Immunological Studies on Malignant Melanomas of Man

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INTRODUCTION

In this paper, we have reviewed current problems in tumor immunology with regard to melanoma and report our studies of a small series of patients with melanoma and other malignancies. A humoral response to malignant melanoma has been well established by the immunofluorescence test (1–5). Cross-reacting cytoplasmic antigens were detected as were cross-reacting and individually specific membrane tumor-associated antigens (5, 6).

Cell-mediated immunity has been shown in patients with progressively growing tumors and in patients clinically free of disease by in vitro techniques (7–12). Cytotoxicity of peripheral lymphocytes was observed in both the autochthonous and allogeneic systems (7, 8, 12). Also, lymphocyte cytotoxicity has been observed to be abrogated by serum-mediated factors in a large number of patients with malignant melanoma (13).

In studying cell-mediated immunity in human malignant melanoma, deVries (12) observed in several patients that lymphocytes appeared to lose their capacity for killing melanoma target cells in the absence of systemic treatment. They did not observe a correlation between the presence of immune lymphocytes and the clinical stage of disease.

After demonstration of humoral antibodies to surface and intracellular antigens by immunofluorescence tests (5), we tried to detect a humoral response to soluble extracts of melanoma tumors by gel diffusion (17). While unable to demonstrate tumor-specific antibodies in sera by immunodiffusion, the extracts prepared as a source of antigen were found to contain substantial amounts of immunoglobulins. Further studies, utilizing antisera to specific human immunoglobulins, revealed that a majority of fluorocarbon-prepared extracts of melanoma tumor and all extracts prepared by the freeze-thaw method contained IgG. A lesser but substantial number also contained IgA. When compared by quantitative radial immunodiffusion with similarly prepared normal tissue extracts, it was found that the tumor extracts had higher concentrations of these immunoglobulins (unpublished data).

Next, attempts were made to elute the immunoglobulins from the surface of

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live melanoma tumor cells. One such eluate was prepared from a recurrent tumor excised from a patient who had undergone long-term active immunotherapy.

The effects of fluorocarbon extracts, freeze-thaw extracts, and eluates on the growth of melanoma cells propagated in vitro were then studied. Fluorocarbon extracts and eluates were also tested for their ability to block the cytotoxic action of sensitized lymphocytes by in vitro cell-mediated immunity tests.

Humoral and cell-mediated immunity studies were carried out utilizing serial serum samples from two patients who were undergoing long-term immunotherapy. The sera of these two patients were first studied for tumor antibody by gel diffusion (Ouchterlony method) using fluorocarbon and freeze-thaw tumor extracts, similarly prepared extracts of tissue-cultured melanoma cells, and extracts of homologous tumor vaccines as the source of antigen.

The serial serum samples were tested for complement-fixing antibody against the soluble antigen preparations, utilizing guinea pig serum as the source of complement.

The immunotherapy-patient sera were finally tested for complement-dependent cytotoxic antibody to cultured melanoma target cells, using rabbit complement diluted with human umbilical cord serum.

For possible correlation of immunological parameters with tumor growth and/or recurrence of disease (15), the immunotherapy patient sera were tested for L-3,4-dihydroxyphenylalanine (dopa) oxidase activity, anticipating that this enzyme may reflect metabolic functions of malignant melanoma in vivo.

MATERIALS AND METHODS

Serum and tumor collection. Blood collected by venipuncture was allowed to clot at room temperature and the serum decanted following centrifugation. The serial blood samples from the immunotherapy patients were obtained just before each injection of vaccine. Normal control sera were obtained from healthy hospital employees and staff who had no history of transfusions or pregnancies.

Melanoma tumors were obtained from fresh surgical specimens and immediately processed.

Specific immunoglobulin antisera were obtained from Hyland, Travenol Division.

Extract and eluate preparation. Extracts of tumor and normal tissue were prepared by homogenization with a VirTis ‘45’ homogenizer equipped with an ice bath. After sedimentation of the debris, supernatant fluids were extracted five times with 1/2 volumes of Genetron 113, as previously described (17).

Freeze-thaw extracts were prepared by homogenization of 20% (w/v) suspensions (as described above) followed by freezing in an acetone/dry ice bath and thawing in water at 37°C. Following centrifugation at 20,000 rpm for 30 min at 4°C, the debris was discarded.

