Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 6114–6118, October 1980
Immunology

Cell surface antigens of human malignant melanoma: Definition of six antigenic systems with mouse monoclonal antibodies

(serology/tumor antigens/hybridomas/glycoproteins)

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ABSTRACT **Eighteen mouse monoclonal antibodies were selected for reactivity with cell surface antigens of the immunizing human melanoma cell line SK-MEL-28. Six distinct antigenic systems were defined by direct serological assays and absorption tests with a panel of 41 cell lines derived from normal and malignant human tissues. Biochemical analysis indicated that two of the antigens are glycoproteins with molecular sizes of 95,000 and 150,000 daltons (gp95 and gp150). Two other antigenic systems (O_5 and the R_{24} group) are associated with heat-stable molecules having the characteristics of glycolipids. The remaining two antigens (M_{19} and R_8) are heat labile, but molecular characterization has not been possible. Each of the antigenic systems has a distinctive pattern of distribution on various cell types, varying from a broad representation to a more restricted occurrence. O_5 appears to be a species antigen, being present on virtually every human cell type tested. gp95, gp150, M_{19}, and R_8 are found on a characteristic proportion of melanomas, astrocytomas, and epithelial cancers and on normal kidney cells. The antigen defined by the R_{24} antibody has the most restricted distribution of all. Reactivity is found with melanomas and astrocytomas, whereas epithelial cell types, fibroblasts, and cells of hematopoietic origin lack R_{24}. Although occurrence of gp95, gp150, M_{19}, and R_8 distinguishes a small subset of melanomas not expressing these antigens, R_{24} is found on all melanoma cells.**

This communication describes the results of our initial analysis of surface antigens of human malignant melanoma by using monoclonal antibodies produced by the hybridoma technology of Köhler and Milstein (1).

MATERIALS AND METHODS

Tissue Cultures. For derivation and culture of melanoma and other cell lines, see refs. 2 and 3. Melanocytes were cultured from postmortem specimens of adult human choroid by A. Houghton.

Serological Procedures. Protein A–mixed hemadsorption (PA-MHA) tests and absorption tests were performed as described (2, 3). For anti-IgG assays (IgG-MHA), indicator cells were prepared by conjugating the immunoglobulin fraction of rabbit anti-mouse Ig (DAKO, Copenhagen) to human O erythrocytes with 0.01% chromium chloride.

Immunizations. (BALB/c × C57BL/6)F_1 female mice were immunized with SK-MEL-28, an established melanoma cell line (2), by seven immunizations over a 7-mo interval with increasing numbers of cells. For the initial immunization, 2×10^6 melanoma cells were injected subcutaneously with Freund's complete adjuvant. The mice were further immunized at 4-wk intervals by intraperitoneal inoculation of melanoma cells. The final immunization consisted of 50×10^6 melanoma cells injected intraperitoneally and intravenously.

Cell Fusion Technique. Spleen cells from an immunized mouse (3 days after the final immunization) were mixed with MOPC-21 NS/1 cells at a ratio of 5–10:1, and the cell mixture (after pelleting by centrifugation) was suspended in 1 ml of 42% (wt/vol) polyethylene glycol in phosphate-buffered saline with 15% (vol/vol) dimethyl sulfoxide. After incubation for 1 min at room temperature, 15 ml of RPMI 1640 medium was added dropwise over a period of 3 min. After this, the cells were centrifuged and resuspended in complete RPMI 1640 medium containing 10% fetal bovine serum (supplemented with streptomycin, penicillin, pyruvate, and glutamine) and 0.2 mM hypoxanthine, 80 μM aminopterin, and 32 μM thymidine (HAT medium). Approximately 2×10^5 cells in 1 ml of HAT medium was added to wells of Costar plates (24 wells per plate) with attached feeder cells (BALB/c spleen). After 10 days in HAT medium, cultures were fed with complete RPMI 1640 medium containing 0.2 mM hypoxanthine and 32 μM thymidine.

