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Monoclonal Antibody-defined Human Lung Cell Surface Protein Antigens[1]

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ABSTRACT

Monoclonal antibodies were used to define four distinct antigens present on the surfaces of human lung tumors. Immunoprecipitation of the four antigens by monoclonal antibodies and sodium dodecyl sulfate:polyacrylamide gel electrophoresis reveals that they have distinct complex structures. Different patterns of expression of these antigens on cells of other than lung tumor origin were detected by the same panel of monoclonal antibodies.

INTRODUCTION

In the past few years, we have developed monoclonal antibodies defining antigens expressed by human melanomas (7) and colorectal carcinomas (3, 8). In the present study, we have directed our efforts to produce monoclonal antibodies reacting with human lung tumors.

In order to generate such hybridomas, BALB/c mice were immunized with tumor cell lines or with cells or membrane preparations isolated from a fresh tumor. Spleen cells from these mice were fused either with P3×63Ag8 (6) or P3×63Ag8-653 (5) myeloma cells, and the resulting hybrids were grown and cloned. This report describes the distribution and structures of four distinct protein antigens identified on the surfaces of these cells.

MATERIALS AND METHODS

Cells Used for Immunization

Bronchogenic carcinoma MBA 9812 and a lung carcinoma A427 (2) were obtained from the Cell Culture Laboratory, Naval Bioscience Laboratory, Oakland, Calif. SW 900 lung carcinoma cell line obtained from a tumor described as a Grade IV squamous cell carcinoma (1) was obtained from A. Leibovitz, The Scott and White Clinic, Temple, Texas.

The three cell lines were maintained in Eagle's MEM[3] supplemented with 10% fetal calf serum.

The WL 1680 cell line was initiated from a fresh moderately differentiated squamous cell carcinoma removed from a 63-year-old man. On the day of removal, the tumor was gently minced in a sterile Petri dish with 20% MEM which contained antibiotics. The cell suspension was centrifuged over fetal calf serum at 1000 × *g*, and the pellet was resuspended in 20% MEM. Some of this cell suspension was distributed into Linbro wells and Petri dishes and incubated at 37° with 5% CO_2. In one of the wells, a colony of tumor cells originated a WL 1680 cell line. The remainder of fresh tumor cells was used to prepare cell membrane antigen for the further immunization of mice.

Normal tissue adjacent to the tumor was processed at the same time as the tumor tissue in order to grow normal lung fibroblasts.

Preparation of Cell Membrane Antigen. Cells from a confluent monolayer of lung tumors (MBA 9812, A427, or SW 900) were trypsinized from the growth surface, washed 3 times in cold PBS, and resuspended (5×10^7 cells/ml) in PBS containing 2 mM phenylmethylsulfonyl fluoride. Cells were disrupted with a Potter-Elvehjem homogenizer at 4°, and nuclei and debris were removed by centrifugation at 600 × *g*. Membranes contained in the supernatant were pelleted at 100,000 × *g*, resuspended in 1 ml of PBS containing 2 mM phenylmethylsulfonyl fluoride, and stored at −70°.

Immunization of Mice

MBA 9812. BALB/c mice were immunized by an i.p. inoculation of 1×10^7 washed live MBA 9812 cells. The mice were boosted 4 months later by an i.v. injection of 0.3 ml MBA 9812 cell membrane suspension.

A427. One BALB/c mouse was immunized by an i.p. inoculation of 1×10^7, 3.5×10^6, and 1×10^6 A427 live cells at monthly intervals. The mouse was boosted 4 months later with 0.2 ml of A427 cell membrane suspension injected i.v. on 3 consecutive days.

SW 900. One BALB/c mouse was immunized by i.p. inoculation of 1.2×10^6 SW 900 live cells and 1.0×10^6 cells 2 months later. The mouse was boosted 3 months later with 0.2 ml of SW 900 cell membrane suspension injected i.v. on 3 consecutive days.

WL 1680. One BALB/c mouse was immunized with freshly prepared tumor cells; 0.25 ml containing 5×10^6 cells was given by i.p. inoculation and 0.1 ml (1×10^6 cells) s.c. A booster injection given 1 month later consisted of 0.2 ml of cell membrane suspension injected i.v.

Production of Hybridomas. In all 4 cases, the mice were sacrificed 3 days after the last booster. Spleen cells were fused to the myeloma cell line P3×63Ag8 or its nonsecreting variant P3×63Ag8-653 using polyethylene glycol 1540 (7). Fused cells were distributed into 24-well Linbro plates in hypoxanthine:aminopterine:thymidine medium and incubated until growth could be detected microscopically. Cells were subsequently cloned by limiting dilution in 96-well culture plates.