Eluates were prepared by teasing excised tumors into a single cell suspension in phosphate buffer with sterile forceps and scalpel. The cell suspension was then put through a 200-mesh wire screen to retain the fibrous elements and remaining clumps. The cell suspension was washed briefly in several volumes of phosphate buffer, pH 7.5, centrifuged to pellet the cells, and the supernatant discarded. The cells were resuspended in 100 vol of buffer and washed for 1 hr at 4°C with continuous stirring. After the extended wash, the cells were resuspended in 10 vol of cold glycine buffer at pH 2.4 and gently agitated for 30 min at 4°C, the supernatant removed, and the pH immediately adjusted to neutral using 1 N NaOH.
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The supernatant was then dialyzed against phosphate buffer, pH 7.5, and concentrated to original packed-cell volume by Amicon diafiltration equipped with an XM-100 (retaining molecules above 100,000 molecular weight) membrane. The eluates were then tested by gel diffusion for immunoglobulin content using specific immunoglobulin antisera and used as the source of antibody in the lymphocyte microcytotoxicity test.

**Complement fixation tests.** Complement fixation studies were carried out according to the microtiter method outlined by Sever (18) on the sera of the immunotherapy patients. Individual melanoma tumor extracts, prepared by fluorocarbon extraction as previously described, and pooled tumor extracts were used as the source of antigen. Similarly prepared extracts of normal tissues and of human sarcomas were used as controls. Guinea pig serum obtained from Hyland, Travenol Division, was used as the source of complement.

**Complement-dependent cytotoxicity tests.** Serum-mediated, complement-dependent cytotoxicity studies were conducted similarly to the test outlined by Wood and Morton (14), on the immunotherapy-patient sera and a battery of normal control sera. Human umbilical cord serum, used to dilute rabbit complement 1:1, was obtained from Cordis Laboratories, Miami, Florida, and by local collection. Cultured melanoma cells, used as target cells, were incubated with test serum for 1 hr at 37°C. Complement was then added to the plates and incubated for an additional 2 hr at 37°C. Complement and serum were then removed, replaced with fresh media, and the plates incubated overnight at 37°C. The target cells were then washed with Hanks BSS buffer, air-dried, stained with Giemsa, and the remaining cells scored microscopically.

**Vaccine preparation.** Vaccines used in immunotherapy were made by preparing a single cell suspension of melanoma tumor in Hanks BSS. The cells were irradiated with 10,000 r to render them nonreproductive. The cell suspension was adjusted to contain $1 \times 10^8$ viable cells/ml and aliquotted in 1 ml ampules. The ampules were stored at $-70°C$ following tests for sterility and reproducibility. Immunization was carried out by intradermal injection of 1 ml of cells at regular intervals.

**Lymphocyte isolation.** Forty milliliters of heparinized peripheral blood was left at room temperature for 1-2 hr to allow the red cells to settle. The supernatant was then decanted, the cells sedimented by a low-speed spin, washed in Hanks BSS, centrifuged, and resuspended in media. This suspension was placed on a 4-in. column of beads and incubated in a water bath at 37°C for 10 min. The lymphocytes were flushed from the column with Eagles' medium containing 10% fetal calf serum. After counting, the cells were resuspended in an appropriate amount of Eagles' medium to give a ratio of from 500 to 1,000 lymphocytes per target cell.

**In vitro lymphocyte cytotoxicity tests.** SH-1 cells, established by Giovanella et al. (21) from a malignant melanoma tumor and shown by previous tests to possess antigenicity to sensitized lymphocytes from melanoma patients, were used routinely in these tests. Line LeCA, strain 26-5 human melanoma cells, established in this laboratory 6 years ago and shown to be sensitive to the cytotoxic action of lymphocytes, were also used routinely. Both human melanoma cell lines have been shown to have similar sensitivities to cytotoxic action of donor lymphocytes.

The lymphocyte microcytotoxicity procedures used in the tests have been reported (8, 20). The sera and eluates to be tested for blocking activity were used
undiluted and were inactivated at 56°C for 30 min. Following incubation with target cells for 1 hr at 37°C, they were poured off, and the lymphocyte suspension was then added. After incubation for 48 hr at 37°C, the lymphocytes and media were removed, and the plates washed and air-dried. The cells remaining in the plates were fixed with methanol, stained with Giemsa, and scored by microscopy.

Serum enzyme studies. Serum proteins were separated by polyacrylamide disc electrophoresis as described by Davis (19). For each column, 0.1 ml of each serum was used. Following electrophoresis, gels were placed in tubes containing 0.3% dopa in 0.1 M phosphate buffer, pH 6.9, and incubated in a 37°C water bath for 2 hr. Gels were then washed in 7% acetic acid and scanned with a spectrophotometer at 400 nm. The area under each dopa-positive peak was calculated by planimetry.