Cloning of Hybridomas and Passage in Mice. After cell colonies 1–2 mm in diameter formed, samples of supernatants were harvested and assayed for antibody by using PA-MHA tests against an initial screening panel of 10 human cell lines—5 melanoma cell lines (SK-MEL-13, -27, -28, -31, -37) and 5 nonmelanoma cell lines (SK-RC-6 and -7, ME-180, EBV-B cells, and skin fibroblasts). Cells from wells containing antibody were passaged at low cell density (two to five cells per well) and, if antibody continued to be detected, the cells were cloned (one cell per well) two consecutive times. Cultures of cloned hybridomas were injected subcutaneously into *nu/nu* mice (Swiss background) and also were stored in liquid nitrogen. Sera from mice with progressively growing tumors were collected, stored at either −20°C or −70°C, and used for serological and biochemical analysis. Sera were obtained from mice prior to tumor inoculation, and only mice with low levels of preexisting natural antibody against human cells were used.

Immunoprecipitation Procedures. SK-MEL-28 cells were labeled as follows: (*i*) surface labeling with ^{125}I by the lactoperoxidase procedure (4), (*ii*) metabolic incorporation of [^{35}S]methionine (Amersham, 1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) by using 250 μCi in 10 ml of methionine-free minimal essential medium containing 1% fetal bovine serum for 16 hr, and (*iii*) metabolic incorporation of [^3H]glucosamine (New England Nuclear, 30–60 Ci/mmol) by using 150 μCi in 10 ml of minimal essential medium with 10% (vol/vol) fetal bovine serum for 72 hr. Labeled cells were extracted with 0.1% Nonidet P-40 in 0.15 M NaCl/0.01 M Tris, pH 7.2 (Tris buffer) containing 0.1 mM phenylmethylsulfonyl fluoride, and the solution was clarified by centrifugation at 100,000 × *g* and filtered through a 0.22-μm filter. Extracts of [^{35}S]methionine-

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Abbreviations: HAT medium, hypoxanthine/aminopterin/thymidine medium; MHA, mixed hemadsorption tests; PA-MHA, protein A–mixed hemadsorption tests.

labeled cells were also fractionated on a concanavalin A-Sepharose (Pharmacia) column, and samples eluted with 0.2 M α-methyl D-mannoside were used in immunoprecipitation experiments. Immunoprecipitation was carried out by mixing a portion of the cell extract (5–10 × 10^5 cpm) with 5 μl of mouse antibody, 15 μl of rabbit anti-mouse Ig (Cappel Laboratories, Cochranville, PA) serum, and 500 μl of 0.1% Nonidet P-40 in Tris buffer with 1% gamma globulin-free fetal bovine serum. [Before use, cell extracts were precleared with 5 μl of normal mouse serum, 15 μl of rabbit anti-mouse Ig, and 300 μl of 10% (wt/vol) *Staphylococcus aureus* suspension (New England Enzyme Center, Boston)]. Immune complexes were isolated by using *S. aureus*, and the labeled components were detected by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography as described (5). Two-dimensional electrophoresis (6) was carried out using a mixture of ampholytes, pH 3.5–10.0 (LKB)/pH 2.5–5.0 (Pharmacia), 3:2 (vol/vol).

Solubilization and Gel Filtration of SK-MEL-28 Antigens. Washed monolayer cultures of SK-MEL-28 cells were incubated for 10 min at 37°C with 0.2% sodium deoxycholate in Tris buffer and 1 mM EDTA (20 ml per 490-cm^2 roller flask). Cellular debris was removed by centrifugation at 2000 × *g* for 10 min, and the supernatant was incubated for 5 min at 37°C with papain (0.8 unit/ml of extract, Worthington), 0.4 M cystine, and 1 mM EDTA. Digestion was terminated with 0.4 M iodoacetamide, and the supernatant was clarified by ultracentrifugation at 100,000 × *g* for 1 hr, dialyzed extensively against Tris buffer, and concentrated by using an Amicon pressure system (PM 10 membrane). Extracts were then applied to Sephacryl S-200 columns (86 × 26 cm) equilibrated with Tris buffer containing 0.02% sodium deoxycholate. Every five fractions (2 ml each) were pooled, concentrated to 1 ml, and tested for antibody inhibitory activity in PA-MHA and IgG-MHA assays (see ref. 7 for inhibition tests and definition of inhibitory units).