Immunoprecipitation and Characterization of Antigens. Cells were surface iodinated with ^{125}I by the lactoperoxidase:glucose oxidase method as described previously (10, 11). Radiolabeled cells were dissolved in Nonidet P-40 buffer and immune complexes were formed and precipitated by methods that have been described in detail previously (10, 11). SDS:PAGE was performed by the discontinuous method of Laemmli (9), and gels were fixed, stained, and dried as described previously (10, 11).

Immunodiffusion. Ouchterlony immunodiffusion was performed in 1% agarose in 0.01 M Tris buffer with 0.1 M NaCl at pH 8.0. Class-specific antisera were purchased from Litton Bionetics, Kensington, Md.

Radioimmunoassay. Tumor cells were detached from the growth surface by brief trypsinization, washed twice with radioimmunoassay buffer (PBS buffer with 2% IgG-free horse serum), and then suspended at a concentration of 10×10^6/ml. Radioimmunoassay was performed as described previously, using ^{125}I-labeled rabbit anti-mouse $F(ab')_2$ IgG (7).

In all experiments, monoclonal antibody 480-1-4 (3) was included as a positive control since this antibody binds to all human cells with the exception of RBC. P3×63Ag8 immunoglobulin from the parental

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[3] The abbreviations used are: MEM, minimal essential medium; SDS:PAGE, sodium dodecyl sulfate:polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline (g/liter: NaCl, 8.0; KCl, 0.20; KH_2PO_4, 0.20; Na_2HPO_4, 1.15; $CaCl_2$ (anhydrons), 0.10 $MgCl_2 \cdot 6H_2O$, 0.10).

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myeloma line (4) was used as a negative control, and the counts bound were subtracted from each value to reveal the specific binding.

RESULTS AND DISCUSSION

Two mice were immunized with cells of line MBA 9812, and following fusion of their splenocytes, 46 hybrid colonies were isolated. Two colonies (16A and 16B) were cloned to yield 6 clones which secreted antibody of the IgG-2a subclass and exhibited identical binding patterns on a panel of target cell lines. Data obtained with clone 16B.13 are presented in Tables 1 to 3 and show that this antibody bound strongly to 5 lung carcinomas, one oat cell carcinoma, all colon carcinomas tested, one breast carcinoma, and one melanoma. This antibody did not bind to normal fibroblasts of 5 cultures tested. It bound strongly to normal lymphocytes and to RBC. Antigens

Table 1

Binding of monoclonal antibodies to lung tumor cell lines

Binding of monoclonal antibodies to 6 lung carcinoma cell lines and to one lung oat cell line was assessed by the method described in the text. Each determination was performed in triplicate and the means of these determinations are presented after subtraction of the P3X63Ag8 control. P3X63Ag8 binding (control) values are shown, and the deviation from the mean did not usually exceed 15% of the count.

Lung tumor cell lines used as targets	Monoclonal antibody (cpm bound/5 × 10^5 cells)					
	9812-16B-13	427-2-f	900-1-7	1680-25-35	480-1-4[a]	P3X63Ag8
Carcinoma						
MBA 9812	4020	689	600	600	1890	110
A427	920	2690	600	660	4570	220
SW900	2250	2870	4790	1000	6850	130
WL 1680-C	5100	2420	0	1700	8790	380
A 549	2320	3200	0	0	8480	280
SW 1271	2290	2060	0	0	3010	160
Oat cell						
DMS.114	3230	0	0	0	5340	550

[a] Positive control; antibody 480-1-4 binds to all human cells.

Table 2

Binding of monoclonal antibodies to carcinoma and melanoma cell lines

Binding of monoclonal antibodies to 5 colon carcinoma lines, 3 breast carcinoma lines, and 5 melanoma lines is shown. The form of the results is described in Table 1.

Tumor cell lines used as a target	Monoclonal antibody (cpm bound/5 × 10^5 cells)					
	9812-16B-13	427-2-f	900-1-7	1680-25-35	480-1-4	P3X63Ag8
Colon carcinoma						
SW 1116	4310	4010	0	680	5600	280
SW 1222	4770	6040	0	0	7690	590
SW 948	4290	4990	0	0	7720	760
SW 403	2840	5810	0	0	7660	430
SW 1083	1280	4490	0	0	7500	330
Breast carcinoma						
SW 1403	2330	1740	1380	0	4390	310
SK BR5	1110	0	0	0	6880	260
MCF 7	3390	530	0	560	6500	330
Melanoma						
WM 9	2820	3960	4270	690	7480	330
WM 28-7	0	3400	4820	0	8420	390
WM 28-9	0	2430	1190	0	8110	460
WM 98-1	0	0	4880	0	5300	310
WM 56	0	0	6330	0	7270	310

Table 3

Binding of monoclonal antibodies to fibroblasts and blood cells

Binding of monoclonal antibodies to 4 fibroblast cell lines, RBC, and peripheral blood lymphocytes is shown. The form of the results is described in Table 1.

