Identification of Tumor-associated Antigens on Ultraviolet Light-induced Tumors Using Antitumor Antibodies Developed in Ascites Fluid

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ABSTRACT

A method is described which leads to the production of large amounts of ascites containing antitumor antibody in small numbers of mice. The antibody was then used to identify and characterize tumor-associated antigens on an ultraviolet light-induced murine skin fibrosarcoma. The antibody showed specific complement-dependent cytotoxicity to the homologous tumor and to an allogeneic tumor line which displayed a glycoprotein viral determinant with a molecular weight of 70,000 on its surface. Absorption of the immune ascites with other tumor cell lines removed the cytotoxicity in relation to the presence of the glycoprotein. Isolation of the tumor cell surface components binding antibody revealed two components with molecular weights of approximately 70,000 and 60,000. The M, 70,000 component was identified as viral gp70 by peptide mapping.

INTRODUCTION

TAA identified by tumor rejection assays and called TATA generally have been considered to be unique for individual chemically induced, radiation-induced, or spontaneous tumors. Common or shared cross-reacting TATA have usually been associated with tumors induced by oncogenic viruses and identified by in vitro assays with the use of antibodies or cytotoxic lymphocytes as specific reagents. In a number of studies during the last few years, common cross-reacting TATA have been identified on chemically induced, radiation-induced, and spontaneous tumors (2, 7, 11, 12, 16, 20). Studies involving the same methylcholanthrene-induced murine sarcomas showed that the common TATA were related to MuLV-associated tumor antigens (18) or to fetal antigens (3). Burton and Warner (2) described common TATA that were not related to either RNA virus antigens or to fetal antigens (3). A larger group of these tumors, both regressors and progressors, have been selected and adapted to tissue culture (10). Although unique TATA have been well documented, the case for common cross-reacting antigens is still being debated. Spellman and Daynes (24) and DeWitt (6) showed unique and common TATA on UV-induced tumors, whereas Kripke (19) could find evidence only of unique TATA. A serological analysis of TAA on UV-induced tumors by DeLuca et al. (5) showed that any cross-reactivity observed could be absorbed by cells containing MuLV-associated antigens.

Our approach for analyzing TAA has been to prepare antisera specific for UV-induced skin tumors in syngeneic mice to use as specific reagents in identifying and analyzing TAA on these tumors. Since obtaining sufficient amounts of murine sera is difficult and time consuming and involves using large numbers of mice, we developed a technique for producing large amounts of ascites containing antitumor antibody that would require immunizing only a few mice. We report here that immune ascites can be induced in large volumes by i.p. injections of tumor cells emulsified in complete Freund’s adjuvant. Antibodies of the ascites reacted with the homologous tumor cell and were used to analyze the TAA on one UV-induced tumor.

MATERIALS AND METHODS

Mice. Inbred C3H/HeN (mammary tumor virus resistant) mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). The mice were 7 to 10 weeks old when used in the experiments.

Tumor Cell Lines. The tumors (1591, 1316, and 2237) used in these experiments were fibrosarcomas induced by UV irradiation in C3H/HeN (mammary tumor virus resistant) mice (19), adapted to tissue culture, and grown as monolayers in tissue culture flasks with Eagle’s minimal essential medium containing 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin (10). NS1 is a nonsecreting clone of plasmacytoma P3x63Ag8 of BALB/c origin obtained from the Cell Distribution Center of the Salk Institute. The cells were grown at 37° in a humidified atmosphere containing 5% CO2. A cell line derived from normal C3H tail epidermis (TSII) was used as a control (10). This cell line was not tumorigenic in normal, immunosuppressed, or nude syngeneic mice. Cell lines were used from passages 1 to 5 after they had been recovered from frozen stocks.