RESULTS

Eighteen monoclonal antibodies detecting cell surface antigens of the immunizing melanoma cell line SK-MEL-28 were analyzed in this series. Antibodies reacting with HLA, Ia, or β_2m antigens were excluded during initial screening. The fine serological specificity of the 18 antibodies was tested on a panel of 41 established human cell lines (16 melanomas, 5 astrocytomas, 13 epithelial cancers, 6 B- and T-cell lines, and 1 fetal cell line) and on early cultures of fetal brain, adult kidney epithelium, skin fibroblasts, and melanocytes. In addition to cultured cells, we examined erythrocytes, peripheral blood leukocytes, and preparations of fetal liver, fetal brain, and adult brain. In most cases, serological analysis consisted of both direct tests and absorption tests. (Cultured melanocytes could not be obtained in sufficient number for absorption tests and preparations of adult human brain are not suitable for direct tests.)

Except for antibody O_5 (see below), no reactions were observed with A, B, AB, or O human erythrocytes in direct hemagglutination tests and in absorption tests. Sheep erythrocytes, lyophilized guinea pig kidney, and purified preparations of human blood groups A, B, and O, Le^a glycoproteins, and pneumococcal XIV polysaccharide were negative in absorption and inhibition tests. The possibility that fetal bovine serum components on cultured cells were being detected was ruled out by growing target cells in human serum for more than three passages before testing; no change in serological reactivity was observed.

These serological studies, in conjunction with immunoprecipitation analysis of radiolabeled cell extracts and antibody inhibition tests with solubilized antigen, indicate that the 18 monoclonal antibodies detect 6 antigenic systems. Two of the

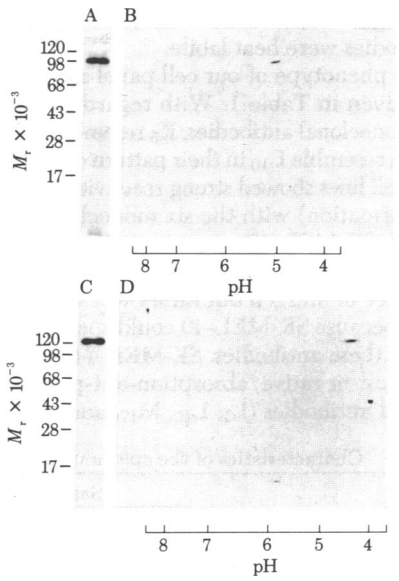

FIG. 1. Autoradiograms of [^{35}S]methionine-labeled glycoproteins from lysates of SK-MEL-28 immunoprecipitated by monoclonal antibody I_{12} (*A* and *B*) and by monoclonal antibody N_9 (*C* and *D*). (*A* and *C*) NaDodSO$_4$/polyacrylamide gel electrophoresis. (*B* and *D*) Two-dimensional polyacrylamide gel electrophoresis. Molecular weight markers: β-galactosidase (120,000), phosphorylase (98,000), bovine serum albumin (68,000), ovalbumin (43,000), concanavalin A (28,000), and myoglobin (17,000).

systems are glycoproteins with molecular sizes of 95,000 daltons (gp95) and 150,000 daltons (gp150), and two systems have characteristics of glycolipid antigens. The biochemical nature of the remaining two antigenic systems has not been determined.

gp95 Antigenic System. Six monoclonal antibodies [$I_{12(\gamma 2b)}$, $K_{5(\gamma 1)}$, $L_{1(\gamma 1)}$, $L_{10(\gamma 1)}$, $M_{17(\gamma 1)}$, and $R_{19(\gamma 1)}$] precipitated a 95,000-dalton component from labeled extracts of SK-MEL-28 (see Fig. 1 for I_{12}). Gel filtration chromatography of antigen solubilized by limited papain digestion of SK-MEL-28 showed inhibitory activity in the same molecular weight range (see Fig. 2 for I_{12} and L_{10}). Because the antigen detected by these monoclonal antibodies bound to concanavalin A and could be labeled by surface ^{125}I iodination and by metabolic incorporation of [^{35}S]methionine or [^{3}H]glucosamine, it was concluded that the 95,000-dalton component is a cell surface glycoprotein (gp95). Both I_{12} and L_{10} precipitated gp95 components with the same isoelectric point, pI 5.0 (see Fig. 1 for I_{12}), even though they showed differences in their serological reactivities (see