Target cells	Hybridoma antibody (cpm bound/5 × 10^5 cells)					
	9812-16B-13	427-2-f	900-1-7	1680-25-35	480-1-4	P3X63Ag8
HS 0853	0	0	760	0	4760	260
WI 38	0	960	700	580	4950	260
MRC 5	0	650	0	0	3910	220
Human foreskin fibroblasts	0	0	0	0	6510	230
RBC						
B	3700	0	0	0	0	480
B	7500	0	0	0	0	500
A	920	0	0	0	0	600
O	1000	0	0	0	0	500
Peripheral blood lymphocytes	6170	5560	0	0	9100	430

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precipitated by the antibody from cells of MBA 9812 and from colon carcinoma cell line SW 948 were fractionated by SDS: PAGE, and the results from SW 948 are shown in Fig. 1. The antigen was fractionated after reduction and contains 2 components of M.W. 37,000 and 19,000 (*A*). The control myeloma P3×63Ag8 (*B*) did not precipitate these components from this cell line.

From a fusion of splenocytes of a mouse immunized with cell line A427, 36 hybrid colonies were isolated. Three colonies (2, 32a, and 26c) were cloned to yield 120 clones which secreted antibody of the IgG-1 subclass and which exhibited identical binding patterns on a panel of target cell lines of various kinds. Data obtained with subclone 427-2-f are presented in Tables 1 to 3 and show that this antibody bound strongly to 5 lung adenocarcinoma lines, one oat cell carcinoma, 5 colon carcinoma lines, one breast carcinoma, 4 melanoma cell lines, and peripheral blood lymphocytes. Line 427-2-f also bound weakly to 2 fibroblast lines; it did not bind to one melanoma (WM 56), cells of foreskin fibroblasts, 2 breast carcinomas, or RBC.

Antibody 427-2-f was used to precipitate antigens from surface-iodinated A427 lung carcinoma cells and SW 1222 colon carcinoma cells. Qualitatively, identical results were obtained in each case, and those obtained with SW 1222 are illustrated in Figs. 2 and 3. Fig. 2 shows the results obtained when antigenic material was fractionated by SDS:PAGE either in the absence (*NR*) or presence (*R*) of the reducing agent β-mercaptoethanol. Without reduction, the antigen migrated as a diffuse complex band which was resolved, after reduction, into 3 components with molecular masses of 145,000, 127,000, and 113,000 daltons. Only the 127,000-dalton component in this complex was heavily iodinated with the other 2 components being much more lightly labeled. To investigate the relationships of the bands obtained before and after reduction, a 2-dimensional fractionation was performed with the first dimension being performed prior to reduction and the second dimension after treatment of the gel strip with β-mercaptoethanol. The results of this experiment are presented in Fig. 3 and show that 4 and perhaps as many as 6 components of this antigen can be resolved.

Three lightly labeled components *a*, *b*, and *c* can be discerned with apparent molecular masses of about 145,000, 127,000, and 113,000 daltons in the second dimension. Two of these, *b* and *c*, migrated more slowly in the first dimension, a phenomenon which suggests that the molecules have decreased in size after reduction. Since the apparent change in molecular mass is small, the dissociation of a small disulfide-bonded component may be suspected and a radioactive spot can be detected, *d*, which migrates below these high-molecular-mass entities at the low-molecular-mass front of the gel which, under these conditions, represents molecules which

