	
					Pos./Test‡	Titer	Pos./Test	Titer	Pos./Test	Titer
AD	45	F	SK-MEL-8§	6(18)	0/3		0/3		0/4	
AH	56	M	SK-MEL-13§	16(24)	41/41	1/20–1/160	6/6	1/20–1/80	17/27	1/20–1/80
AI	49	M	SK-MEL-14§	5(9)	0/3		0/3		0/4	
BO	61	M	SK-MEL-15	1(0)	0/4		0/4		0/3	
AL	50	M	SK-MEL-19	5(12)	0/4		0/4		2/8	1/4
AO	59	M	SK-MEL-22	6(9)	0/3		0/4		4/6	1/4
AP	67	F	SK-MEL-23	6(6)	3/3	1/4	1/3	1/4	2/5	1/4
AQ	67	M	SK-MEL-24§	15(5)	0/3		0/3		0/3	
AR	43	F	SK-MEL-25§	4(3)	0/6		4/7	1/4	1/4	1/4
AS	53	F	SK-MEL-26§	6(4)	0/4		0/4		0/4	
AT	52	F	SK-MEL-27§	12(6)	4/5	1/4–1/16	5/6	1/4–1/32	8/10	1/4–1/32
AU	51	M	SK-MEL-28§	9(14)	0/3		0/6		6/12	1/4–1/32
AV	19	M	SK-MEL-29§	4(2)	1/3	1/4	9/10	1/4–1/16	4/6	1/4–1/16
AW	67	M	SK-MEL-30	1(0)	0/4		0/5		0/4	
AX	33	F	SK-MEL-31	6(5)	7/7	1/4–1/16	0/3		0/5	
BC	38	M	SK-MEL-36	13(12)	0/5		0/5		0/4	
BD	65	M	SK-MEL-37§	16(10)	1/3	1/4	29/29	1/8–1/96	25/27	1/8–1/96
BI	78	M	MeWo§	3(1)	0/3		0/3		0/3	

* Temperature for incubation of cells with autologous sera in IA assay.

‡ Number of positive tests/number of tests performed. Each test performed on all available serum specimens from individual patients.

§ Companion autologous skin fibroblast lines also established.

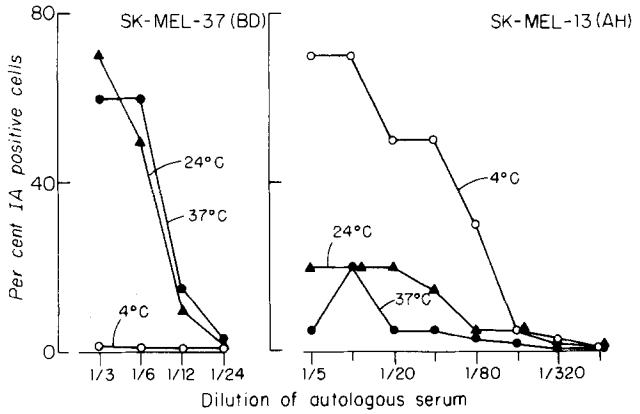

FIG. 1. Influence of temperature on IA reactivity. (1*a*, left) Reaction of BD serum on autologous SK-MEL-37 cells is optimal at 24 or 37°C; no reaction is seen at 4°C. (1*b*, right) AH sera shows stronger reactivity with autologous SK-MEL-13 cells at 4°C than at higher temperatures.

IA Assays on Autologous Skin Fibroblasts

Skin fibroblasts have been cultured from 11 patients in this study (Table I). No autologous serum reactivity has been observed with fibroblasts, including repeated tests with the serum of patients having antibody to autologous melanoma.