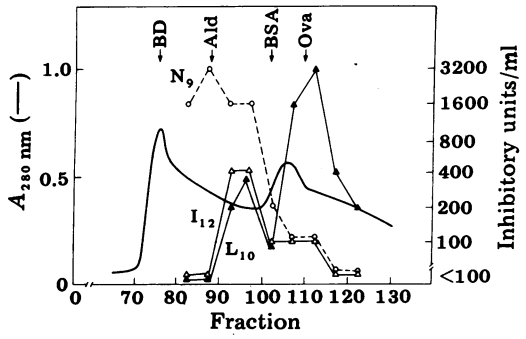

FIG. 2. Chromatography of sodium deoxycholate/papain extracts of SK-MEL-28 cells on Sephacryl S-200 columns. Fractions were assayed for antibody inhibitory activity with I_{12}, L_{10}, and N_9 monoclonal antibodies. Molecular weight markers: BD (blue dextran), 2,000,000; Ald (aldolase), 158,000; BSA (bovine serum albumin), 68,000; and Ova (ovalbumin), 43,000. O---O, N_9; △—△, I_{12}; ▲—▲, L-10.

below). The gp95 determinants detected by these six monoclonal antibodies were heat labile.

The gp95 phenotype of our cell panel as analyzed with I_{12} and L_{10} is given in Table 1. With regard to the other gp95-detecting monoclonal antibodies, K_5 resembles I_{12}, whereas L_1, M_{17}, and R_{19} resemble L_{10} in their pattern of reactivity. Eleven melanoma cell lines showed strong reactivity (titer and strength of rosette formation) with the six monoclonal antibodies detecting gp95. SK-MEL-40 was not reactive with I_{12}, M_{17}, and R_{19} in direct tests. Absorption tests indicated that this was not due to absence of antigen but rather to a low level of antigen expression, because SK-MEL-40 could specifically absorb the reactivity of these antibodies. SK-MEL-44 also demonstrated this direct-test-negative/absorption-test-positive phenotype with selected antibodies (L_1, L_{10}, M_{17}, and R_{19}). Three other melanoma lines (SK-MEL-29, -31, and -42) appeared to lack any gp95 expression as indicated by both direct tests and absorption tests. Comparable analysis of other human cell types showed that gp95 determinants can be demonstrated on a wide range of cell types, both normal and malignant. As in the case of melanoma cell lines, levels of gp95 ranged from easily detectable (e.g., T-24 and normal kidney epithelium) to detectable only by absorption tests (e.g., HT-29) to not demonstrable (e.g., AlAb and SK-OV-3).

Table 1. Characteristics of the gp95 antigenic system*

Cells	Serologic tests: Ab $I_{12(\gamma 2b)}$ Titer $\times 10^{-3}$†	Abs‡	Ab $L_{10(\gamma 1)}$ Titer $\times 10^{-3}$†	Abs‡
Melanomas				
SK-MEL-28,19,57,64,94	300	+	300	+
SK-MEL-13,23,37,61,79,90	12	+	60	+
SK-MEL-40	−	+	60	+
SK-MEL-44	−	−	−	+
SK-MEL-29,31,42	−	−	−	−
Astrocytomas				
AS,U138MG,U373MG	12	±	12	+
AN,AJ	−	−	−	−
Epithelial cancers				
Renal: SK-RC-2	60		300	
SK-RC-9	12		300	+
SK-RC-6	−	−	2	±
SK-RC-7	−		−	
Breast: BT-20, MCF-7	−	−	−	+
AlAb	−	−	−	−
Bladder: T-24	60	+	60	+
Ovarian: SK-OV-3	−	−	−	−
Colon: HT-29	−	+	−	+
Cervix: ME-180	−	−	−	+
Lung: SK-LC-LL	−	−	−	−
Testicle: SK-GR-1	−	−	−	+
Lymphoblastoid cells				
T cell: MOLT 4		−		+
T-45		−		−
EBV-B cell§: AH,BD,BT	−	−	−	+
Normal cells				
Melanocytes	−		−	
Fibroblasts	−	−	−	+
Kidney epithelium: 1,2	60		300	
Brain		±		±
Leukocytes		−		+
Fetal fibroblasts	−	±	−	±
Fetal brain	−	−	−	−
Fetal liver		−		−

* Antibody (Ab) subclass was determined on culture supernatants by double diffusion in agar with anti-Ig heavy chain specific reagents (Bionetics, Kensington, MD). Heat stability of antigenic determinants: labile (cells were exposed to 100°C for 5 min and assayed for residual antigen activity in absorption tests). M_r by immunoprecipitation: 95,000. M_r by gel filtration: 70,000–100,000 (inhibitory activity was also found in the M_r 35,000–55,000 region, see text).