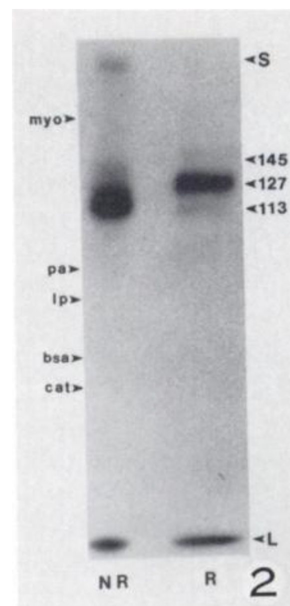

Fig. 2. An autoradiograph prepared from a SDS:PAGE gel of iodinated antigens precipitated from SW 1222 cells by monoclonal antibody 427-2-f. Gel concentration was 7% with a 38:1 acrylamide:bisacrylamide ratio. Antigen preparations were either prepared in the absence of β-mercaptoethanol (*NR*) or reduced with this reagent prior to electrophoresis (*R*). *S*, position of the stacking gel interface; *L*, position of the low-molecular-weight band (about 20,000 or less in this case). Molecular weight standards were run and their positions are shown: *myo*, myosin (M.W. 210,000); *pa*, phosphorylase *a* (M.W. 92,500); *lp*, lactoperoxidase (M.W. 77,500); *bsa*, bovine serum albumin (M.W. 66,000); *cat*, catalase (M.W. 60,000). The molecular masses of the components of the antigen are shown in thousands of daltons.

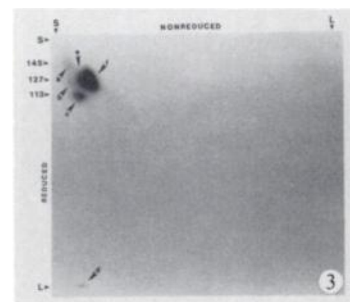

Fig. 3. An autoradiograph of a 2-dimensional gel in which antigens precipitated by 427-2-f were first fractionated on a 10% gel without reduction and subsequently fractionated at right angles after scission of disulfide bonds with β-mercaptoethanol. The designations *S*, *L*, etc. are explained in Fig. 2, and the complexity of the antigen as discussed in the text is indicated.

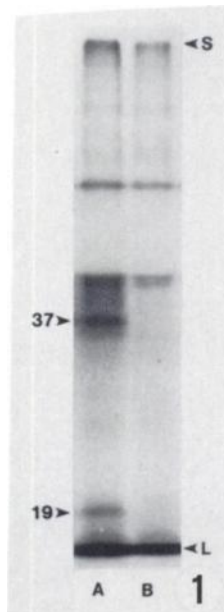

Fig. 1. An autoradiograph of iodinated antigens precipitated by antibody 9812-16B-13 (*A*) and by the control P3×63Ag8 from cell line SW 948. Antigens were separated on a 10% gel, and the positions of the 2 components of the antigen (M.W. 37,000 and 19,000) are indicated. Molecular weight standards were run, and the designations are as explained in Fig. 2.

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have molecular masses of less than about 20,000 daltons. The shape of the heavily radiolabeled spot at 127,000 daltons appears to be consistent with the presence of 2 overlapping entities, *e* and *f*. One or both of these molecules migrates more slowly in the second dimension than in the first. This type of behavior is characteristic of proteins that contain internal disulfide bonds which constrain the molecule in a compact configuration. When in the reduced form, molecules with these characteristics unfold and exhibit greater apparent molecular weights.

The splenocytes of a mouse immunized with cell line SW 900 yielded 27 hybrid colonies after fusion, and one colony was cloned to yield 11 clones with identical binding reactivity, each of which secreted antibody of the IgG-1 subclass. Antibody 900-1-7 bound strongly to the immunizing cell line SW 900 but bound minimally to all other carcinomas except one breast carcinoma which reacted weakly. In contrast, this antibody bound strongly to all 5 melanomas tested. RBC and peripheral blood lymphocytes were unreactive. Three adenocarcinomas and one oat cell carcinoma were unreactive as well as all colon carcinoma and one breast carcinoma cell lines.

Monoclonal antibody 900-1-7 was used to precipitate iodinated antigens from the parental lung carcinoma line to SW 900. Fig. 4 shows an autoradiogram of antigens fractionated by SDS:PAGE under reducing conditions. Precipitates were made with 900-1-7 (*A*) and the control myeloma protein from P3×63Ag8 (*B*). The antigen recognized by 900-1-7 appears as a diffuse lightly iodinated single band with an apparent molecular mass of about 126,000 daltons.

The fusion performed with the splenocytes from the mouse immunized with cells of freshly removed squamous cell carcinoma WL 1680 yielded 150 colonies, 4 of which secreted antibody which bound to cells of the fibroblast-like culture isolated from WL 1680 tumor. One colony (No. 25) was cloned, and clone 1680-25-35 was selected for detailed study. This clone secreted antibody of the IgG-1 subclass which bound to WL 1680, the carcinoma cell grown from the tumor. Antibody 1680-25-35 also bound at a lower level to 2 other lung carcinomas, 2 colon carcinomas, one breast carcinoma, and one melanoma. This antibody did not bind to RBC or peripheral blood lymphocytes.

