Purification of Soluble Human Melanoma-associated Antigens

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SUMMARY

Soluble extracts of melanoma specimens were prepared by 3 M KCl extraction. Delayed cutaneous hypersensitivity reactions to these antigens were noted in 25 of 39 melanoma patients and 7 of 30 patients with other neoplasms. Only 4 of 34 melanoma patients reacted to an extract of autologous muscle. Maximum reactivity to these antigens occurred at 24 hr and was demonstrated histologically by skin biopsy. Chromatography on Sephadex G-150 resulted in two fractions that elicited delayed cutaneous hypersensitivity reactions in melanoma patients. These fractions were subjected to polyacrylamide gel electrophoresis. The first region of the Sephadex I gel reacted in 8 of 13 melanoma patients and only 1 of 7 patients with other neoplasms. Some activity was found in other regions of the gel. Skin test activity was confined to the second polyacrylamide gel electrophoresis region of the Sephadex II gel, to which 7 of 9 melanoma patients reacted compared with 1 of 7 patients with other tumors. Recovery of antigenic activity in excess of the total present in the original extract after partial purification indicated that inhibitors of delayed cutaneous hypersensitivity reactions present in the crude KCl extract were removed. A 20-fold increase in antigenic activity per unit protein was achieved.

INTRODUCTION

A number of antigens associated with human tumor cells have been demonstrated by a variety of immunological techniques. Some of these antigens, such as carcinoembryonic antigen and α-fetoprotein, are not immunogenic in the host and can be detected in a wide variety of benign and neoplastic disease states (4, 16, 29). This limits the diagnostic and therapeutic usefulness of these substances. Other substances are specifically associated with tumor cells and elicit in vivo and in vitro host immune responses (6, 10, 11, 19, 21, 22, 24, 30, 32). Melanomas, for example, possess antigens that elicit specific in vitro and in vivo responses (1, 3, 6, 8, 9, 13, 15, 17, 23). These tumor-associated antigens are potentially useful as immunodiagnostics and immunotherapeutic agents. Purified tumor-associated antigens could be used to develop more sensitive diagnostic serological tests, such as a radioimmunoassay for circulating tumor antigens, and they might be useful in augmenting the host immune response against the tumor. Recently, Holinshead et al. (13) and Char et al. (3) isolated a partially purified melanoma-associated antigen from low-frequency sonic extracts. Using a similar purification scheme, we have attempted to purify melanoma-associated antigens solubilized by 3 M KCl extraction. The purpose of this study was to solubilize melanoma-associated antigens from fresh tumor specimens, to purify these antigens, and to monitor the antigenicity of these preparations by testing for DCHR in melanoma patients.

MATERIALS AND METHODS

Source of Antigens. A 3 M KCl extract of 2 s.c. melanoma metastases from a single patient was used throughout the study. The patient had a prolonged clinical course. He developed axillary metastases 1 year after excision of his primary melanoma. One year later, he developed 2 s.c. metastases, which were excised. At this time, muscle was excised for use as a control tissue. After 5 months, he developed small bowel metastases and expired 10 months later, a total of 3 years after the development of his primary melanoma. Because of the limited nature of the surgical procedures, only muscle adjacent to the tumor was available for a control tissue.

Solubilization of Antigens. Fresh surgical specimens of melanoma were extracted using the 3 M KCl technique (26) as modified by Roth et al. (14, 28). In brief, specimens were weighed and finely minced. A single-cell suspension of an aliquot of tissue was prepared by passing it through a fine mesh screen, and the number of cells per g of tissue was determined by counting the cells in a hemocytometer. The tissue was then suspended in 3 M KCl adjusted to a pH of 7.4 with PBS (Grand Island Biological Co., Berkeley, Calif.) at a concentration of 3 × 10^7 cells per ml. This suspension was mixed constantly for 16 to 24 hr at 4 °C. All subsequent procedures were performed at 4 °C. The mixture then was centrifuged at 136,000 × g for 1 hr in a Spincso L3-50 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) and the supernatant was dialyzed against 200 volumes of PBS with 3
changes in 24 hr. The precipitate formed during dialysis was removed by centrifugation at 136,000 × g for 1 hr.

Normal tissue extracts were prepared in the same manner.

**Gel Exclusion Chromatography.** Fresh tissue KCl extracts in PBS were concentrated with an Amicon ultrafiltration device (Amicon Corp., Lexington, Mass.) that had a UM2 membrane. The concentrate was dialyzed in 1 m NaCl + 0.01 m Tris-HCl, pH 8.0, containing 0.16% NaN₃, and 20 ml (600 mg of protein) were applied to a Sephadex G-150 column (5 × 60 cm) equilibrated with the same buffer. The flow rate was maintained at 24 ml/hr. Fractions were pooled as shown in Chart 1 and concentrated with an Amicon ultrafiltration device equipped with a PM-10 membrane.