† −, No reaction in direct tests at a serum dilution of 1:50.

‡ Abs, absorption tests. See ref. 3 for details. Serum (diluted according to end point) was absorbed with the indicated cell type and tested for residual activity for SK-MEL-28 target cells. +, Complete absorption; ±, partial absorption; −, no absorption.

§ For direct tests with EBV-B cells, PA-MHA indicator cells were used.

Although the overall pattern of serological reactivity of the six different monoclonal antibodies detecting gp95 was similar, there were evident differences. For instance, the gp95 determinant detected by L_{10} on several epithelial cancers in absorption tests was not detected by I_{12}. Further indication that the specificities of I_{12} and L_{10} were different comes from antibody inhibition tests with sodium deoxycholate/papain-solubilized preparations from SK-MEL-28. Fractionation of this material by gel filtration showed that inhibitory activity for I_{12} was confined to one peak, whereas inhibitory activity for L_{10} was found in two molecular weight ranges (Fig. 2). These results suggest that at least two distinct determinants on the gp95 molecule are recognized by this series of monoclonal antibodies.

gp150 Antigenic System. Four monoclonal antibodies [$N_{9(\gamma 1)}$, $R_{23(\gamma 1)}$, $Q_{14(\gamma 1)}$, and $Q_{24(\gamma 1)}$] precipitated a 150,000-dalton component from labeled extracts of SK-MEL-28 (see Fig. 1 for N_9). A fifth antibody that did not have precipitating activity [$J_{11(\gamma 1)}$] is provisionally included in this group because of its related serological reactivity. Gel filtration chromatography of antigen solubilized by limited papain digestion of SK-MEL-28 showed inhibitory activity in the range 110,000–150,000 daltons (see Fig. 2 for N_9). Surface labeling with ^{125}I, metabolic labeling with [^{35}S]methionine and [^{3}H]glucosamine, and concanavalin A binding indicate that the 150,000-dalton component is a cell surface glycoprotein (gp150). Both N_9 and Q_{24} precipitated gp150 components with the same isoelectric point, pI 4.2 (see Fig. 1 for N_9), even though they showed differences in their serological reactivities (see below). The gp150 determinants detected by these five monoclonal antibodies were heat labile.

The gp150 phenotype of our cell panel, as analyzed with N_9 and Q_{24}, is given in Table 2. R_{23} and Q_{14} resembled N_9 in their pattern of reactivity. The distribution of gp150 determinants is clearly distinguishable from that of gp95 (e.g., SK-MEL-29 and -42, two melanoma cell lines that are gp95-negative, are strongly gp150-positive; SK-MEL-79, which is gp150-negative, is strongly gp95-positive). gp150 determinants were found on a wide range of other cell types of normal and malignant origin. As with gp95, levels of gp150 on different cell types (even on those derived from the same cell lineage) varied from easily demonstrable in direct tests to demonstrable only by absorption to not detectable. Absorption analysis indicated that at least two distinguishable determinants were recognized on gp150 by this series of monoclonal antibodies. The gp150 determinant detected by N_9, R_{23}, and Q_{14} appeared to be closely related or identical and could be distinguished from that detected by Q_{24} on the basis of absorption tests with ME-180, SK-OV-3, and SK-LC-LL epithelial cancer lines.

M_{19} and R_8 Antigenic Systems. The reactivity of $M_{19(\gamma 1)}$ and $R_{8(\gamma 1)}$ in direct serological tests and absorption analysis (Table 3) defined two distinct antigenic systems unrelated to gp150, gp95, or the other surface antigens detected by our present battery of monoclonal antibodies. The antigenic determinants recognized by M_{19} and R_8 were heat labile. In the case of M_{19}, gel filtration chromatography of antigen solubilized by limited papain digestion of SK-MEL-28 showed inhibitory activity for M_{19} antibody in the 50,000–70,000 molecular weight range.