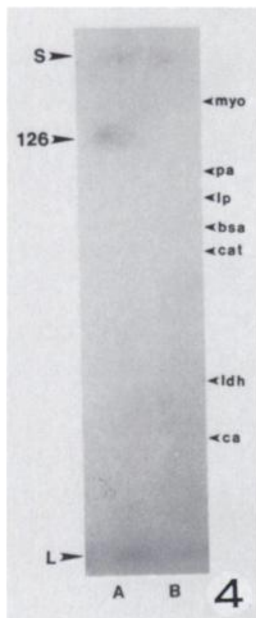

Fig. 4. An autoradiograph of iodinated antigens precipitated by antibody 900-1-7 (*A*) from cell line SW 900 and by a P3×63Ag8 control (*B*). Antigens were fractionated on a 10% gel, and the designations are as explained in Fig. 2 with the addition of the positions of 2 additional standards: *ldh*, lactic dehydrogenase (M.W. 36,000); *ca*, carbonic anhydrase (M.W. 29,000).

Monoclonal antibody 1680-25-35 was used to precipitate antigens from iodinated cells of WL 1680, SK MEL 37, WM 56, a 1680-25-35-negative melanoma, and WM 9, a melanoma line positive for this antigen (Table 2). The results (Fig. 5) show that 1680-25-35 precipitates an antigen which contains 2 heavily iodinated components with apparent molecular weights of 149,000 and 119,000 (Fig. 5, *Lane 1*). Antibody 1680-25-35 precipitates an antigen from WM 9 (*Lane 4*) which appears to be qualitatively identical to that on WL 1680. Consistent with the results obtained by radioimmunoassay, WM 56 does not appear to express this antigen (*Lane 3*). However, a faint trace of the upper most heavily iodinated component may be detectable in SK MEL 37 (*Lane 2*).

Each antigen appears to be expressed by the tumor cell lines and one fresh culture and not only on cells of isologous tumor but also on a variety of different cell lines as assessed by the binding in a radioimmunoassay.

Some interesting patterns of cross-reactivity can, however, be distinguished. For instance, in addition to binding to antigen(s) expressed by all tumor cells investigated, 2 of the antibodies (9812-16B-13 and 427-2-f) bind to peripheral blood lymphocytes and one (9812-16B-13) to erythrocytes, with highest binding to erythrocytes of Group B. This particular antibody does not bind to normal human fibroblasts.

Antigens isolated from different cell lines are indistinguishable by SDS:PAGE immunoprecipitation with the same monoclonal antibody despite the fact that the individuals from which

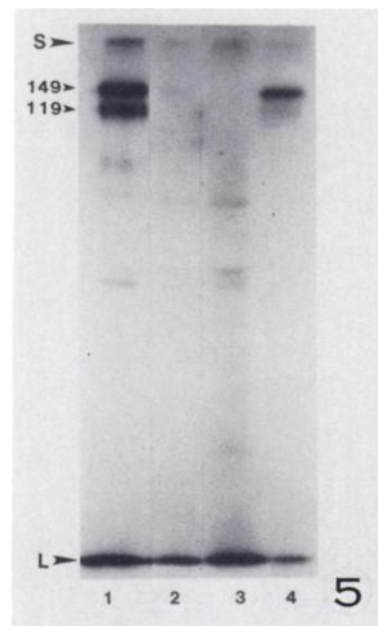

Fig. 5. An autoradiograph of antigens precipitated from various cell lines by monoclonal antibody 1680-25-35. *Lanes 1* and *2*, antigens precipitated from WL 1680 and SK MEL 37 by 1680-25-35. *Lanes 3* and *4*, show a similar pair of precipitations from 2 melanomas (WM 56 and WM 9, respectively) prepared with antibody 1680-25-35. The positions and molecular weights of the 2 components of the antigen precipitated by 1680-25-35 are shown. The designations *S* and *L* are described in the legend to Fig. 2.

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the tumors are derived are unrelated.

The expression of each of these molecules undoubtedly requires the activation of the appropriate structural genes, and it seems possible that the presence of the antigen may be a marker for a particular state of differentiation. These molecules may be oncofetal or oncodevelopmental markers. We have not excluded at present the possibility that 2 of the monoclonal antibodies may recognize blood group associated antigens also expressed strongly on tumor cells. In any event, studies on the occurrence and structure of these and other surface markers of tumor cells and normal cells will improve our understanding of the structural changes which accompany and underlie the neoplastic transformation which gives rise to the disease we call cancer.

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