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DEMONSTRATION AND ISOLATION OF MURINE MELANOMA-ASSOCIATED ANTIGENIC SURFACE PROTEINS

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SUMMARY: Melanomas are antigenic and induce the formation of antibodies in both syngeneic and xenogeneic species. The nature of melanoma-associated antigens remains problematic, however. We found that xenogeneic (goat) antiserum to the mouse (C57BL/6) B16 melanoma, following appropriate absorptions with nonmelanoma cells, showed specificity for melanoma-associated surface antigens of B16 and one other murine melanoma. The antibody to B16 did not react with histocompatibility antigens, mouse-specific xenoantigens, viral antigens or melanin. The IgG fraction of the goat antibody was cross-linked covalently to protein A-Sepharose using dimethylsuberimidate. This immunoadsorbent was used to isolate shed antigens from cultures in which B16 cells had been grown and from detergent extracts of biosynthetically labeled (^{3}H-leucine) B16 cells. The immune-affinity purified antigen preparation contained two major components of apparent molecular weight 60,000 and 50,000 daltons as assessed by SDS-polyacrylamide gel electrophoresis. Immunization of rabbits with immune-affinity purified B16 antigens induced antibodies which bound specifically to B16 cells.

Studies of both human (1-3) and murine (4) melanomas show that these tumors are immunogenic and possess antigens which can be demonstrated using syngeneic and xenogeneic antisera. The exact nature of the melanoma-associated antigens is not clear at present, and questions can arise whether or not such antigens are of viral origin (murine systems), are associated with the histocompatibility complex, are oncofetal or are strictly associated with melanomas. In this study we present results of serological and biochemical investigations using goat antiserum to the murine B16 melanoma. We provide evidence that goats when immunized with B16 melanoma cells produce antiserum that, when appropriately absorbed, recognizes a melanoma-associated antigen or antigens that are serologically distinguishable from murine xenoantigens, histocompatibility complex antigens, viral antigens and melanin. In addition, we report partial characterization of antigenic proteins resident on the B16 melanoma.

MATERIALS AND METHODS:

Animals, Cells and Cell Culture: Inbred C57BL/6 mice were obtained from the Experimental Animal Production Area, Frederick Cancer Research Center,

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Frederick, Maryland. Tumor cells, unless otherwise specified, were maintained as monolayer cultures in complete minimum essential medium supplemented with 10% fetal calf serum as described previously (5). The tumor cells used are as follows: B16 melanoma variants F0, F0-U1, F0-U2, F10, F10^{Lr}, obtained from Dr. I. J. Fidler; melanoma 1735 Mel 8, ultraviolet light induced fibrosarcomas UV-112 and 2237, obtained from Dr. M. L. Kripke; AKR low-passage fibroblasts, AKR adenocarcinoma, and DMBAII fibrosarcoma, obtained from Dr. I. J. Fidler. The relevant characteristics of these cells are given in Table I below.

Goats and Immunization: The B16 melanoma and UV-112 fibrosarcoma syngeneic to the C57BL/6 mouse were grown in vivo following subcutaneous (s.c.) inoculation. When tumors reached a size of 1.5-2.5 cm they were removed aseptically, minced in cold Hanks' balanced salt solution (HBSS) and dispersed mechanically to prepare single cell suspension. Viability as measured by trypan blue exclusion ranged from 25-35%. The cell suspension was mixed with Complete Freund's Adjuvant (CFA) at 4:1 ratio. Each goat was injected intradermally (i.d.) at 4 different sites with 0.5 ml of cell:CFA mixture. A total of 2 x 10^7 viable cells per goat was injected. Two weeks later, the injection of 2 x 10^7 viable cells/goat was repeated without CFA. Seven days thereafter, each goat was injected intravenously (i.v.) with 1 x 10^6 viable cells and bled 2 weeks later. Thereafter goats were given a booster injection i.v. of 1 x 10^6 viable cells and were bled on alternate weeks (total of 3 bleedings). The serum was filtered through a 0.2 μm Millipore filter and stored frozen in liquid nitrogen until absorption. This method is similar to that described for production of goat-anti-mouse macrophage serum (6).

