ABSTRACT

We reported previously the partial purification of detergent-solubilized specific tumor rejection antigen of a chemically induced sarcoma, Meth-A. During the course of the study, rabbit antiserum against a partially purified specific tumor rejection antigen preparation was raised and rendered specific by in vivo absorption. In this report we show that an antigenic molecule defined by in vivo-absorbed rabbit antiserum, which we tentatively refer to as tumor-specific surface antigen, was solubilized by detergent Nonidet P40, and extensive attempts at purification were carried out by a sequence of procedures including gel filtration, isotachophoresis, and polyacrylamide gel electrophoresis. The most highly purified tumor-specific surface antigen retained some specific tumor rejection antigen activity, suggesting an association of the two activities. Both antigens were shown to have a molecular weight of about 60,000 and an electrophoretic mobility of α-globulin.

INTRODUCTION

TSTA's are associated with most chemically induced neoplasms. These antigens can elicit tumor rejection in syngeneic or autochthonous hosts. In addition, there are other tumor cell surface antigens detected principally by in vitro assays that are immunogenic in the syngeneic host but that may or may not mediate tumor rejection (14). We have previously reported that the TSTA of a chemically induced sarcoma, Meth-A, can be solubilized from cell membranes by the detergent NP40 and has a molecular weight near 70,000 in the presence of detergent with an electrophoretic mobility of α-globulin. The tumor rejection antigen was found to be well separated from H-2 antigens by lectin affinity chromatography and was shown to be specific for sarcoma Meth-A (13).

During the course of the study, we raised an antiserum in rabbits against the partially purified TSTA preparation and obtained a specific rabbit antiserum by absorbing the antiserum in vivo. The xenogenic activity detected by this in vivo-absorbed rabbit antiserum was found in the final product of TSTA, had been purified following in vivo tumor rejection activity (TSTA) and had been shown to contain only 3 major bands on analytical polyacrylamide gel electrophoresis. An important question was therefore whether the antigen defined by this xenogenic activity, TSSA, is the TSTA molecule or is related to the TSTA. We report here that the most highly purified antigen defined by in vivo-absorbed rabbit antiserum still retained TSTA activity, which suggests an association of the xenogenic activity with TSTA activity.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 8 to 12 weeks old, were obtained from the production unit of the NIH.

Tumors. Sarcoma Meth-A was originally provided by Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York, N. Y. The tumor was developed into and has been maintained in this laboratory in ascitic form by serial passage in BALB/c mice. The dose of tumor cells that would produce progressively growing tumors in 50% of recipients is 10⁶ cells for Meth-A. An SV40-induced sarcoma, mKSA (9, 10), and a methylcholanthrene-induced sarcoma, MCA-6, both in BALB/c mice, were used as specificity controls.

Preparation of Rabbit TSSA Antiserum and in Vivo Absorption. Rabbit TSSA antiserum was raised in adult female New Zealand rabbits by immunizing with the lectin unbound fraction (LcH-I) that had previously been isolated from the NP40-solubilized material and shown to be immunogenic in syngeneic mice (13). LcH-I fraction (230 μg) was mixed with Freund's complete adjuvant (230 μg) and injected into foot pads. Four weeks later, rabbits were boosted s.c. with Freund's incomplete adjuvant. Seven days after the second immunization, blood was taken and serum was separated. The antiserum was absorbed in vivo by the method described originally by Shigeno et al. (16). Briefly, a 1.5-ml portion of the antiserum was injected i.p. into normal female BALB/c mice; 16 hr later the mice were bled out and serum was recovered. The rabbit anti-TSSA antiserum thus absorbed in vivo was shown to be specific for sarcoma Meth-A (Table 1). An antiserum against LcH-I fraction of an unrelated syngeneic sarcoma, mKSA, was raised in rabbits and absorbed in vivo as described previously. This antiserum was used as a control. Normal rabbit serum was also passed through normal BALB/c mice and used as a nonimmunized control serum.

Other Antisera. Rabbit anti-H-2 antiserum used was described fully in a previous report (13). An alloantiserum, anti-H-2.4 (B10.AKM × 129 anti-B10.A) was obtained from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md. Rabbit anti-mouse β₂-microglobulin

359

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3 The abbreviations used are: TSTA, specific tumor rejection antigen; NP40, Nonidet P40; TSSA, tumor-specific surface antigen; DATD, diallyltartardiamide.

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Radioimmunochemical Isolation of an Antigen of Sarcoma Meth-A Defined by \textit{in Vivo}-absorbed Rabbit Antiserum. A 50-μl (33-μg) portion of the final product [a fraction (Fraction NP40-SA), and subsequent fractions obtained by gel filtration, isothachophoresis, and polyacrylamide gel electrophoresis. The methods have been described in detail previously (13). Proteins throughout were measured by the method of Lowry et al. (11).

**RESULTS**

Immunochemoical Characteristics of TSSA from Meth-A Membranes. The specificity of the radioimmunoassay is shown in Tables 1 and 2, and most of the activity was found exclusively on Meth-A cells with less than 10% on other cells. The antigen (TSSA) of sarcoma Meth-A was radioimmunochemically isolated from the \textsuperscript{125}I-labeled Meth-A preparation by binding to \textit{in vivo}-absorbed rabbit anti-TSSA antiserum.

