Production of Antisera with Specificity for Malignant Melanoma and Human Fetal Skin

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SUMMARY

Complement-dependent cytotoxic antibodies to common cell surface antigens of cultured melanoma cells were produced in guinea pigs. At appropriate dilution, melanoma antisera were cytotoxic only to melanoma target cells. Following absorption with pooled lymphoid cells, additional absorption with melanoma cells but not absorption with fibroblasts or carcinoma cells was found to remove all cytotoxic activity from melanoma antisera. Absorption with human fetal skin cells but not with autologous fetal visceral cells was found to remove cytotoxicity from melanoma antisera. Tissue type-specific antigens may be shared by human malignant melanomas and fetal skin of black racial origin (at 16 to 18 weeks of gestation). The methods may be useful in the production of xenogeneic antisera with "operational monospecificity" for common melanoma-specific antigens. Sera from 47 patients with malignant melanoma failed to evidence specific cytotoxicity for melanoma target cells.

INTRODUCTION

In malignant melanomas, as well as in many other human cancers, the existence of common tumor-associated antigens, shared by tumors of similar histological type, has been demonstrated by tests for cell-mediated immunity (1, 4, 7, 8, 11, 13, 14, 16, 26-28, 32, 37-39), by immunofluorescence (16, 30, 35, 36, 38, 42, 46), and by complement fixation (23, 34). The nature of these common tumor-associated antigens has yet to be elucidated (12), although (e.g., in human colon carcinomas) common tumor-specific antigens of the embryonal type have been demonstrated (21) and their complex glycoprotein structure well characterized (43). Further serological studies in malignant melanoma may provide a clue as to the genetic origin of the information coding for the presence of common melanoma-associated cell surface antigens, since human melanoma cell lines have been shown to contain particles with some properties in common with RNA tumor viruses (40) and since viral-induced tumors of rodents are known to share common tumor-associated antigens (22). "Blocking" factors (either circulating tumor antigen or immune complexes) in sera of melanoma patients (10, 11, 24-27, 31) might be detected by a serological reagent with specificity for common melanoma-associated antigens. If specific antisera to common melanoma-associated antigens became available, the isolation and purification of melanoma-associated antigens (4, 7, 14, 28, 41) might be facilitated by absorption onto antibody-coated affinity columns. Purified melanoma antigens and specific antisera might be used as reagents for the identification and quantitation of serum-blocking factors by radioimmunoassay. Such testing might be useful in the diagnosis of patients with tumors of unknown primary and/or might aid in the follow-up of patients known to have melanoma.

Therefore, we attempted to produce, in animals, antisera to malignant melanoma, the specificity of which could be determined by a simple, rapid, and sensitive serological microcytotoxicity test (18, 20). We elected to raise antisera in animals for the following reasons: (a) carcino-embryonic antigen had been detected using xenogeneic antisera (21); (b) uniformly high titers of cytotoxic antibodies can be expected in xenogeneic antisera; (c) there is no need to depend on selected human donors, and attempts by Metzgar (33) and ourselves to demonstrate melanoma-specific antibodies in allogeneic sera from patients with malignant melanoma have been disappointing.

MATERIALS AND METHODS

Tumor Cell and Fibroblast Cultures. Seven melanoma cell lines and 2 adenocarcinoma cell lines were established in tissue culture by primary explant from surgical specimens obtained from 7 patients with malignant melanoma, a patient with gastric carcinoma, and a patient with carcinoma of the colon. A 2nd carcinoma of the colon cell line, HT-29M, was kindly provided by Dr. E. Bloom, Department of Microbiology and Immunology, UCLA, Los Angeles, Calif. The ovarian carcinoma cell line, 2008, was obtained, in 1972, from the USC-LA County Medical Center, Los Angeles, Calif. BT-20 breast carcinoma cells were provided by Contract E-73-2001-No. 1 within the Special Virus-Cancer Program, NIH, USPHS, through the courtesy of Dr. W. Nelson-Rees.