Growth and fixation of EL4 cells for absorption of antisera: EL4 lymphoma cells, syngeneic to C57BL/6 mice, were grown in spinner culture to early stationary phase. A pellet of 1 x 10^9 glutaraldehyde-fixed EL4 cells was prepared by washing 3 times in a solution of 1 mg/ml fraction V bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO). Five ml of goat anti-B16 melanoma or goat anti-UV-112 fibrosarcoma serum was diluted with 20 ml of PBS and added to the EL4 pellet. The suspension was incubated for 1 hr at 37° then 2 hr at 4°, centrifuged at 500 x g and the supernatant was retained for testing. This cycle was repeated until no anti-C57BL/6 erythrocyte activity (below) was observed. Two to four absorptions were routinely necessary.

Complement-dependent cytotoxicity: Once the absorbed antisera demonstrated no complement-dependent lysis of C57BL/6 erythrocytes, their specificity was assessed using the various tumor targets listed above. The cytotoxicity was determined using a Coulter counter assay described previously (7) and verified by a direct binding measurement described below.

Target cells were harvested by brief trypsinization from the monolayer, washed twice in HBSS (Grand Island Biological), and resuspended at a concentration of approximately 1 x 10^6/ml. One-tenth ml of cell suspension was mixed with 0.1 ml of antiserum appropriately diluted in PBS and incubated at 37° in a 12 x 75 mm polypropylene tube (Falcon Plastics, Oxnard, CA). The suspension was agitated every 10 min to prevent the cells from plating out on the walls of the tube. After 30 min, 0.02 ml of freshly thawed guinea pig complement was added and the incubation was continued for an additional 4-5 min. The interaction was terminated by moving the cells to an ice bath while maintaining the agitation. The cells were washed from the incubation tubes with 10 ml of counting electrolyte (Isoton, Coulter Electronics, Hialeah, FL) and the cell numbers were determined. The Coulter counter (model ZBI) was fitted with a 100 μm aperture and the setting of the lower discriminator was varied according to the target cell used. The data represent the means of duplicate observations of duplicate determinations of

at least two separate experiments. Percent cytotoxicity was calculated by subtracting the number of cells remaining in the incubation tube following antiserum treatment from the number of cells in the PBS blank divided by the number of cells in the PBS blank.

Antibody binding assay: Goat IgG was purified from the absorbed serum by affinity chromatography on a column of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously (8). B16-F10 or UV-112 cells were inoculated into a 96 well flat bottom microtest plate (Falcon Plastics, Oxnard, CA) at a concentration of 1-10 x 10^3 cells/0.2 ml/2311 and allowed to grow to confluency. The wells were washed twice with PBS and the monolayers fixed to the plastic with glutaraldehyde according to the method of Segal and Klinman (9). The fixation solution was 0.1 M potassium phosphate buffer pH 7.0 to which glutaraldehyde (Ladd Research Industries, Burlington, VT) was added to a final concentration of 0.15% (W/V). After 5 min incubation at room temperature, the wells were washed twice with a solution of fraction V BSA, 1.0 mg/ml containing 0.1% NaN_3. The plates were stored at 4^o until use.

Immediately prior to use, the plates were warmed to room temperature and the wells were washed twice with PBS to remove residual BSA and NaN_3. Fifty µl of appropriately diluted IgG were overlaid on the monolayer and incubated for 2 hr at room temperature. The wells were washed twice more wih PBS and overlaid with 100 µl of protein A from S. aureus (Pharmacia Fine Chemicals, Piscataway, NJ) which had been prelabeled with ^{125}I by the chloramine T method (10). The protein A solution contained 100-200 ng protein A/ml of 1 mg/ml BSA. Following overnight incubation of the plates at 4^o, unbound radioactivity was removed by two washes with PBS. The monolayers were harvested and counted for radioactivity in a Searle model 1185 gamma counter equipped with a 2 x 2-inch well-type NaI(Tl) detector.

Immunoadsorbents and isolation of antigen: The IgG fraction of normal goat serum, goat antiserum to UV-112 or absorbed antiserum to B16 was coupled covalently to a matrix of S. aureus protein A-Sepharose as described elsewhere (11). Briefly, 5 ml of serum was reacted with 3 ml of swollen protein A-Sepharose and washed exhaustively with PBS. The bound IgG was covalently coupled to the matrix using the cross-linker dimethylsuberimidate. The immunoadsorbent was used as a column. A sample was loaded and washed through with PBS until protein absorbance at A_{280} or ^{125}I-radioactivity fell to background levels. Specifically bound material was eluted with 0.01 M acetic acid/0.15 M NaCl pH 2.9.