Table 2. Characteristics of the gp150 antigenic system*

Cells	Serological tests: Ab $N_{9(\gamma 1)}$ Titer $\times 10^{-3}$†	Ab $N_{9(\gamma 1)}$ Abs‡	Ab $Q_{24(\gamma 1)}$ Titer $\times 10^{-3}$†	Ab $Q_{24(\gamma 1)}$ Abs‡
Melanomas				
SK-MEL-28,13,19,23,29, 37,40,42,57,64,90.94	1500	+	1500	+
SK-MEL-61	60	+	60	+
SK-MEL-44	–	+	–	–
SK-MEL-31,79	–	–	–	–
Astrocytomas				
AS,U138MG	1500	+	1500	+
U373MG	300	+	60	+
AN	60	+	–	+
AJ	–	–	–	–
Epithelial cancers				
Renal: SK-RC-6	300		60	
SK-RC-2,7	–		–	
SK-RC-9	–	–	–	–
Breast: BT-20,MCF-7,AlAb	–	–	–	–
Bladder: T-24	60	+	60	+
Ovarian: SK-OV-3	–	+	–	–
Colon: HT-29	60	+	60	+
Cervix: ME-180	–	+	–	–
Lung: SK-LC-LL	–	+	–	–
Testicle: SK-GR-1	–	+	–	–
Lymphoblastoid cells				
T cell: MOLT 4		+		–
T-45		–		–
EBV-B cell§: BD,BT	–	+	–	+
AH	–	+	–	–
Normal cells				
Melanocytes	1		1	
Fibroblasts	2	+	0.5	+
Kidney epithelium: 1,2	1500		1500	
Brain		+		±
Leukocytes		–		–
Fetal fibroblasts	–	+	–	+
Fetal brain	300	+	60	+
Fetal liver		+		+

* Methods, layout, and footnotes as in Table 1. Heat stability: labile. M_r by immunoprecipitation: 150,000. M_r by gel filtration 110,000–150,000.

Table 3. Characteristics of the M_{19} and R_8 antigenic systems*

Cells	Serological tests: Ab $M_{19(\gamma 1)}$ Titer $\times 10^{-3}$†	Ab $M_{19(\gamma 1)}$ Abs‡	Cells	Serological tests: Ab $R_{8(\gamma 1)}$ Titer $\times 10^{-3}$†	Ab $R_{8(\gamma 1)}$ Abs‡
Melanomas			**Melanomas**		
SK-MEL-28,64	1500	+	SK-MEL-19,28, 42,90	1500	+
SK-MEL-23,29,37	60	+	SK-MEL-13,64	60	+
SK-MEL-44,57,61, 90,94	12	+	SK-MEL-23,57,79	12	+
SK-MEL-31,79	–	+	SK-MEL-29,37,40	0.5	±
SK-MEL-13,19,40,42	–	–	SK-MEL-31,61	–	+
			SK-MEL-44,94	–	–
Astrocytomas			**Astrocytomas**		
AS,U138MG, U373MG	60	+	AS,U138MG	2	+
AN,AJ	–	+	AJ,AN,U373MG	–	–
Epithelial cancers			**Epithelial cancers**		
Renal: SK-RC-6,9	60	+	Renal: SK-RC-2,6, 7,9	60	+
SK-RC-2	–				
Breast: AlAb, BT-20, MCF-7	–	–	Breast: AlAb, BT-20, MCF-7	–	–
Bladder: T-24	–	–	Bladder: T-24	60	+
Ovarian: SK-OV-3	12	+	Ovarian: SK-OV-3	–	–
Colon: HT-29	–	–	Colon: HT-29	–	–
Cervix: ME-180	–	–	Cervix: ME-180	–	–
Lung: SK-LC-LL	–	–	Lung: SK-LC-LL	–	–
Testicle: SK-GR-1	–	–	Testicle: SK-GR-1	–	–
Lymphoblastoid cells			**Lymphoblastoid cells**		
T-cell: MOLT 4, T-45		–	T-cell: MOLT 4, T-45		–
EBV-B cell§:			EBV-B cell§:		
BD,BT	–	+	BD,BT	–	–
AH	–	–	AH	–	–
Normal cells			**Normal cells**		
Melanocytes	1		Melanocytes	0.1	
Fibroblasts	12	+	Fibroblasts	–	–
Kidney epithelium: 1,2	12		Kidney epithelium: 1,2	0.5	
Brain		–	Brain		+
Leukocytes		+	Leukocytes		+
Fetal fibroblasts	2	+	Fetal fibroblasts	–	±
Fetal brain	–	–	Fetal brain	–	–
Fetal liver		–	Fetal liver		–

* Methods, layout, and footnotes as in Table 1. Heat stability: labile. M_r of M_{19} by gel filtration: 50,000–70,000.