The tumor cell lines were designated as follows and studied in the passages indicated in brackets: (a) melanomas,
Bacillus Calmette-Guérin; HL-A, histocompatibility antigen.
malignant melanomas; RI-CA, CA-2008, HT-29M, WIL-CA, BT-20, cell cultures.
Fibroblasts were grown in tissue culture following primary explant from biopsies of normal skin and muscle obtained from melanoma patients ROB, VEG, and lung cancer patient PEARs. Fibroblast cultures were studied in Passages 5 to 10. All cultures were maintained in Roswell Park Memorial Institute Medium 1640, supplemented with 20% fetal calf serum, 0.15% glutamine, 0.25 mg amphotericin B per ml, 100 IU penicillin per ml, and 100 µg streptomycin per ml (all from Flow Laboratories, Los Angeles, Calif.).

All cell cultures were free of contamination with bacteria and mycoplasma.

Human Sera. Sera from 47 malignant melanoma patients at various stages of disease, sera from 7 patients with cancers other than melanoma, sera from 5 melanoma patients who had been treated for 8 to 12 months by alternate weekly immunotherapy with BCG and a melanoma cell vaccine (derived from a single melanoma of patient ED), and sera from patient ED (with progressively growing metastatic melanoma) were obtained from the Division of Oncology, Department of Surgery, UCLA School of Medicine.

Xenogeneic Antisera. Antisera were prepared in a separate group of 2 to 5 adult female Hartley guinea pigs by immunization with tumor cells from confluent tissue culture monolayers. Single cell suspensions of tumor cells were prepared by 5- to 10-min exposure to 0.25% trypsin followed by 3 washings in Roswell Park Memorial Institute Medium 1640. Tumor cells (10⁵) suspended in 1 ml of Roswell Park Memorial Institute Medium 1640 were mixed with an equal volume of complete Freund’s adjuvant and injected into each of the 4 footpads. Control guinea pigs were immunized with complete Freund’s adjuvant and injected into each of the 4 footpads. Control guinea pigs were immunized with complete Freund’s adjuvant serum only. Sera were collected from each group of guinea pigs 2 weeks after immunization, heat inactivated for 30 min at 56°C, and frozen in aliquots at −76°C. In early experiments, antisera were pooled within each experimental group. In later experiments, selected antisera from individual guinea pigs were used. Antisera were designated according to the tumor cell type used for immunization, e.g., ED-MEL, ROB-MEL, RI-CA, and CA-2008 serum.

Absorption of Xenogeneic Antisera. In the initial series of experiments, 0.3 ml of pooled guinea pig serum (diluted 1:20 in Roswell Park Memorial Institute Medium 1640) was incubated with counted numbers of packed viable cells in a gyratory shaking water bath at 37°C for 60 min. Packed cells were obtained from tissue cultures of the various tumors and fibroblasts as described above. In addition, lymphoid cells from 12 healthy human donors were isolated on Ficoll-Hypaque gradients (6), pooled, and used for absorption.

A black male fetus was obtained at 16 to 18 weeks of gestation immediately after 0.9% NaCl solution-induced abortion. Under sterile conditions the fetus was freed from adherent placental tissue. No gross abnormalities could be detected. Skin and adherent muscles were dissected from the skeleton, cut in fine pieces, pressed gently through 40 and then 80 mesh stainless steel sieves, and washed 5 times in Hanks’ balanced salt solution. The resulting single cell suspension was designated as “fetal skin.” Separately, a single cell suspension was prepared from a pool of all fetal internal organs by the same procedure, and designated as “fetal viscera.” Fresh fetal cells were used for absorption.

In a 2nd series of experiments, 0.5 ml of a melanoma antiserum (ROB-MEL serum) and 0.5 ml of an ovarian carcinoma antiserum (CA-2008 serum) were absorbed separately with 0.5 ml of packed human lymphoid cells derived from the pool of human donors described above. Following this absorption, 0.3 ml of ROB-MEL serum and 0.3 ml of CA-2008 serum (each diluted 1:25) were additionally absorbed with counted numbers of packed cells derived from cultures of tumors or fibroblasts.