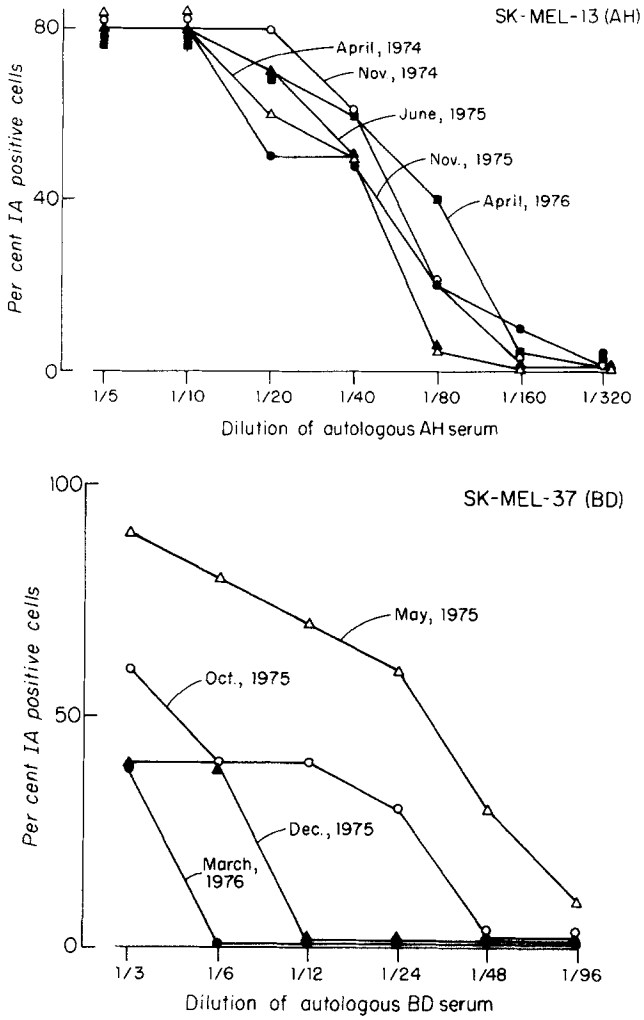

FIG. 2. Reactivity of sequential serum samples on autologous melanoma cells. (2*a*) Serum collected from patient AH over a period of 24 mo. (2*b*) Serum collected from patient BD over a period of 10 mo.

Analysis of AH and BD Sera: Definition of Two Serologically Distinct Cell Surface Antigens of Melanoma

In this series, sera from patients AH and BD were most strongly and consistently reactive against autologous melanoma cells and were therefore selected for detailed study.

REACTIVITY OF SEQUENTIAL SERUM SAMPLES ON AUTOLOGOUS MELANOMA CELLS. Simultaneous titrations of individual serum specimens collected from patients AH and BD over a period of 1–2 yr are shown in Fig. 2. The reaction of AH sera remained essentially unchanged over a 24-mo period (Fig. 2*a*). Sera from patient BD, however, showed a progressive fall in autologous melanoma reactivity (Fig. 2*b*).

QUALITATIVE ABSORPTION ANALYSIS OF AH AND BD SERA. Fig. 3 illustrates

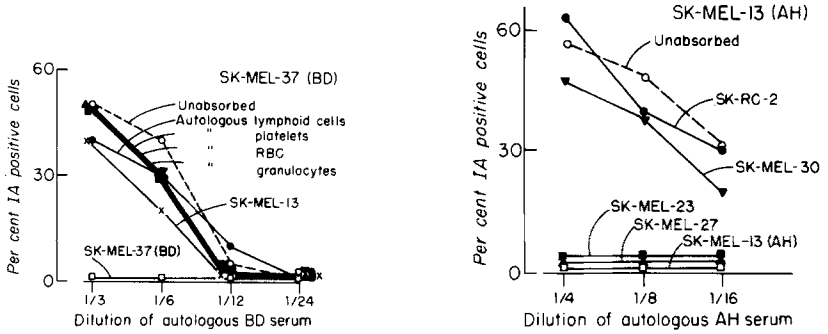

FIG. 3. Qualitative absorption analysis of BD and AH sera. Examples of individual absorption tests. (3*a*) Absorption of BD sera with autologous normal cells, autologous SK-MEL-37 cells, and allogeneic SK-MEL-13 cells. Only autologous SK-MEL-37 cells absorb IA reactivity. (3*b*) Absorption of AH sera with autologous SK-MEL-13 cells, allogeneic melanoma cell lines, and SK-RC-2 cells. Autologous melanoma and two allogeneic melanoma cell lines, SK-MEL-23 and SK-MEL-27, absorb IA reactivity.

single absorption tests with AH and BD sera, and Tables II and III summarize the extensive series of qualitative absorptions which have been performed with these sera. The antigen defined by the reaction of BD sera on autologous SK-MEL-37 cells has been found on no other cell type, autologous, allogeneic, or xenogeneic (Fig. 3*a* and Table II). The antigen defined by the reaction of AH sera on autologous SK-MEL-13 cells has been detected on 4 of the 11 allogeneic melanoma cell lines, but on no other normal or neoplastic cell tested (Fig. 3*b* and Table III).