RESULTS

Gel diffusion studies to demonstrate immunological reactivity between putative tumor antigens in melanoma extracts and patient sera were not positive (17). Sera collected serially from the two long-term immunotherapy patients and tested against extracts of melanoma tumors, including those from which the vaccines were prepared, did not demonstrate tumor antibody reactivity by gel diffusion. All melanoma tumor extracts, however, were found to have immunoglobulin G above the level observed in corresponding normal tissues by immunodiffusion with specific immunoglobulin antiserum. IgA was also demonstrated in a majority of tumor extracts.

An eluate obtained from a tumor of a patient (T.C.) who had not received prior systemic treatment was shown to contain IgG, while tumor eluate from the patient (J.M) undergoing immunotherapy demonstrated both IgG and IgA. The eluate (T.C.) containing only IgG had greater enhancing capacity than the eluate with both immunoglobulins (Table 1). While melanoma tumor eluates enhanced the growth of target cells, eluates from sarcoma tumor cells inhibited their growth. An eluate prepared from normal muscle had little effect on cell growth.

Melanoma tumor extracts prepared by freeze-thaw cleavage were inhibitory to cell growth. Tumor extracts prepared by fluorocarbon extraction were found to

| Preparation | Patient | Tumor type  | % Enhancement | % Inhibition |
|-------------|---------|-------------|---------------|--------------|
| Eluate      | T.C.    | melanoma    | 400           | none         |
| Eluate      | J.M.    | melanoma (IT) | 116          | none         |
| Eluate      | F.B.    | sarcoma     | none          | 52           |
| Eluate      | K.F.    | sarcoma     | none          | 43           |
| Eluate      | S.C.    | normal muscle | none         | 7            |
| Extract     | N.M.    | melanoma    | none          | 41           |
| Extract     | L.R.    | melanoma    | none          | 22           |
| Extract     | A.C.    | melanoma    | none          | 45           |
| Extract     | C.H.    | melanoma    | 122           | none         |
| Extract     | J.M.    | melanoma (IT) | 120          | none         |

a Extracts were prepared by freeze-thaw extraction 5 times.

b Extracts were prepared by Genetron 118 extraction for a total of 5 times.

c Immunotherapy patient.
immunotherapy against a pool of melanoma tumors are shown in Charts 1 and stimulate growth. The different effect is presumed to be owing to a higher concentration of particulate protein in the freeze-thaw extracts than in the fluorocarbon preparations, the latter containing only lipid-free soluble proteins. Sera from patients with melanoma usually stimulated the growth of cultured melanoma cells.

Complement-fixation titers of sera from each of the two patients undergoing 2. Patient M.I. (Fig. 1), after sporadic positive titers at the initiation of immunotherapy, had no titer some 5 months after immunotherapy was started. The titer became positive 8 months later, but reverted to negative even with the introduction of a new vaccine. It was still negative when recurrence of clinical disease was observed 11 months later.

The second immunotherapy patient (J.M.) demonstrated complement-fixing antibody during the initial period of his therapy regime (Fig. 2). The introduction of a different vaccine may have been responsible for the disappearance of a complement-fixation titer. When another vaccine was subsequently utilized, however, his titer again became elevated. Recurrence of clinical disease was observed while his
titer remained high. It may be relevant that a complement-fixation titer was not registered during the time when both patients were receiving the same vaccine.

Cytotoxic antibody was not observed at any time during the course of immunotherapy when the sera were examined by the complement-dependent microcytotoxicity tests. In fact, target cells appeared to thrive in the presence of patient serum and complement. The growth of the target cells was slightly enhanced or not affected by serum alone. Enhanced growth was presumed to be mediated by the human cord serum used to dilute the rabbit complement.

Table 2 depicts the frequency of lymphocyte cytotoxicity to cultured melanoma target cells observed in these tests. While three of four patients with untreated melanoma had cytotoxic lymphocytes, they were not present in the two patients following long-term immunotherapy. It is not known whether either immunotherapy patient had cytotoxic lymphocytes before treatment. Results obtained from studies on patient M.I.'s sera are shown in Fig. 3. There was no blocking activity in his sera at the commencement of immunotherapy; he did, however, develop blocking factors 5 months later which were maintained for 7 months. During this period, there was no complement fixing antibody detected, and dopa-positive reactions were near levels obtained with normal serum. Shortly after the blocking factors disappeared, there was a striking increase in dopa-positive activity concomitant with a rise in complement-fixing antibody titer. The complement-fixation reactivity

![Graph](image.png)

**Fig. 3.** Dopa-positive reactivity of sera from patient M.I. NV = new vaccine; R = recurrence of disease; C-F = complement fixing antibody.
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then declined while the dopa-positive reactivity persisted up to the time when recurrent disease was clinically detectable.