Microcytotoxicity Test for Cytotoxic Antitumor Antibody. The method used was a modification of the procedure originally reported by Cohen et al. (9) and has been described in detail previously (18, 20). Briefly, 3 to 5 x 10⁶ viable tumor cells or fibroblasts prelabeled with [³²P]iododeoxyuridine were placed in each well of a Falcon Microtest II plate (Falcon Plastics, Oxnard, Calif.) and incubated overnight to allow the cells to adhere to the well bottoms. The next morning, medium was aspirated from the wells and 40 µl of serum were added to each well followed by incubation for 1 hr at 37°C. The serum was gently removed by aspiration, and 40 µl of pooled lympholysed rabbit complement (diluted 1:13) (Hyland Laboratories, Costa Mesa, Calif.) were added to each well. Complement alone was not cytotoxic at this dilution (17). After 1 hr of incubation with complement, the plates were washed twice, aspirated dry, and sprayed with a plastic film. Each serum dilution was tested in 4 or 6 replicate wells. The individual wells of each plate were separated with a band saw, and residual radioactivity in each well (a measure of the number of surviving cells) was measured with a γ counter. Means and S.D.’s for each set of replicate wells were calculated. Results are expressed in terms of percentage of cytotoxicity calculated according to the formula:

\[
\% \text{ cytotoxicity} = \frac{\text{cpm control serum} - \text{cpm test serum}}{\text{cpm control serum}} \times 100
\]

Controls in all experiments included diluted rabbit complement. In calculating the percentage of cytotoxicity, we chose to compare the cpm recorded following incubation with test sera to the cpm obtained following incubation with diluted rabbit complement alone in order to permit the determination of cytotoxic activity in control (i.e., normal) sera. Experimental groups were compared for significance using Student’s t-test for unpaired data.
RESULTS

**Human Sera.** Initially, sera from 47 melanoma patients at various stages of the disease were screened for cytotoxicity to target cells from 3 different melanoma cultures. All sera were allogeneic with respect to the target cells. Most sera from patients with melanoma, as well as sera from 7 patients with various cancers other than melanoma, showed less than 20% cytotoxicity for all 3 melanomas. The sera from 4 melanoma patients were significantly cytotoxic for all 3 melanoma cell lines. These sera were tested by the microlymphocytotoxicity test on a panel of lymphocytes obtained from 33 healthy Caucasian donors and were found to contain a broad spectrum of anti-HL-A reactivities (E. Zeller and Dr. R. L. Walford, Department of Pathology, UCLA, personal communication).

Sera from 5 melanoma patients (including Stages I, II, and III) were screened for cytotoxic activity on target cells from 3 melanomas and 3 carcinomas prior to and during the course of immunotherapy with BCG and allogeneic melanoma cell vaccine (provided by Dr. Wendell Winters, Division of Oncology, Department of Surgery, UCLA). In 3 of the 5 sera, cytotoxicity to ED-MEL target cells increased significantly during treatment with ED-MEL cell vaccine. However, autologous sera from patient ED, who was not vaccinated with his own melanoma cells, evidenced no change in cytotoxicity to target cells from his own tumor. Sera from the 2 vaccinated patients who showed the most impressive increase in cytotoxicity to ED-MEL target cells evidenced no change in cytotoxicity to target cells from 2 other melanomas. Therefore, the increase in cytotoxicity during immunotherapy with BCG and melanoma cell vaccine was found to be confined to target cells derived from the immunizing melanoma cell line. Since these results suggested that specific cytotoxic activity to common melanoma-associated antigens was not detected in sera of melanoma patients even during immunization with allogeneic melanoma cell vaccine, these data are not presented in detail. We then proceeded to study xenogeneic antisera.

**Xenogeneic Antisera.** The characterization of the xenogeneic antisera was approached in 2 ways. Melanoma antisera were diluted until they appeared to lyse melanoma target cells selectively. At these and higher dilutions, the sera were tested prior to and following absorption with cells from various tissues. In the second approach, antisera were absorbed with packed pooled human lymphoid cells to remove antiserum and anti-HL-A reactivity. Then, these sera were tested prior to and following additional absorption with cells from various tissues.