Thus, absorption analysis has defined two serologically distinct cell surface antigens of melanoma; the BD antigen representing an individually distinct (private) melanoma antigen and the AH antigen representing a common (but not universal) melanoma antigen.

ESTIMATION OF AH ANTIGEN EXPRESSION BY AUTOLOGOUS AND AH$^+$ ALLOGENEIC MELANOMA CELLS. Fig. 4 illustrates quantitative absorption tests with 4 AH$^+$ melanoma cell lines. As measured by absorption capacity per cell, SK-MEL-23 cells and SK-MEL-29 cells have 10 times more AH antigen than autologous SK-MEL-13 cells; MeWo melanoma was found to have 10 times less.

Further Characteristics of AH and BD Melanoma Antigens

(*a*) Quantitative absorption tests show that AH and BD antigens are not destroyed by 0.25% trypsin or 0.05% EDTA; (*b*) Viable melanoma cells after controlled freezing in 10% dimethyl sulfoxide have sharply reduced levels of AH and BD antigens; the antigens promptly return on further culture; (*c*) In contrast to the marked fluctuation in expression of AU melanoma antigen noted in our previous study (10), AH and BD antigens show little fluctuation in antigen expression from passage to passage or within a single passage generation; (*d*) Melanoma cells passaged in normal human serum rather than FBS continue to express AH and BD antigens, as demonstrated by direct IA assays and absorption tests; and (*e*) Serum from 20 normal adults having no history of transfusion or pregnancy were negative for IA antibody to AH$^+$ cells and BD$^+$ cells.

TABLE II

Absorption of IA Reactivity from Serum of Patient BD Tested Against Autologous Melanoma Cells (SK-MEL-37)

Positive absorption	Negative absorption	
Autologous cultured melanoma cells:	Autologous normal cells:	Allogeneic cultured melanoma cells:
SK-MEL-37 (BD)	Lymphoid cells	SK-MEL-8 (AD)
	Platelets	SK-MEL-13 (AH)
	Granulocytes	SK-MEL-15 (BO)
	Erythrocytes	SK-MEL-19 (AL)
		SK-MEL-22 (AO)
	Autologous cultured normal cells:	SK-MEL-23 (AP)
	BD Fibroblasts	SK-MEL-27 (AT)
		SK-MEL-28 (AU)
	Allogeneic normal cells:	SK-MEL-29 (AV)
	Pooled buffy coat leukocytes	SK-MEL-30 (AW)
	A erythrocytes	SK-MEL-36 (BC)
	B erythrocytes	MeWo (BI)
	AB erythrocytes	
	O erythrocytes	Allogeneic cultured nonmelanoma cells:
		SK-RC-2 (renal cell ca)
	Xenogeneic cells and serum:	SK-RC-4 (renal cell ca)
	Sheep erythrocytes	T-24 (bladder ca)
	Guinea pig kidney	J-82 (bladder ca)
	Fetal bovine serum	ME-180 (cervical ca)
		SK-LC-LL (lung ca)
	Oncornaviruses:	SK-L7 (leukemia)
	SSV-1	Sal III (breast ca)
	RD-114	
	MuLV(AKR)	Allogeneic cultured normal cells:
		AT fibroblasts
	Microorganisms:	
	BCG	Allogeneic cultured fetal cells:
		WI-38 (fetal lung fibroblasts)
		PHEL-6 (fetal lung fibroblasts)