**Preparative PGE.** Preparative PGE was performed essentially as described in System B of Rodbard and Chrambach (27). About 3 mg of protein were placed in each of 6 gels (15 × 500 mm) in tubes of a semipreparative electrophoresis device (Buchler Instruments Co., Fort Lee, N. J.). Electrophoresis was performed by applying 15 ma/gel with the temperature kept at 4° by a cooling bath and was discontinued after the bromphenol blue tracking dye had reached the anodic end of the gel. The gels were then removed from the glass tubes and cut into 1-cm slices with a razor blade. Slices were homogenized in an equal volume of PBS and agitated at 4° for 24 hr. Eluates were concentrated against 50% sucrose in PBS with 0.16% NaN₃. The sucrose and NaN₃ were removed by dialysis against PBS prior to skin testing.

**Protein Determination.** Protein was determined by the method of Lowry et al. (18), by UV adsorption at 280 nm, and by nitrogen determination (7).

**Tests for Bacterial Antigen.** Surgical specimens were removed by sterile procedures. All specimens were cultured routinely for common bacteria, and all cultures were negative. The tumor antigens were reacted against 36 different typing sera for several common species of bacteria (Lederle Laboratories, Pearl River, N.Y.), including types of pneumococci, streptococci, *Escherichia coli*, and others in Ouchterlony plates by means of the standard gel diffusion technique (25). The antigen did not form precipitin bands with any of these antisera. In addition, all antigens were cultured for *Mycoplasma*-like organisms (pleuropneumonia-like organisms), and all cultures were negative.

**Skin-testing Procedure.** All skin tests were performed at least 1 month after patients underwent surgical procedures. All patients (both melanoma patients and control patients with other neoplasms) were skin tested for their ability to develop DCHR to tumor antigens only if they could be sensitized to 2,4-dinitrochlorobenzene or if they reacted with at least 10 mm of induration to 1 or more recall antigens. All patients were sensitized to 2,4-dinitrochlorobenzene as previously described (5) and were also skin tested with recall antigens: purified protein derivative (Connaught, Englewood, N. J.), Varidase (Lederle Laboratories), mumps (Eli Lilly & Co., Indianapolis, Ind.), and *Monilia* (Hollister-Stier, Burbank, Calif.). The antigen preparations used in the skin tests were sterilized by passage through a 45-µm Millipore filter (Millipore Corp., Bedford, Mass.). The osmolality of each preparation was measured and adjusted to 300 mOsmoles to eliminate nonspecific inflammatory responses following i.d. injection. One-tenth ml of tumor antigen was injected i.d. into the volar surface of the forearm. Erythema and induration were read at 24 and 48 hr, and the mean measurement for 2 perpendicular diameters was recorded. Ten mm of induration at 24 hr were considered a positive response. Punch biopsies of reactions were taken, fixed in formalin, stained with hematoxylin and eosin, and read without prior knowledge of the antigens tested.

**Patient Population.** Forty-one melanoma patients were studied. There were 32 males and 9 females with an average age of 45 (range, 22 to 78). Fifteen patients were Stage I (local disease), 21 were Stage II (regional disease), and 5 were Stage III (disseminated disease). Twenty-three patients had undergone surgical excision of their melanomas and had received no further treatment. Fourteen patients had surgery and were receiving BCG by the tine technique (31). Four patients were receiving chemotherapy and were the only melanoma patients who had clinically evident tumor at the time of testing.

Thirty-one patients with other neoplasms served as controls. There were 24 males and 7 females in this group, with an average age of 58 (range, 21 to 81) years. The precise clinical stage could not always be determined, but no patient in this group had disseminated (Stage III) disease. All patients had surgical excision of their primary neoplasm and were clinically free of tumor at the time of testing. Four were receiving BCG. Thirteen patients had carcinoma of the lung, 6 had squamous cell carcinoma of the head and neck region, 4 had breast carcinoma, 4 had soft tissue sarcomas, 2 had colon carcinoma, 1 had renal cell carcinoma, and 1 had squamous cell carcinoma of the esophagus.

**RESULTS**

Two s.c. metastases from a single melanoma patient were extracted separately. Crude and partially purified fractions were prepared from each. DCHR were similar for both extracts, and the results were therefore pooled. A 3 m KCl extract of muscle from the same patient served as a control.
A dose of 500 \( \mu g \) of crude melanoma extract or control extract was injected for skin testing. A dose greater than 500 \( \mu g \) resulted in nonspecific inflammatory responses in all patients. A dose of less than 250 \( \mu g \) failed to elicit skin test reactivity in any patients, and thus the 500-\( \mu g \) dose was chosen.