Initially, the cytotoxicity of antisera to 2 melanomas (ED-MEL, ROB-MEL) and a gastric carcinoma (RI-CA) was tested on target cells from 3 melanomas and 3 carcinomas. The results obtained are illustrated in Chart 1. Both melanoma antisera were cytotoxic to all 3 melanomas over a wide range of serum dilutions, whereas complete Freund’s adjuvant serum and gastric carcinoma antisera were completely nontoxic. At dilutions of 1:8 to 1:64, both melanoma antisera were also cytotoxic to the carcinoma target cells. However, at 1:128 and higher dilutions, both melanoma antisera showed less than 20% cytotoxicity to all 3 carcinoma target cells (Chart 1, arrows). The gastric carcinoma antisera (RI-CA) was found again to be cytotoxic only to RI-CA target cells (19).

Then we studied whether absorption with melanoma cells would remove more cytotoxic activity from melanoma antisera than would absorption with pooled lymphocytes or gastric carcinoma cells. These results are summarized in Chart 2. Prior to absorption, both melanoma antisera (ED-MEL, ROB-MEL) were cytotoxic to target cells from 3 melanomas. Whereas gastric carcinoma cells or pooled lymphocytes failed to remove significant cytotoxicity, comparable numbers of cells from 5 different melanoma cultures uniformly reduced the cytotoxicity of both melanoma antisera significantly. The melanoma cells used in these absorption experiments could have removed cytotoxicity simply by expressing stronger species and/or HL-A antigens than the other tissue cells. Therefore, we studied whether more cytotoxicity was removed from both melanoma antisera by melanoma cells than by autologous skin fibroblasts. The results of this experiment are illustrated in Chart 3. Whereas absorption with gastric carcinoma cells or pooled lymphocytes failed to remove more than 20% cytotoxicity, in most cytotoxicity tests (5 of 6), absorptions with autologous melanoma cells were found to remove significantly more cytotoxicity from both melanoma antisera than did identical numbers of autologous fibroblasts. In 1 experiment, fibroblasts and autologous melanoma cells removed similar quantities of cytotoxicity. In this instance, the antiserum might have been too dilute to produce different absorption between melanoma cells and autologous fibroblasts. Most important, significantly more cytotoxicity was removed from ROB-MEL antiserum by absorption with ROB-MEL melanoma cells than by ROB fibroblasts in tests on ROB-MEL melanoma target cells.

Since melanoma cells might share antigens with human fetal tissue, both melanoma antisera were absorbed with increasing numbers of cells derived from either skin and muscle or internal organs of a fetus of black racial origin. The results obtained are illustrated in Chart 4. Absorption with fetal skin cells as well as melanoma cells was found to remove all cytotoxicity, whereas absorption with fetal visceral cells or pooled lymphocytes removed significantly less cytotoxicity from both melanoma antisera.

We attempted to obtain similar results by absorbing antisera first with pooled lymphoid cells to remove antiserum species and/or anti-HL-A reactivity, followed by additional absorption with cells from cultures of fibroblasts, carcinomas, or melanomas. The results of this 2nd series of experiments are illustrated in Charts 5 to 9. Xenogeneic antisera were collected from individual guinea pigs following immunization with cells from each of 3 melanomas or carcinomas. These sera were selected for high cytotoxicity to ED-MEL melanoma target cells. The data depicted in Chart 5 illustrate that, at dilutions of 1:8 and 1:32, all antisera, except the RI-CA antiserum, were found to be cytotoxic to ED-MEL target cells. Cytotoxicity was complement dependent, since heat-inactivated rabbit complement abrogated cytotoxicity completely. One melanoma antiserum (ROB-MEL) and 1 ovarian carcinoma antiserum (CA-2008) were each absorbed with packed pooled lymphoid cells at a cell to serum ratio of 1:1. Prior to and following this absorption, both antisera were tested on tar-
Melanoma-specific Antisera

Chart 1. Percentage of cytotoxicity obtained with 2 xenogeneic melanoma antisera (ED-MEL and ROB-MEL), a gastric carcinoma antiserum (RI-CA), and an antiserum collected after immunization with complete Freund’s adjuvant (CFAS). Target cells are derived from 3 melanomas (top) or 3 carcinomas (bottom).