Immunization of rabbits: Rabbits were immunized using the immune-affinity melanoma antigen preparation isolated from material released into serum-free medium. The fraction was eluted from the immunoadsorbent using acetic acid (0.01 M)/saline (0.15 M), dialyzed against PBS and passed through protein A-Sepharose to remove γ-globulin which might have eluted from the matrix. Two rabbits were each given 2 injections consisting of 200 µg of purified antigen preparation. The first injection was given in complete Freund's adjuvant; the second was given 3 weeks later using incomplete Freund's adjuvant. Serum was obtained two weeks following the second injection. In addition, serum samples were prepared prior to immunization.

SDS-polyacrylamide gel electrophoresis: This was essentially the method of Laemmli and Favre (12) using conditions and standards described in detail previously (13).

RESULTS:

Demonstration of antibodies to melanoma antigens: Goat antiserum to B16 melanoma was absorbed with fixed EL4 lymphoma cells until no complement-dependent hemolysis was observed against C57BL/6 erythrocytes. The absorbed serum was then assayed for complement-dependent cytotoxicity against B16 line F10 and the syngeneic fibrosarcoma UV-112. Figure 1 shows that the antiserum had strong lytic activity towards B16-F10, but appreciable lysis of UV-112 was not observed. Goat antiserum raised against UV-112 and absorbed with EL4 was used to ensure the susceptibility of B16-F10 to lysis with goat serum. Less than 15% lysis was observed at any concentration. Based upon the titration curve in Figure 1, we chose an antiserum dilution of 1:100 in order to obtain further information regarding the specificity of the antiserum. Reactivity against various syngeneic, allogeneic and xenogeneic tumors was determined (Table I). Consistent with the titration data, only B16 lines are lysed by the antiserum. Although the UV-112 cells showed 11% lysis this is not a significant value. This tumor generally gives high background lysis which is invariant with antibody concentration.

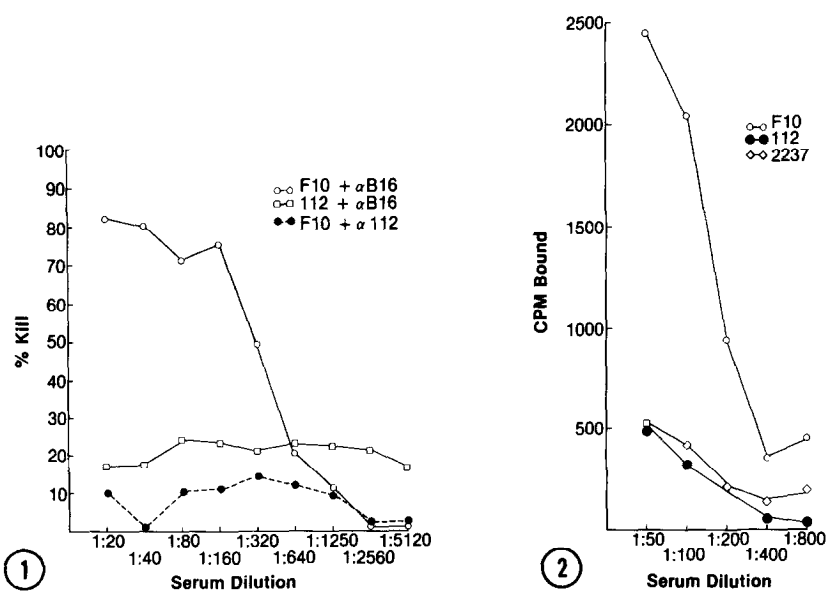

Fig. 1. Cytotoxicity assay of goat antiserum to murine melanoma B16. F10, B16 line F10; αB16, goat antiserum to B16, absorbed with the lymphoma EL4; 112, fibrosarcoma UV-112, syngeneic to B16; α112, goat antiserum to UV-112, absorbed with EL4 cells. Assay was performed as described by Gately and Mayer (7).

Fig. 2. Radioactive binding assay of goat antiserum to B16 melanoma and to syngeneic and allogeneic fibrosarcomas. Ordinate gives ^{125}I-radioactivity of labeled S. aureus protein A used to detect the F_c region of cell-bound IgG. Absorbed αB16 was used. F10, B16 line F10; 112, UV-112, syngeneic to B16; 2237, UV-induced fibrosarcoma 2237, allogeneic (C3H) to B16.