No inhibitory activity of solubilized antigen was found with R_8 antibody. As yet, it has not been possible to immunoprecipitate antigen from radiolabeled extracts of SK-MEL-28 with either M_{19} or R_8 antibodies.

O_5 Antigenic System. The determinant recognized by $O_{5(\gamma 1)}$ was present (in variable amounts) on every human cell type tested, including erythrocytes. The O_5 determinant was heat stable. No antigenic activity could be detected in soluble extracts either by antibody inhibition tests or by immunoprecipitation.

Antigens Defined by the R_{24} Monoclonal Antibody Group. Four monoclonal antibodies [$K_{9(\gamma M)}$, $I_{24(\gamma M)}$, $C_{5(\gamma 3)}$, and $R_{24(\gamma 3)}$] defined antigens that were heat stable, and initial biochemical characterization indicated that glycolipid determinants were involved. They are grouped together because of their related serological reactivity, although it is not known whether they detect a single determinant or a family of determinants. The cellular distribution of antigens detected by the R_{24} group (see Table 4 for K_9 and R_{24}) was more restricted than the distribution of gp95, gp150, R_8, M_{19}, and O_5 antigens. Melanomas and astrocytomas were the most reactive cell types, with other cells expressing little or no antigen. The virtual absence of these antigens on epithelial cancers was particularly striking. The antigen defined by the R_{24} antibody was the most restricted of all, reactivity with cell lines being confined to melanomas and two of five astrocytomas. Further studies are needed to determine the significance of the weak reactions observed with melanocytes and the partial absorptions observed with normal brain.

DISCUSSION

Now that the technical aspects of generating hybridomas and screening for antibody activity are becoming relatively straightforward, the major challenge confronting the cell surface serologist is in defining the specificity of the resulting monoclonal antibodies. This task is greatly simplified if the antibodies being sought are directed against antigenic systems that have been previously defined by conventional immune sera. In the case of monoclonal antibodies to new antigenic systems that are not alloantigenic, one of the key problems is to distinguish presence vs. absence of antigen from high vs. low expression of antigen, and this problem is of particular concern to the tumor immunologist in his search for tumor-specific

Table 4. Characteristics of the R_{24} antigenic system*

Cells	Serological tests: Ab $K_{9(IgM)}$ Titer $\times 10^{-3}$†	Ab $K_{9(IgM)}$ Abs‡	Ab $R_{24(\gamma 3)}$ Titer $\times 10^{-3}$†	Ab $R_{24(\gamma 3)}$ Abs‡
Melanomas				
SK-MEL-28,19,29 42,57,64,94	25	+	25	+
SK-MEL-13,37,40,90	2	+	25	+
SK-MEL-23,44,79	1	+	5	+
SK-MEL-31,61	−	+	1	+
Astrocytomas				
AJ	5	+	5	±
AN	−	+	−	+
AS,U138MG,U373MG	−	−	−	−
Epithelial cancers				
Renal: SK-RC-7	−		−	
SK-RC-2,6,9	−	−	−	−
Breast: BT-20, MCF-7,AlAb	−	−	−	−
Bladder: T-24	−	−	−	−
Ovarian: SK-OV-3	−	−	−	−
Colon: HT-29	−	−	−	−
Cervix: ME-180	−	−	−	−
Lung: SK-LC-LL	−	−	−	−
Testicle: SK-GR-1	−	−	−	−
Lymphoblastoid cells				
T-cells: MOLT 4		±		−
T-45		−		−
NALL	−	−	−	−
EBV-B cell§: AH,BD,BT	−	−	−	−
Normal cells				
Melanocytes	−		0.1	
Fibroblasts	−	±	−	−
Kidney epithelium: 1,2	−		−	
Brain		+		±
Leukocytes		+		−
Fetal fibroblasts	−	±	−	−
Fetal brain		+		−
Fetal liver		+		−

* Methods, layout, and footnotes as in Table 1. Heat stability: stable.

antigens. What has been shown repeatedly with conventional immune sera or naturally occurring antibodies in animal and human systems is that direct serological tests are frequently less sensitive than absorption tests in typing cells for presence or absence of antigen. Cells that do not react with a particular antibody may nevertheless specifically absorb antibody reactivity, indicating that cells do express antigen but not at a sufficient level to score positive in direct tests. A number of examples of this direct-test-negative/absorption-test-positive phenotype have been found in the present analysis of monoclonal antibodies to melanoma cells. Thus, although direct tests with monoclonal antibody are useful in identifying quantitative differences among different cell populations, they cannot be relied on to establish the antigenic phenotype of nonreactive cells. For this purpose, absorption tests are of great value.