Figure 4 depicts the results obtained in studies of patient J.M.'s serial serum samples. Blocking activity and complement-fixing antibodies were present at first. Blocking factors, however, disappeared after 6 months of therapy with the same vaccine. The complement-fixing activity was lost after the administration of a new vaccine preparation and remained absent until after the administration of a third vaccine preparation. Thereafter, complement-fixing antibody levels maintained at a high level and serum dopa-positive reactivity increased. Prolonged absence of a complement-fixing antibody titer was observed during the administration of the same vaccine preparation in both patients M.I. and J.M.

An eluate prepared from J.M.'s recurrent tumor mass following immunotherapy was repeatedly found to completely block lymphocyte cytotoxicity and also to enhance the growth of melanoma target cells. An eluate similarly prepared from an untreated melanoma patient had the capacity to block lymphocyte cytotoxicity by 80%; in the absence of lymphocytes, however, it had a greater enhancing ability on target cell growth.

DISCUSSION

While gel diffusion studies have revealed the presence of specific immunoglobulins in tumor extract preparations above that found in normal tissues, the biological role of these proteins is not known. It seems plausible that these immunoglobulins are associated with or function in response to some determinant on the tumor cell membrane. In this respect, they may facilitate tumor cell growth by masking of tumor specific membrane antigens and thereby protect cells from cytotoxic lymphocytes.

Attempts to identify the function of these immunoglobulins requires that they be isolated from other cellular products which may influence tumor target cells. Consequently, the eluates, which primarily contain immunoglobulins, enhance melanoma target cell growth, while less pure extracts of tumor tissue can inhibit
growth. The eluates contain immunoglobulin G, often IgA, and possibly dissociated antigen. Extracts presumably contain antigen-antibody complexes in addition to a myriad of proteins. The specificity of the eluates from melanoma tumors toward melanoma target cells is supported by failure to show an enhancing effect with either human sarcoma or muscle eluate preparations in the identical test system.

These preliminary observations do not permit one to consider which immunoglobulin may play the more prominent role in enhancement. We did observe, however, that the eluate prepared from the patient undergoing immunotherapy blocked the cytotoxic action of sensitized lymphocytes slightly more effectively than did the eluate prepared from an untreated patient. The former contained both IgG and IgA; the latter contained only IgG. Further experiments will be necessary in an attempt to identify mechanisms operative in this relationship between cells and immunoglobulins.

The complement-fixing antibody studies suggest that the host may well respond to antigenic stimulation provided by the vaccine preparation, even though the lack of specificity associated with this test precludes a correlation of titer to clinical activity of the tumor in vivo. It may be noteworthy, however, that during periods when complement-fixation titers were registered, serum dopa-oxidase reactivity was prominent. Identification of a possible correlation between these has not yet been shown.

Blocking of sensitized lymphocytes by sera from patients undergoing immunotherapy cannot in itself imply that the therapy is the causative factor. The demonstration of blocking was intermittent, though neither patient demonstrated blocking activity during the year preceding the actual observance of recurrent disease. The patients' own lymphocytes examined at this time were not sensitized to cultured melanoma cells, and an eluate prepared from the tumor of one of the patients was shown to effectively block sensitized lymphocyte cytotoxicity in an allogeneic system.

It is now generally accepted that active immunotherapy has been of little, if any, value. The possibility of enhancement of tumor growth in vivo is an ever-present threat. Tumor growth may be facilitated through the elicitation of a humoral response to melanoma vaccines which could result in serum-mediated blocking of lymphocyte recognition and cytotoxicity. Tumor antigens in live cell vaccines may also be masked from recognition by lymphocytes by an immunoglobulin coating, thereby invoking a response to serum proteins in homologous immunization or possibly a state of tolerance in the autologous situation.

If these mechanisms are real, methods could be sought first to remove such immunoglobulins from tumor cells, then to use them for immunization to permit antigen recognition by lymphocytes. Also, the humoral response to active immunization could be suppressed to prevent or modify the formation of blocking factors responsible for abrogating lymphocyte cytotoxicity.

These considerations concerning the interaction of humoral and cellular immunity in solid tumors may offer new approaches to the therapy of human malignant melanoma.

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