Induction of Ascitic Fluids and Antisera. The technique of Tung et al. (27) was modified for the use of tumor cells as antigen. Mice were given injections of 4 or 5 i.p. 0.2-ml doses of complete Freund's adjuvant, H37 Ra (Difco Laboratories., Detroit, Mich.) emulsified with HBSS in a 9:1 ratio. For antitumor antibody production, 1 × 105 tumor cells were added to the 1 part HBSS. Emulsions were prepared by agitation with a mechanical shaking device (Spex Industries, Inc., Metuchen, N. J.). Except for a 2-week interval between the first and second injections, the emulsion was administered weekly. Ascites, which first appeared after the third injection, were tapped when the abdomen was distended. Fluids were collected by inserting a 19- or 20-gauge needle into the peritoneal cavity and allowing the fluid to drain into a plastic centrifuge tube, which was then frozen. Upon thawing, fibrin clots were removed by centrifugation. The antiserum to...
purified gp70 was obtained from Dr. Jack Gruber, Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Md. BALB/c anti-C3H serum was prepared by a C3H skin graft to BALB/c mice followed by 4 weekly i.p. injections of C3H spleen cells. Mice were bled 2 weeks after the last injection.

**Indirect Immunofluorescence.** Target cells were seeded onto 4-chambered tissue culture slides (Lab-Tek, Naperville, Ill.) at 3 x 10^5 cells in 1-ml volumes and incubated overnight. The medium was decanted, and the cell monolayer was washed with HBSS and fixed for 5 min with 1% formalin:phosphate-buffered saline. The slides were washed with HBSS and incubated on ice for 30 min with a 1:10 dilution of the appropriate ascitic fluid. After 3 washes with HBSS, the slides were incubated on ice for 20 min with a 1:5 dilution of fluorescein-conjugated IgG fraction of goat anti-mouse IgG (Cappel Laboratories, Cochranville, Pa.). The slides were washed 3 times with HBSS and examined with a fluorescence microscope outfitted with Ploem vertical illumination.

**Antibody-mediated, Complement-dependent Cell Lysis Assay.** The 2-stage cytotoxicity procedure of Fathman (9) was used. The assays were performed in microtiter plates with the appropriate target cells incubated in turn with ascites fluid or antisera and then with rabbit complement (Cappel Laboratories). Live versus dead cells were counted following addition of trypan blue. Medium and complement controls were included.

**Production of Immune Ascites.** Cell suspensions of each tumor line were plated in 16-mm wells at confluence and incubated overnight in media. The media was removed from a single well of each cell line, the monolayer was washed with HBSS, and 0.5 ml of anti-1591 ascites was added and incubated for 30 min at 37°. The process was repeated 5 times, transferring the ascites to another well each time. The absorbed ascites were used in the cell lysis assay.

**Radioiodination and Peptide Mapping.** Cells were seeded into 25-cm tissue culture flasks and allowed to grow to approximately 95% confluency for 42 to 48 hr. Cell surface radioiodination using 1 mCi carrier-free ^125^I, 13 to 17 mCi/µg (Amersham-Searle Corp., Chicago, Ill.), was catalyzed by lactoperoxidase according to the method of Hynes (14). Cellular proteins were then solubilized with Nonidet P40, adsorbed with anti-sera or ascites, and resolved using 10% polyacrylamide gel as described by Takemoto et al. (26). The dried gels were exposed to XRP-1 film, and the radioactive bands were excised, digested with trypsin, and mapped in 2 dimensions. All procedures were exactly as outlined by Elder et al. (8). Purified virus was obtained from Dr. Jack Gruber.

**RESULTS**

**Ascites Production.** Ascites were produced in mice by a technique modified after that of Tung et al. (27). When tumor cells were emulsified in the adjuvant used for injection, the ascites contained antitumor antibody (Table 1). Immune ascitic fluid (64 ml) was obtained from 4 mice from a total of 24 individual tappings over a period of 6 weeks. Individual tappings yielded from 1 to 6 ml per mouse, an average of 2.7 ml/tap. Balanced salt solution emulsified in adjuvant and used for injection resulted in more ascitic fluid, an average of 9.5 ml/tap. A total of 38 ml of control ascitic fluid was produced in 5 mice.

**Analysis of Ascites.** The control and tumor cell-induced ascites were tested for the presence of IgG by immunodiffusion in which goat anti-mouse IgG was used as the reagent. Both ascites preparations formed precipitates with the antoglobulin reagent (data not shown). Antitumor antibody in individual ascitic fluids was analyzed by indirect immunofluorescence (Table 1). There was variability in antibody-binding activity between both individual mice and individual tappings from a single mouse. There was no relationship between the volume of fluid or the time when the fluid was collected and the amount or intensity of immunofluorescence on the homologous tumor cell. Fifty-eight % of the tappings had strong binding activity (3+ or greater), while only 4% were negative for binding activity. The tappings that gave a binding activity of 4+ were pooled for further studies. The control ascitic fluid contained no binding activity to the tumor cells when tested by immunofluorescence.