The results of skin tests with crude melanoma extract and autologous muscle are shown in Table 1. Significantly more melanoma patients reacted to melanoma antigen than to muscle antigen. The reactivity of melanoma patients to melanoma antigen was significantly greater than that of patients with other neoplasms.

Skin test reactions were read at both 24 and 48 hr. Readings were compared for a total of 55 skin tests with crude and purified melanoma antigens in 18 patients (Chart 2). Maximum reactions were noted at 24 hr. An induration response of 10 mm in diameter, or greater, in 24 hr discriminated best between melanoma and nonmelanoma patients. Most of these tests were still 5 mm in diameter, or larger, at 48 hr. However, because the larger responses were easier to quantitate and were unequivocally positive, a diameter of 10 mm induration at 24 hr was considered a positive test. Twenty punch biopsies of these responses in 16 melanoma patients demonstrated perivascular mononuclear cell infiltration consistent with DCHR. Twenty of the 47 melanoma patients were receiving BCG at the time of testing. Because of recent reports (2, 20) indicating possible cross-reactivity between BCG and melanoma-associated antigen, BCG-treated patients and those not receiving BCG were compared. Seven of the 14 melanoma patients receiving BCG had DCHR to either crude or purified melanoma antigen. This did not differ significantly from melanoma patients not receiving BCG (17 of 27).

Patients at all clinical stages of diseases reacted similarly to the crude melanoma antigen, including patients with disseminated disease (Table 2). Three of the 4 Stage III melanoma patients had clinically apparent tumor and 2 were receiving chemotherapy. Reactivity to the melanoma extract did not correlate with ABO and Rh blood type, blood transfusion history, or multiparity.

The crude melanoma extract was concentrated and applied to a G-150 Sephadex column. Fractions were pooled as indicated in Chart 1, and these were adjusted to equal protein concentrations (1.3 mg/ml). The results of DCHR to these antigens (130 \( \mu g/dose \)) are shown in Chart 2. The most pronounced skin test reactivity was found in Sephadex Fractions I and II.

Sephadex Fractions I and II were further purified by PGE. Gels were cut into 5 equal regions (1 cm each), and the antigen eluted from each region was adjusted to a protein concentration of 0.25 mg/ml. DCHR for PGE of Sephadex Fractions I and II are shown in Table 3. For Sephadex Fraction I, PGE Region 1 was most reactive, but some antigenic activity was also noted in Regions 3 to 5. For Sephadex Fraction II, antigenic activity was concentrated in a single PGE region (R, 0.2 to 0.4), with 7 of 9 melanoma patients having positive reactions at 24 hr.

Table 4 summarizes the purification steps indicating increases in specific antigenic activity, recovery of activity from each phase, and degrees of purification.

**DISCUSSION**

Extraction of melanoma tumor tissue by the 3 M KCl method of Reisfeld et al. (21) yielded a soluble preparation with a high degree of antigenic activity, as measured by the DCHR of melanoma patients. In contrast, a much lower degree of DCHR activity was exhibited by the crude preparation in patients with other neoplasms. Crude extracts of tumors obtained by low-frequency sound elicited activity only after further purification (3, 12), leading to the conclusion that inhibitors or blocking factors may be present in the crude preparations. Crude extracts obtained by the 3 M KCl method apparently contain such inhibitors, as discussed...
due to multiple antigens, or aggregation to higher-molecular-weight moieties. The method allowed a 20-fold increase in activity per unit protein as measured by the DCHR. The partially purified antigen of Sephadex Fraction II PGE 2 appears comparable. Activity found in other fractions, such as Sephadex Fraction I PGE 1, may be due to multiple antigens, or aggregation to higher-molecular-weight moieties. The method allowed a 20-fold increase in activity per unit protein as measured by the DCHR. The use of analytical PGE in the presence of urea and sodium dodecyl sulfate, however, showed this material to be electrophoretically complex.

It is most interesting to note that, during the purification procedure, we obtained greater than 200% recovery of antigenic activity in the gel filtration step, with a slight additional increase with PGE (Table 3). These data clearly support the previously postulated concept of inhibitors or blocking factors in crude extracts (13). A selective remixing of fractions might be attempted to characterize the inhibitor(s).

The possibility of bacterial or pleuropneumonia-like organism antigenic activity in these preparations was eliminated by careful preparation and monitoring, as described in "Materials and Methods." Also, no correlation between DCHR to melanoma antigen and ABO or Rh blood type existed. Histocompatibility antigens (HL-A) did not appear to play a role either, as multiparity or history of blood transfusion did not correlate with DCHR to melanoma antigens. Furthermore, patients reacted more often to melanoma antigens than to an extract of autologous muscle tissue, which was shown to have the same HL-A profile as the tumor tissue (unpublished results).