TABLE I

SPECTRUM OF COMPLEMENT-MEDIATED CYTOTOXICITY AGAINST RODENT TARGETS BY GOAT ANTI-B16 MELANOMA ANTISERUM

Target cell designation (Ref)	Strain of origin	C-type virus[a]	% Cytotoxicity[b]
B16-F10 melanoma (5)	C57BL/6 mouse	positive	57 ± 6
B16-F10^{Lr-6} melanoma (18)	C57BL/6 mouse	positive	32 ± 3
UV-112 fibrosarcoma (19)	C57BL/6 mouse	negative	11 ± 2
UV-112 fibrosarcoma (19)	C57BL/6 mouse	positive	0 ± 0
UV-2237 fibrosarcoma (19)	C3H mouse	negative	3 ± 1
AKR low-passage fibroblasts (20)	AKR mouse	negative	4 ± 1
AKR adenocarcinoma (20)	AKR mouse	positive	0 ± 0
DMBAII fibrosarcoma (20)	F344 rat	negative	5 ± 1

[a]The presence of endogenous C-type virus (MuLV) was determined by radioimmune precipitation assay (20).

[b]Data represent mean ± standard error of pooled data of 2 experiments, each one carried out in triplicate. Data are rounded off to integral numbers.

These data indicate that activity to B16 cells is not directed against mouse-specific antigens, alloantigens or antigens specified by C-type viruses.

To further refine quantitative assessment of the specificity of the goat antiserum to B16 melanoma, we used a radioimmunoassay which measures the binding of IgG to the target cells. ^{125}I-labeled protein A of *S. aureus*, which exclusively recognizes the Fc portion of IgG antibodies (14), was used to quantitate the amount of antibody bound. The titration curve in Figure 2 indicates specific binding to line B16-F10, but not to fibrosarcoma UV-112 (syngeneic to B16) or the allogeneic fibrosarcoma UV-2237.

Further comparison data illustrating the capacity of several variant lines derived from B16 melanoma and melanoma 1735 syngeneic to the C3H mouse to bind goat anti-B16 melanoma antibodies are given in Table II. All of the B16 sublines bound the antiserum, but quantitative variation was apparent. For example, in three separate experiments the following quantitative order was observed: F10 = F10^{Lr-6} > F0 > F0 - U_1 = F0 - U_2. Since F_0 - U_1 and F_0 - U_2 (which are paired pigmented and nonpigmented variants) showed comparable binding, the antiserum does not appear to be directed against melanin components. This result is consistent with the work of Cannon et al. (15) who report that extracts of melanotic and amelanotic melanoma cells give equal reactivity in cell-mediated immune reactions specific for human

TABLE II

BINDING OF GOAT ANTI-B16 MELANOMA ANTISERUM TO SURFACE OF MURINE MELANOMAS

Target cell designation	Origin	Description	CPM Bound[a]
B16-F0	C57BL/6	Parent tumor to which antiserum was raised, pigmented, low metastasis	1620 ± 40
B16-F0-U1	C57BL/6	Pigmented variant, low metastasis	1360 ± 130
B16-F0-U2	C57BL/6	Nonpigmented variant, low metastasis	1220 ± 130
B16-F10	C57BL/6	Selected in vivo, pigmented, high metastasis	2050 ± 180
B16-F10^{Lr}	C57BL/6	Selected in vitro, pigmented, resistant to lysis by syngeneic lymphocytes, low metastasis	1960 ± 180
UV-112	C57BL/6	Fibrosarcoma syngeneic to C57BL/6	330 ± 18
1735 Mel 8	C3H	Melanoma syngeneic to C3H mouse	1460 ± 170
UV-2237	C3H	Fibrosarcoma syngeneic to C3H mouse	405 ± 20

[a]Data represent mean ± S.E. of three observations in at least two separate experiments.

melanoma antigens. Binding, moreover, does not correlate with metastatic potential of B16 cells because F10 is highly metastatic, whereas F10^{Lr-6} lacks the capacity to metastasize (18). Since line F10^{Lr-6} is resistant to lysis by syngeneic lymphocytes and F10 is sensitive to killing by such lymphocytes, the possibility arises that the goat antiserum recognizes a murine melanoma surface determinant distinct from that involved in lymphocyte-mediated cytolysis. Further evidence for this comes from the observation that the C3H melanoma 1735 bound significantly more antibody than the C3H fibrosarcoma UV-2237.