Discussion

On the basis of this study and our previous analysis (10), three distinctive antigens (or systems of antigens) can now be defined on the surface of melanoma cells. Two of these, AU and BD, belong to the category of individually unique antigens, i.e., present only on a single melanoma line. The other, AH, has a more widespread distribution, being detected on 5 out of 12 melanomas, and can be considered a common antigen shared by a class of melanomas. These methods are being applied to a growing series of melanomas and should, in time, provide a serological classification of the surface antigens of melanoma cells. Typing of new melanoma cell lines is presently being done by determining whether the cells specifically absorb reactivity from reference AU, BD, or AH sera, and the system will be extended as new melanoma surface antigens are recognized. Although this procedure of typing is both sensitive and accurate when dealing with a single antigenic determinant, it may well provide misleading information if our typing system is detecting a complex of determinants on a single molecule or a system of antigens on different molecules. Positive absorption simply signifies that the antigens being detected on the target cell are also present on the absorbing cell; if common as well as unique antigens are present on the target cell, then a common antigen on the absorbing cell (provided these cells lack the unique antigen) may well be missed. For this reason, absorption tests should be supplemented whenever possible by direct tests. This was possible in the case of AU typing serum since it lacked demonstrable alloanti-

TABLE III

Absorption of IA Reactivity from Serum of Patient AH Tested Against Autologous Melanoma Cells (SK-MEL-13)

Positive absorption	Negative absorption	
Autologous cultured melanoma cells:	Autologous normal cells:	Oncornaviruses:
SK-MEL-13 (AH)	Lymphoid cells	SSV-1
	Platelets	RD-114
Allogeneic cultured melanoma cells:	Granulocytes	MuLV(AKR)
SK-MEL-23 (AP)	Erythrocytes	
SK-MEL-27 (AT)		Microorganisms:
SK-MEL-29 (AV)	Autologous cultured normal cells:	BCG
MeWo (BI)	AH fibroblasts	Corynebacterium parvum
	Allogeneic normal cells: (from patients whose cultured melanoma cells absorb reactivity from AH serum)	Allogeneic cultured melanoma cells:
	AT and AV:	SK-MEL-8 (AD)
	Lymphoid cells	SK-MEL-15 (BO)
	Platelets	SK-MEL-19 (AL)
	Granulocytes	SK-MEL-22 (AO)
	Erythrocytes	SK-MEL-28 (AU)
	AP:	SK-MEL-30 (AW)
	Lymphoid cells	SK-MEL-37 (BD)
	Allogeneic cultured normal cells: (from patients whose cultured melanoma cells absorb reactivity from AH serum)	Allogeneic cultured nonmelanoma cells:
	AT fibroblasts	SK-RC-2 (renal cell ca)
	AV fibroblasts	SK-RC-4 (renal cell ca)
	BI fibroblasts	T-24 (bladder ca)
		J-82 (bladder ca)
	Allogeneic normal cells:	SK-LC-LL (lung ca)
	Pooled buffy coat leukocytes	SK-LU1 (lung ca)
	A erythrocytes	Sal III (breast ca)
	B erythrocytes	AlAb (breast ca)
	AB erythrocytes	ME 180 (cervical ca)
	O erythrocytes	HeLa (cervical ca)
		SK-OV-3 (ovarian ca)
	Xenogeneic cells or serum:	HT-29 (colon ca)
	Sheep erythrocytes	
	Guinea pig kidney	Allogeneic cultured normal cells:
	Fetal bovine serum	AU fibroblasts
		FB fibroblasts
		FB normal kidney cells
		Allogeneic cultured fetal cells:
		WI-38 (fetal lung fibroblasts)
		PHEL-6 (fetal lung fibroblasts)

bodies (10). Tests on 22 allogeneic melanoma cell lines, including those derived from patients BD and AH, were negative, confirming the conclusion from absorption tests that AU antigen was restricted to a single melanoma cell line. In cases where typing sera contain alloantibody, it should theoretically be possible to render these sera suitable for direct tests on particular melanoma cell lines by prior absorption with corresponding autologous leukocytes, platelets, or fibroblasts. Even under these circumstances, however, caution should be exercised in concluding that a positive reaction signifies common antigens, since melanomas may possess differentiation alloantigens not present on other autologous cell types. Undoubtedly, the biochemical characterization of these melanoma antigens, which is now feasible with the serological reagents that are available, will define in more precise terms what we mean by individually unique (private) versus common antigens, just as comparable studies of histocompatibility antigens have clarified these issues. Equally important, such studies may give insight into the genetic origin of these specific surface components of melanoma cells.