The reactivity of the melanoma patients to the extract of autologous muscle is worthy of further comment, as none of the patients with other neoplasms showed DCHR to the preparation. The disease-specific nature of the DCHR in this case would seem to indicate a tumor-specific antigen, and it is possible that the extract contained tumor antigen, since the excised muscle was necessarily located near the tumor site. However, additional tests of melanoma patients with an extract of skin did reveal a low level of nontumor-specific response, with 2 of 9 patients who exhibited positive DCHR. Tissue-specific, fetal, or viral-specific antigens may be present in the partially purified melanoma antigen preparations, and the presence of several antigens could account for the reactivity exhibited by patients with other tumors.

Recent reports have demonstrated cross-reactivity between BCG and some types of neoplastic cells including melanoma (2, 20). However, we were unable to show an increased incidence of reactivity among melanoma patients receiving BCG. Four patients with other types of neoplasms were also receiving BCG and 1 reacted to melanoma antigen, an incidence no higher than that found in a large group of patients receiving no BCG.

Our criterion for a positive DCHR was 10 mm of induration at 24 hr following injection. Others have considered 5 to 6 mm of induration at 24 hr a positive test (3, 13). Because the selection of such criteria is to some degree arbitrary, we

### Table 2

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Positive/tested</th>
<th>% positive&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7/13</td>
<td>54</td>
</tr>
<tr>
<td>II</td>
<td>11/16</td>
<td>69</td>
</tr>
<tr>
<td>III</td>
<td>3/4</td>
<td>75</td>
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<sup>a</sup> A reaction of 10 mm or more of induration at 24 hr is considered positive.

### Table 3

<table>
<thead>
<tr>
<th>Gel region</th>
<th>Number positive/total no. of patients</th>
</tr>
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<tbody>
<tr>
<td>Sephadex G-150 I</td>
<td>Melanoma patients</td>
</tr>
<tr>
<td>−R&lt;sub&gt;r&lt;/sub&gt; 0.0-0.2</td>
<td>8/13</td>
</tr>
<tr>
<td>0.2-0.4</td>
<td>0/6</td>
</tr>
<tr>
<td>0.4-0.6</td>
<td>2/6</td>
</tr>
<tr>
<td>0.6-0.8</td>
<td>5/13</td>
</tr>
<tr>
<td>+R&lt;sub&gt;r&lt;/sub&gt; 0.8-1.0</td>
<td>2/5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sephadex G-150 II</th>
<th>Melanoma patients</th>
<th>Patients with other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>−R&lt;sub&gt;r&lt;/sub&gt; 0.0-0.2</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
<td>0.2-0.4</td>
<td>7/9</td>
<td>1/7</td>
</tr>
<tr>
<td>0.4-0.6</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
<td>0.6-0.8</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>+R&lt;sub&gt;r&lt;/sub&gt; 0.8-1.0</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Scheme of purification of melanoma-associated antigen solubilized by 3 M KCl extraction of melanoma tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg)</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>3 M KCl extract</td>
</tr>
<tr>
<td>Sephadex Fraction 1 (Stage I)</td>
</tr>
<tr>
<td>Sephadex Fraction 2 (Stage II)</td>
</tr>
<tr>
<td>Stage I-PGE Fraction 1</td>
</tr>
<tr>
<td>Stage II-PGE Fraction 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> UD (unit dose), µg of protein injected to obtain a positive DCH reaction.

<sup>b</sup> Total protein divided by the UD.
selected a time interval when reactions were largest, thus facilitating measurements and reducing error. All biopsies of reactions at this time demonstrated histological evidence of DCHR. In addition, 24% of positive responses at 24 hr would have been less than 5 mm at 48 hr and therefore overlooked.

Patients at all clinical stages of disease demonstrated DCHR to the melanoma antigens. Bluming et al. (1) reported similar results in their test with autologous membrane extracts. Hollinshead et al. (13) found decreasing DCHR reactivity with increasing dissemination of disease. Unexpectedly, we noted an increase in incidence of reactivity with advancing stages of disease. However, there were only a small number of patients in each group and, therefore, statistical analysis is not possible. It may be that the inhibitors of DCHR that are removed during purification of the melanoma antigens act to prevent immunological rejection of tumor in vivo, in spite of the constant antigenic stimulation as evidenced by the intact immune response in some of the late-stage melanoma patients studied here. Thus, an augmented DCHR to partially purified melanoma antigen could be noted even though the immunological reactivity thus indicated is inhibited in vivo. Characterization of the inhibitor(s) as well as the melanoma antigens and elucidation of their interaction are both clearly required and would be of potential diagnostic and therapeutic usefulness.

REFERENCES

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