Chart 2. Percentage of cytotoxicity obtained with 2 xenogeneic melanoma antisera, ED-MEL (right), and ROB-MEL (left), prior to and following absorption with cells from: (1) gastric carcinoma; (2) a pool of lymphocytes; (3 to 7) 5 different melanomas. Each serum was tested on melanoma target cell ED-MEL, ROB-MEL, and AL-MEL. N.T., not tested.
get cells derived from cultures of 2 melanomas (ED-MEL, ROB-MEL) or fibroblasts (VEG). At dilution of 1:100 or 1:200, the absorption with packed lymphocytes was found to remove 40 to 60% of the cytotoxicity for all 3 target cells. The ovarian carcinoma antiserum became completely non-cytotoxic to all 3 target cells, whereas the melanoma antiserum was still significantly cytotoxic to ED-MEL melanoma cells (Chart 6). When the ROB-MEL melanoma antiserum was additionally absorbed with ROB-MEL cells, the cytotoxicity to ED-MEL target cells was removed completely. By contrast, absorption with 3 types of carcinoma cells failed to reduce cytotoxicity further. Since the ovarian carcinoma antiserum already became noncytotoxic, it remained noncytotoxic following additional absorption with ovarian carcinoma cells (Chart 6).

We then studied whether additional absorption of ROB-MEL melanoma antiserum with melanoma cells would remove more cytotoxicity than would additional absorption with fibroblasts. The results obtained are illustrated in Chart 7. Additional absorption of ROB-MEL antiserum with cells from 3 different melanomas was found to remove all cytotoxicity, whereas absorption with cells from 3 fibroblast cultures removed significantly less cytotoxicity.

Finally, we absorbed ROB-MEL antiserum additionally with carcinoma cells, fibroblasts, or melanoma cells. Prior to and following absorption, this antiserum (diluted 1:50) was tested on target cells from 2 recently established melanoma cell lines (CAMER-MEL, Passage 5; VEG-MEL, Passage 6). The results depicted in Charts 8 and 9 show that fibroblasts and carcinoma cells absorbed significantly less additional cytotoxic activity than did melanoma cells. In these absorptions, VEG fibroblasts were autologous to the VEG-MEL melanoma target cells (Chart 9), and ROB fibro-

**Chart 3.** Percentage of absorption obtained with each of 2 xenogeneic melanoma antisera, ED-MEL (top), and ROB-MEL (bottom), using cells from gastric carcinoma (RI-CA), pooled lymphocytes, autologous fibroblasts, or autologous melanoma cells (both from patient ROB). Each serum was tested on 3 melanoma target cells.

**Chart 4.** Percentage of cytotoxicity obtained with 2 xenogeneic melanoma antisera, ED-MEL (top), and ROB-MEL (bottom), prior to and following absorption with increasing numbers of cells derived from human fetal skin and muscle (Fetal Skin), fetal internal organs (Fetal Viscera), pooled lymphocytes (Pooled Lymphos.), or ED-MEL melanoma cells. Each serum was tested on target cells from 3 melanomas: ED-MEL (left), ROB-MEL (center), and AL-MEL (right).
tion with equal volumes of packed pooled lymphoid cells and following absorption with CA-2008 antiserum was absorbed additionally with CA-2008 cells. Xenogeneic melanoma antiserum (ROB-MEL) or xenogeneic ovarian carcinoma antiserum (CA-2008), each diluted 1:200, prior to and following absorption with cells from 3 carcinomas or ROB-MEL melanoma. Did absorption with cells from any of the other tissues. Findings suggested that, following absorption, these antisera reacted preferentially with common melanoma-specific antigens expressed by cultured melanoma cells.

In addition, we noted that fresh cells from human fetal skin (and muscle) shared with cultured melanoma cells the ability to remove more cytotoxic activity from melanoma antisera than could autologous fetal visceral cells or pooled lymphocytes (Chart 4). Since fetal skin cells and fetal visceral cells had been obtained from the same fetus, HL-A antigens probably do not account for the difference noted in absorption with both tissues. However, species and/or HL-A antigens might be expressed on fetal skin at an earlier stage of development or at higher concentration than on internal organs. Fossati et al. (15) found that lymphocytes from 6 of 10 melanoma patients were cytotoxic to epithelial-like cells cultured from human embryos at 1 to 2 months of gestation. However, this cell-mediated cytotoxicity was not specific for lymphocytes from melanoma patients. Fossati et al. (15) found also that lymphocytes from 5 of 5 melanoma patients failed to be cytotoxic to fibroblast-like cultures derived from lung and heart of 2 4- to 5-month-old fetuses. Thus, our observation that absorption with fetal cells from internal organs failed to remove cytotoxicity from melanoma antisera might gain support by inspection of earlier data.