**Antibody Specificity.** The specificity of the antibody in the ascites was determined by testing the immunofluorescent binding activity of the antitumor pool against the homologous tumor cell line, a heterologous tumor cell line, and a cell line control from normal syngeneic mouse tissue. The immune ascites displayed strong binding activity to the homologous tumor cells, weaker binding to the heterologous tumor cells, and none to the control cell line (Table 2). The control ascites had no binding activity to any of the cell lines tested. The intensity of fluorescence of the homologous tumor cells was also greater than that of the heterologous tumor cells.

A test was then made for biological activity of the ascitic fluid with an antibody-mediated, complement-dependent cell lysis assay. The results of that assay showed that the immune ascites had high cytotoxic reactivity to the homologous tumor cell line but not to 2 heterologous tumor cell lines or the control cell line (Table 3). The control ascites had no reactivity against any of the cell lines. Antisera to the C3H H-2^b^ antigens killed all of the C3H tumor lines but not the allogeneic line, indicating that they were sensitive to lysis by antibody and complement.

The UV-induced tumor line 1591 was shown previously to contain the viral antigen gp70 on its surface, whereas the tumor lines 1316 and 2237 were negative for this antigen (5, 15). Electron micrographs of each of the cell lines showed obvious viral particles in only the allogeneic line, NS1 (data not shown). The role of viral antigens in the anti-1591 ascites reactivity was shown in 2 ways: (a) antisera to gp70 was tested for cytotoxicity against each cell line. The antisera lysed the 1591 and NS1 cell lines but not line 1316. The other syngeneic tumor line, 2237, showed reactivity only at the lowest dilution.
Analysis of TAA

Table 3
Cytotoxic reactivity of antibodies directed against surface antigens of tumor cell lines

<table>
<thead>
<tr>
<th>Ascites or serum</th>
<th>1591</th>
<th>1316</th>
<th>2237</th>
<th>TSII</th>
<th>NS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ascites</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Anti-1591</td>
<td>320</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>BALB/c anti-C3H serum</td>
<td>30</td>
<td>10</td>
<td>20</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Anti-gp70 serum</td>
<td>320</td>
<td>0</td>
<td>5</td>
<td>ND</td>
<td>640</td>
</tr>
</tbody>
</table>

* Cytotoxic end point equals the reciprocal of the dilution of ascites or antiserum which lysed 50% of the targets in an antibody-mediated, complement-dependent cell lysis assay.
* ND, not done.

To confirm that the M, 70,000 band of the surface antigen isolated with the immune ascites was the viral antigen gp70, the band was excised from the gel and digested with trypsin, and the tryptic peptides were mapped in 2 dimensions. This map was compared to the tryptic peptide map of purified viral glycoprotein (AKR gp70) (Fig. 2). All the radiolabeled peptides present in the band isolated with the immune ascites were present in the AKR gp70 peptide map.

**DISCUSSION**

The technique of Tung et al. (27) was developed in order to produce large amounts of antihapten and antiprotein antibodies in individual mice. We have extended this technique to include cell surface antigens on tumor cells. The only alteration to the original technique was to add $1 \times 10^6$ tumor cells to the 0.2-ml volume of the 9:1 emulsion mixture of complete Freund’s adjuvant and balanced salt solution. Large volumes of immunoglobulin-containing ascites were produced in both control and antigen-immunized mice. The mice remained healthy and continued to produce ascites as the immunization regimen was continued.

It appeared necessary to test individual tappings for antibody reactivity, since there was wide variability in the binding reactivity of individual tappings even from the same animal. In this study, the samples from the collections with the highest binding activity (4+) were pooled.
Specificity studies using the immunofluorescence assay indicated that, even though there was a high level of binding to the homologous tumor, there was also a low level of binding to the heterologous tumor. Since common antigens on UV-induced tumors have been suggested by others (24), the cross-reactivity was not unexpected.