Characterization of affinity purified melanoma-associated antigens: The preceding data indicated that goat antibodies to the B16 melanoma recognize melanoma-associated surface component(s) which are present on murine melanomas B16 and 1735. In order to obtain the melanoma-associated components for serological and biochemical characterization, we prepared immune-affinity matrices by derivatizing the IgG fraction of the goat antiserum to Sepharose-bound S. aureus A protein using the cross-linking agent dimethylsuberimidate (11). Three preparations made from B16 and UV-112 cells were tested for the presence of components which bound specifically to the immunoadsorbent. First, cells were washed free of fetal calf serum and allowed to shed components

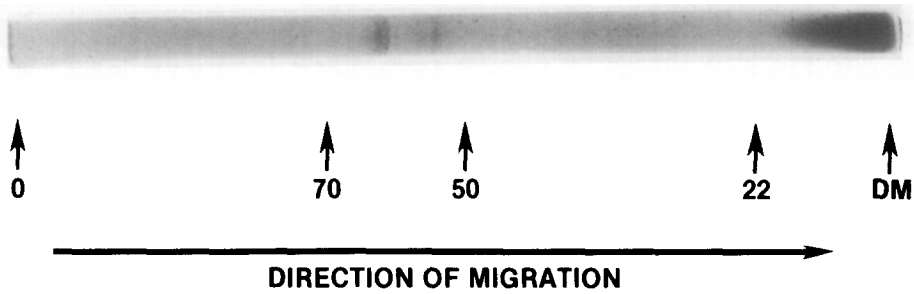

Fig. 3. Analysis by polyacrylamide gel electrophoresis in SDS-containing buffer of B16 melanoma-associated xenoantigen preparation isolated by immune affinity chromatography on goat antibody/Sepharose from culture fluid of *in vitro* grown B16 cells. 0, origin; 70, position of immunoglobulin μ chain standard (70,000 daltons); 50, position of immunoglobulin γ chain standard (50,000 daltons); 22, position of immunoglobulin light chain standard (22,000 daltons); DM, dye marker. In control experiments, (a) material from B16 culture fluid was reacted with an immunoadsorbent consisting of EL4 absorbed normal goat IgG. No components were eluted, and (b) culture fluid of UV-112 was reacted at equal concentration with the goat αB16 immunoadsorbent. No detectable components were isolated.

into serum-free medium. The released material was dialyzed against distilled H_2O, freeze-dried and radioiodinated for binding studies. In a typical experiment using radioiodinated released material, no specific ^{125}I-labeled material was bound from the UV-112 preparation (830 cpm bound to the anti-B16 immunoadsorbent), whereas 1300 cpm bound to the normal IgG control matrix. By contrast, 44,700 cpm from the B16 preparation bound to the anti-B16 immunoadsorbent, while 11,600 cpm bound to the normal goat IgG matrix. When analyzed by polyacrylamide gel electrophoresis under reducing conditions, three components were resolved in the specifically bound and eluted material as shown by both label and protein staining with Coomassie brilliant blue. Figure 3 is an electrophoretogram of antigen preparation isolated specifically from 17.2 mg of lyophilized B16 "shed" material, and represents approximately 10 μg of antigen. The major component comprises two closely spaced bands with mobilities compatible with a mass of about 60,000 daltons. A minor band of about 50,000 daltons is clearly present and a trace component is also seen in the vicinity of the 20,000-dalton marker. This preparation was used to immunize rabbits (see below). No specific components were isolated from the UV-112 preparation (not shown). Similar components were isolated from an unlabeled Triton X-100 lysate of B16 cells and from lysates of B16 cells which had biosynthetically incorporated ^3H-leucine into cell protein.