Our data are consistent with the hypothesis that tissue type-specific antigens may be shared by malignant melanomas and black fetal skin. Fetal tissues of other races must be studied before firm conclusions can be drawn.

In a 2nd series of experiments, xenogeneic antisera were first absorbed with pooled lymphoid cells to remove antispecies and/or anti-HL-A reactivity. Then, these antisera were
Chart 7. Percentage of cytotoxicity for ED-MEL target cells obtained with xenogeneic melanoma antiserum ROB-MEL prior to and following absorption with pooled lymphoid cells and following additional absorption with increasing numbers of cells from 3 cultures of fibroblasts or melanomas.

Chart 8. Percentage of cytotoxicity for CAMER-MEL target cells (Passage 5) obtained with xenogeneic melanoma antiserum ROB-MEL prior to and following absorption with pooled lymphoid cells (PACKED LYMPHOS.) and following additional absorption with increasing numbers of cells from cultures of 3 fibroblasts, 2 carcinomas, or ED-MEL melanoma.

Additionally absorbed with cells from cultures of fibroblasts, carcinomas, and melanomas (Charts 5 to 9). Although one cannot be certain that all antispecies and anti-HL-A reactivity was eliminated from these antisera by absorption with pooled lymphoid cells, our results show reductions in cytotoxicity of from 40 to 60%. In fact, at identical dilutions, an ovarian carcinoma antiserum became completely noncytotoxic to 2 melanoma cell lines and 1 fibroblast line, whereas a melanoma antiserum continued to exhibit significant cytotoxicity for melanoma target cells (Chart 6). Moreover, additional absorptions with melanoma cells were found to remove significantly more cytotoxic activity from this melanoma antiserum than did absorptions with cells from any of the other tissue types (Charts 7 to 9).

Although serological cross-reactivity based on reactions to membrane antigens related to fetal calf serum (29) cannot be entirely ruled out, we suggest that this explanation is unlikely. All tumor cells and fibroblasts were cultured under identical conditions in medium containing 20% fetal calf serum, but the cross-reactivity was observed only among the melanomas. The cross-reactivity could be due to common HL-A antigens or other non-HL-A normal cell surface antigens. However, we have demonstrated previously that the 3 melanomas most often used in this study (ED-MEL, ROB-MEL, AL-MEL) have individual HL-A antigens without any major cross-reacting specificity being shared by any 2 of these cell lines (17).

Although new alloantigenic systems have been described on human lymphocytes (45), the HL-A system is the strongest histocompatibility system in man (44), and may be identi-
cal with or closely related to the human species antigens (2). Therefore, weaker non-HL-A normal antigens are probably not responsible for the cross-reactivity between melanomas observed in this study. Moreover, pooled lymphoid cells were used to absorb xenogeneic melanoma antisera extensively. Blood Group A-like substances have been detected in some human tumors (3), but it appears unlikely that the serological cross-reactivity among all melanoma cell cultures should be due to the same blood group antigens. We cannot rule out that these serological reactions might be due to antigens specified by a virus, since virus particles with some properties in common with RNA tumor viruses have been detected in some human melanoma cell lines (40).

Our results essentially confirm and extend the observations of Metzgar et al. (33). Following immunization of 2 monkeys with 2 human melanoma cell lines, Metzgar et al. (33) detected (by complement-dependent cytotoxicity and mixed agglutination) specific antibodies to common melanoma-associated membrane antigens in these xenogeneic antisera. Although absorptions with human fetal tissues were not done, they speculated that common melanoma-associated antigens might be of the fetal type.

In previous studies, we demonstrated that common cell surface antigens might be shared by carcinogen-induced murine sarcomas (20). By using similar methods to produce and absorb xenogeneic tumor antisera, we have now detected common tumor-associated antigens on cultured human melanoma cells and provided some evidence for their possible relationship to antigens of fetal skin. We suggest that our methods may be useful to produce and to quantitate xenogeneic melanoma antisera with “operational speci-
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