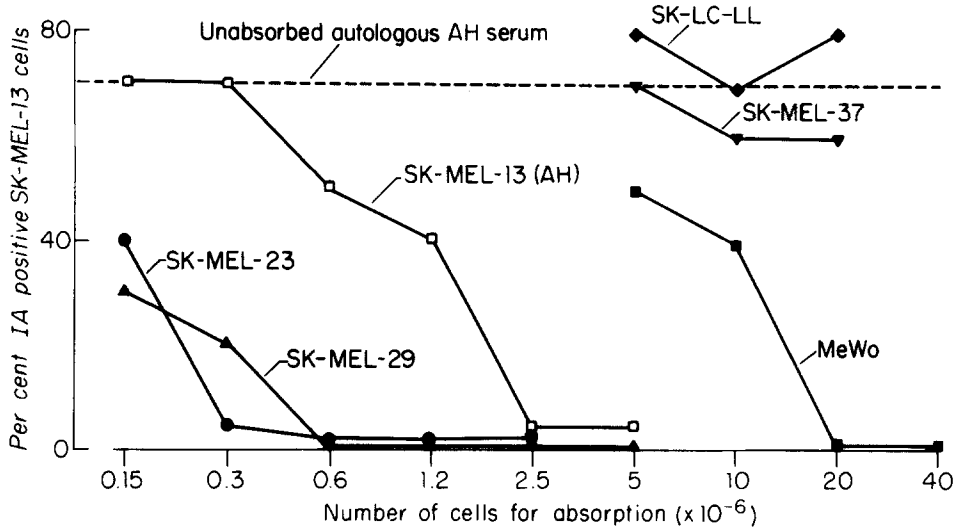

FIG. 4. Quantitative absorption test to compare expression of AH antigen by four AH+ melanoma cell lines. Absorption system (see text); aliquots of 1/5 diluted AH sera absorbed with counted numbers of autologous and allogeneic melanoma cells and tested on autologous SK-MEL-13 cells. Dotted line represents reactivity of unabsorbed diluted AH serum. SK-MEL-13 cells, SK-MEL-23 cells, and SK-MEL-29 cells are of similar size (0.1 ml packed cells = 2×10^7 cells); MeWo cells are smaller (0.1 ml packed cells = 4×10^7 cells). On the basis of absorption capacity per cell, allogeneic SK-MEL-23 cells and SK-MEL-29 cells express approximately 10 times more AH antigen than autologous SK-MEL-13 cells. MeWo cells absorb 10 times less.

As our serological analysis of melanoma surface antigens proceeds, it will become possible to determine whether the serological findings relate to clinical parameters, e.g., correlation of clinical status and course with presence and titer of antibody, temperature characteristics of antibody, and presence of common or private melanoma antigens. Although it is tempting to see if such associations could be made at this point, we feel that it would be misleading to do so until the serological classification is further advanced. If human cancer serology is to aspire to the accuracy, reproducibility, and usefulness of tissue typing and blood bank serology, the antigenic systems of human cancer must first be established with the precision that characterizes HLA serology and blood typing. When this has been accomplished, the application of immunological approaches to the study and therapy of human cancer will have been greatly facilitated.

Summary

Immune adherence assays revealed that 10 out of 18 melanoma patients had demonstrable antibody to surface antigens of autologous cultured melanoma cells, with serum titers ranging from 1/4 to 1/160. Autologous fibroblasts showed no reactions with these sera. Antibody from individual patients showed reproducible temperature preference for maximal reactivity. Two new melanoma antigenic systems were defined in this study. The first, BD, was restricted to autologous melanoma and could not be demonstrated in absorption tests on 12 allogeneic melanoma cell lines. The other, AH, was found on 5 of 12 melanomas

and represents a class of shared melanoma surface antigens. Neither BD nor AH antigen was found on normal cells from autologous, allogeneic, or xenogeneic sources or on any nonmelanoma tumor cell line. Methods are now available to develop a comprehensive serological classification of the surface antigens of melanoma.

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