It is clear from the results of the cytotoxic assay that the TAA gp70 was present on the surface of the 1591 tumor cells and that the major, if not total, cytotoxic antibody response was specific for this antigen. Attempts to absorb the gp70 antibodies with tumor lines resulted in parallel loss of reactivity to the 1591 tumor line whenever gp70 was present on the adsorbing line. Even though reactivity of the immune ascites to the 2237 tumor line was not detected in the cytotoxic assay (<50% cytotoxicity at 1:5 dilution), the absorption studies and reactivity with anti-gp70 serum showed that the tumor line does produce low levels of this TAA. This has apparently happened with time in tissue culture since earlier studies with radiimmunoassay were negative for gp70 on 2237 (15). Viral determinants can be a major problem when analyzing for TAA of mice which contain the genome(s) of retroviruses (22). The tumors may display viral determinants when induced, such as the case with line 1591, or produce them as the tumor is passed in culture, as the case with line 2237. Furthermore, the virus may be produced in some strains of normal mice, and therefore the virus, viral antigens, or antibodies to these components may be found in mice even before tumorigenesis.

The immunofluorescence and cytotoxicity assays, while demonstrating antibody activity, shed no light on the number or types of antigenic determinants on the surface of the 1591 tumor cells that bound antibody. Polyacrylamide gel electrophoresis and autoradiography, used to analyze immunosorbants from 125I-surface-labeled 1591 tumor cells, indicated that there were at least 2 cell surface components binding antibody. The one component was identified as viral gp70 by peptide mapping. The second component of M, 60,000 was not present on the control cell line TSII. Preliminary results indicate that this component is not present on the tumor lines 1316 and 2237, suggesting the possibility that it is found only on the surface of the 1591 cell line. On the other hand, there is still no evidence that this component is the same as the unique TATA identified by tumor rejection assays (19) and in vitro assays of cell-mediated immunity (10). Monoclonal antibodies against cell surface antigens are needed to unambiguously ascertain these relationships.

Analysis of tumor-specific antigens in other systems has shown the presence of components with molecular weights ranging from 8,500 to 240,000 depending on the system and the method of solubilization (4, 12, 17, 21, 25). Membrane glycoproteins in the M, 72,000 to 120,000 range isolated by KCl extraction from another UV-induced tumor showed some tumor protection when used as an immunogen (23).

The etiology of common cross-reacting antigens on tumors is still unresolved. Those determinants have been documented by tumor rejection assays in a number of systems and have been defined as MuLV-associated antigens (13), fetal antigens (3), or tumor-associated antigens (2, 16). In the UV-induced tumor system, the original studies identified unique TATA using primary-single immunization techniques (19). These studies showed that cytotoxic lymphocytes specific for cell line 1316 would not kill 2237 or 1591 cells. Later studies using procedures of hyperimmunization showed cross-protection among UV-induced tumors as well as a benzo(a)pyrene-induced syngeneic tumor (24), but no attempts were made to determine the relationship to MuLV-associated or fetal antigens. In general, it has been difficult to generate antibodies specific for TAA. Hyperimmunization with tumors usually results in a high level of transplantation resistance but few measurable amounts of antibodies specific for TAA. A number of studies have attempted to characterize TAA with specific antisera. Unique and common TAA have been identified and associated with MuLV antigens (1, 18), fetal antigens (17), or tumor antigens (5). An antibody-binding study in the UV-induced tumor system showed that there was some cross-reactive binding to heterologous UV-induced tumors and chemically induced tumors (4). Such cross-reactivity could be adsorbed by MuLV antigen-bearing tumor cells of AKR origin in most cases; however, some non-MuLV cross-reactions were observed in the only antiserum that was prepared by hyperimmunization with tumor cells. The elegant serological studies of DeWitt also indicated that UV-induced tumors bear both unique and common or cross-reactive antigenic specificities; however, the role of viral determinants was not studied (6).

It is of interest that, in the present study, most of the antigen isolated by the ascites was gp70. The viral surface antigens appeared to be much more immunogenic than was the TAA, and this could be a function of either the quality or quantity of the antigenic determinants. It is possible that other cell surface TAA may exist that label poorly or not at all with 125I or that gp70 may pick up more 125I than the M, 60,000 antigen.

In our studies, cross-reactivity was shown to be a function of the viral-related TAA gp70, both by the cytotoxic assay and by
the antigen isolation experiment. These experiments illustrate the need for determining the role that retrovirus expression might play in the interactions of chemically and physically induced tumors with their hosts.

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REFERENCES

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