The 18 monoclonal antibodies analyzed in this study identify 6 distinguishable systems of melanoma surface antigens. Two of these antigenic systems (gp95 and gp150) are glycoproteins, as judged by lectin binding and radiolabeling with amino acid and carbohydrate precursors. Another, the R_{24} antigenic system, has properties of a glycolipid, as indicated by heat stability and antibody inhibition tests with glycolipid fractions of reactive cells (C. Pukel, personal communication). The other three antigens are not as well characterized, but initial indications are that O_5 is a glycolipid antigen. Each of these antigenic systems has a distinctive pattern of expression on different cell types. Some are widely represented, whereas others show characteristics of more restricted differentiation antigens. Of particular interest are those that define subsets of melanomas (gp95, gp150, M_{19}, R_8) and those that show a high degree of restriction to melanoma (e.g., R_{24}). None, however, can be considered melanoma-specific, as even R_{24} is found on astrocytomas and may also be present in normal brain and on melanocytes. Because of the different serological methodology and the use of different cell panels, it is not possible to make any direct comparisons between the monoclonal antibodies defined in this report and those described by other investigators in studies of melanoma (8–10). For this, an exchange of reagents will be necessary. However, it appears likely that the p97 melanoma surface antigen described by Woodbury *et al.* (10) is related to the gp95 antigen identified by six of our monoclonal antibodies.

Continued application of hybridoma methodology should result in a comprehensive picture of the surface antigenic structure of melanoma and, in conjunction with the analysis of somatic cell hybrids, provide ways to define the genetic determinants involved in the construction of the melanoma cell surface. Of course, a central aim of the analysis is to determine whether the mouse recognizes antigens that are melanoma-specific. Another aim is to see whether mouse monoclonal antibodies are formed against melanoma antigens that have been defined by human serum, such as AU (2), AH (11), or Mel 1 (12). When techniques for the production of human monoclonal antibodies are perfected, comparable questions can be asked about the full range of melanoma antigens that are recognized by humans.

This work was supported by grants and contracts from the National Cancer Institute (CA 08748, CA 19765-03, CA 21445, and CB 74124) and the Oliver S. and Jennie R. Donaldson Charitable Trust. W.G.D. is recipient of a Fellowship from the Deutsche Forschungsgemeinschaft.

1. Köhler, G. & Milstein C. (1975) *Nature (London)* **256**, 495–497.
2. Carey, T. E., Takahashi, T., Resnick, L. A., Oettgen, H. F. & Old, L. J. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3278–3282.
3. Ueda, R., Shiku, H., Pfreundschuh, M., Takahashi, T., Li, L. T. C., Whitmore, W. F., Oettgen, H. F. & Old, L. J. (1979) *J. Exp. Med.* **150**, 564–479.
4. Phillips, D. R. & Morrison, M. (1971) *Biochemistry* **10**, 1766–1771.
5. Lloyd, K. O., Travassos, L. R., Takahashi, T. & Old, L. J. (1979) *J. Natl. Cancer Inst.* **63**, 623–634.
6. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
7. Carey, T. E., Lloyd, K. O., Takahashi, T., Travassos, L. R. & Old, L. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2898–2902.
8. Koprowski, H., Steplewski, Z., Herlyn, D. & Herlyn, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3405–3409.
9. Yeh, M.-Y., Hellström, I., Brown, J. P., Warner, G. A., Hansen, J. A. & Hellström, K. E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2927–2931.
10. Woodbury, R. C., Brown, J. P., Yeh, M.-Y., Hellström, I. & Hellström, K. E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2183–2187.
11. Shiku, H., Takahashi, T., Resnick, L. A., Oettgen, H. F. & Old, L. J. (1977) *J. Exp. Med.* **145**, 784–789.
12. Houghton, A. N., Taormina, M. C., Ikeda, H., Watanabe, T., Oettgen, H. F. & Old, L. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4260–4264.