In order to establish that the affinity-purified fraction contained specific B16-associated antigens, two rabbits were immunized with the putative antigen fraction isolated from shed material using the anti-B16 immunoadsorbent. Samples of serum were also taken from the rabbits prior to

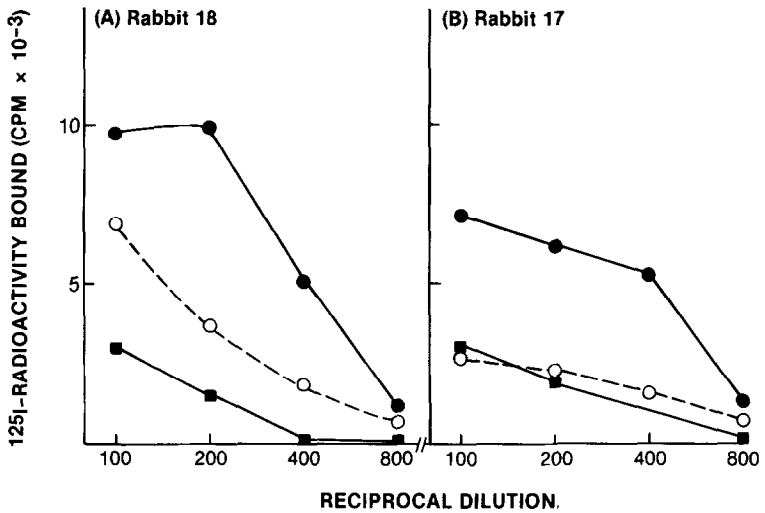

Fig. 4. Binding to B16 and UV-112 target cells of rabbit antisera produced against the melanoma-associated xenoantigen preparation isolated using goat antibodies (Figure 3). (A) Rabbit #18. ●——●, antiserum tested on B16 cells; o-----o, preimmunization serum tested on B16 cells; ■——■, antiserum tested on UV-112 cells. (B) rabbit #17. ●——●, antiserum tested on B16 cells; o----o, preimmunization serum tested on B16 cells; ■——■, antiserum tested on UV-112 cells. ^{125}I-labeled S. aureus A protein was used to detect bound IgG. All sera were absorbed with UV-112 cells to remove activity directed against mouse-specific xenoantigens.

immunization to serve as a control for naturally occurring antibodies. Both preimmune sera and antisera were absorbed with UV-112 cells to remove anti-mouse activity and then titrated for binding to B16 and UV-112 cells. As shown in Figure 4, immunization with the specifically purified antigen fraction induced antibodies binding to B16, but not to UV-112 cells. Rabbit 18 possessed some specific activity against B16 cells prior to immunization, but binding to B16 was substantially increased by immunization.

DISCUSSION: We have used a two-stage xenoimmunization approach to prepare antisera specific for surface antigens of B16 melanoma. Goat antiserum produced against intact melanoma cells detected a melanoma specific antigen shared by B16 and an allogeneic murine melanoma 1735. Studies involving binding to various tumors demonstrated that the component(s) detected did not appear to be mouse-specific xenoantigens, histocompatibility antigens, viral antigens or melanin. An immunoadsorbent made with the IgG fraction of this goat antiserum was used to recover an antigen-containing fraction from shed surface components and detergent lysates of the B16 melanoma. As assessed by polyacrylamide gel electrophoresis in SDS - containing buffers, the antigen fraction recovered from shed material, bulk triton homogenates or metabolically labeled material contained a major component with an apparent mass

of 60,000-68,000 daltons, a second component of approximate mass 50,000 daltons and a minor component of about 20,000 daltons. The fact that this immune-affinity purified preparation contained B16-specific antigens was demonstrated by immunizing rabbits with the purified fraction. The rabbits produced antibodies directed specifically against B16 cells. Although the only tumors which express this antigen are melanomas, it is possible that some embryonic tissues such as melanocyte precursors might also express the surface component(s).

It is noteworthy that the major component of the melanoma-associated surface antigen preparation is comparable in size to tumor surface antigens recently isolated from methylcholanthrene-induced sarcomas (16,17) and 4-dimethylamino-azobenzene-induced hepatomas (16). The migration of these tumor-associated antigens on SDS-PAGE is distinct from that of major histocompatibility antigens (21) and immune response, gene-associated (Ia) antigens (22). Moreover, the B16 xenoantigens described differ markedly in apparent mass from those of surface proteins (95,000 daltons; 100,000 daltons) which are correlated with the capacity of brain-selected B16 lines to colonize the brain (23). The affinity purified material from B16 cells possessed the capacity to induce rabbits to produce antibodies to these cells. It is important to ascertain the relationship of the B16 components to each other and to the antigens of other tumors.

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