<table>
<thead>
<tr>
<th>Specificity of rabbit anti-TSSA antiserum before and after in vivo absorption</th>
<th>Radioiodinated antigen preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extent of binding (%) with rabbit anti-TSSA antiserum</td>
<td>Before absorption (a)</td>
</tr>
<tr>
<td>(\textsuperscript{125}I)-Labeled Meth-A (c)</td>
<td>9.26 (d)</td>
</tr>
<tr>
<td>(\textsuperscript{125}I)-Labeled mKSA (e)</td>
<td>3.03</td>
</tr>
<tr>
<td>(\textsuperscript{125}I)-Labeled H-2, (f) (15,000 cpm)</td>
<td>3.57</td>
</tr>
</tbody>
</table>

\(a\) Ten μl of antiserum were incubated with a given amount of the radioiodinated antigens preparations overnight at 4°, and the soluble immune complexes were precipitated by an excess of goat anti-rabbit IgG antiserum. The radioactivity found in the precipitates expressed as a percentage of radioactivity added.

\(b\) The antiserum was absorbed \textit{in vivo} as described in "Materials and Methods." Twenty μl of \textit{in vivo} absorbed antiserum were used for the binding assay.

\(c\) The \textsuperscript{125}I-labeled Meth-A preparation was prepared as described in "Materials and Methods" from the final product of TSTA previously described (13).

\(d\) Binding with 10 μl of normal rabbit serum or with 20 μl of \textit{in vivo}-absorbed normal rabbit serum ranged from 1.0 to 1.8% and was subtracted from the values.

\(e\) The LcH I (lectin unbound fraction) fraction of sarcoma mKSA had been prepared (18) and radioiodinated.

\(f\) The \textsuperscript{125}I-labeled H-2 antigen preparation was the gift from Dr. O. Henriksen of this laboratory.

Preparation of Immunoabsorbent Column. An immunoglobulin-containing fraction of the \textit{in vivo}-absorbed rabbit antiserum was coupled to cyanogen bromide-activated Sepharose 4B. The sample was allowed to incubate for 24 hr at 4° and was then eluted with glycine-HCl (pH 2.2) buffer containing 0.2% NP40 and 0.05% bovine serum albumin. The eluted preparation is referred to as \textsuperscript{125}I-labeled TSSA antigen.

Radiooimmunoassay. The radioimmunoassay was done essentially by the double antibody technique described by Tanigaki et al. (17). Radioimmunoassays for H-2 and mouse β₂-microglobulin were carried out as described previously (13). Polyacrylamide Gel Electrophoresis. The electrophoresis on 7% polyacrylamide gel at pH 9.2 was carried out by the method of Davis (4). Polyacrylamide gel electrophoresis on 5% stacking gel (15% DATD) and 7% resolving gel (5% DATD) was carried out in the later experiments with 1 No. 2860 buffer system selected from the Multiphasic Buffer Systems Output (8). Electrophoresis on 10% polyacrylamide gel containing 0.1% SDS and 0.5 M urea was done essentially by the method of Shapiro et al. (15).

Isotachophoresis. Isotachophoresis was accomplished essentially by the method of Baumann and Chrambach (2). Briefly, stacking gel of 5% total monomer cross-linked with 15% DATD was used. Isotachophoresis was carried out in a 6 x 120-mm glass tube at 4° and 100 V for 5 hr in the No. 2860 buffer system.

Quantitative \textit{In Vivo} Tumor Rejection Assay. Tumor rejection (TSTA) activity was assayed quantitatively with the use of the crude membrane, the detergent-solubilized fraction (Fraction NP40-SA), and subsequent fractions obtained by gel filtration, isothachophoresis, and polyacrylamide gel electrophoresis. The methods have been described in detail previously (13). Proteins throughout were measured by the method of Lowry et al. (11).
in parallel and stained. The molecular weight of this antigen was estimated to be 60,000 with the use of a heavy chain of mouse IgG (m.w. 52,000), a light chain of mouse IgG (m.w. 23,000), and RNase (m.w. 13,800) as size standards.

In control experiments in which normal rabbit serum replaced the rabbit antisera in the immunoabsorbent column, 4% of the radioactivity applied was retarded in the column, and eluted material gave no radioactivity peak corresponding to m.w. 60,000 peak on SDS-polyacrylamide gel electrophoresis. As shown in Chart 2, the TSSA antigen obtained from the immunoabsorbent column by acid elution did react well with in vivo-absorbed rabbit anti-TSSA antisera. The extent of binding was 54% with 30 μl of the antisera. This value appeared to represent near-maximum binding because (a) further addition of the antisera failed to precipitate radioactivity from the supernatant and (b) radioactivity found in the supernatant gave no peak on SDS-polyacrylamide gel electrophoresis.

Binding activity with other antisera including anti-H-2 antisera was assayed next. A constant amount (8000 cpm) of 125I-labeled Meth-A antigen was allowed to react with 10 μl of in vivo-absorbed rabbit anti-Meth-A antisera, in vivo-absorbed rabbit anti-mKSA antisera, rabbit anti-H-2 antisera, and anti-H-2.4 alloantisera. Results are shown in Table 3. The recovered antigen bound with in vivo-absorbed rabbit anti-TSSA antisera (43%) but not significantly with in vivo-absorbed rabbit anti-mKSA antisera or with rabbit anti-H-2 antisera; nor did it bind with anti-H-2.4 alloantisera. These results indicate strongly the distinctive xenoantigenic activity of the TSSA antigen.

Purification of the TSSA and TSTA Activity. Since the TSSA antigen was found in a fraction that had been shown previously to be highly immunogenic as detected by tumor rejection in syngeneic mice (13), an important question was whether TSSA defined by this xenoantiserum represents TSTA or a part of the molecule. In order to solve the question, we attempted to purify the TSSA by following both the xenoantigenic activity and in vivo tumor rejection (TSTA) activity.

Both tumor rejection activity and the xenoantigenic activity were effectively solubilized with non-ionic detergent, NP40, from crude membrane preparation of Meth-A cells. As shown in Table 4, this NP40-SA antigen contained 46 and 86% of TSTA activity and xenoantigenic activity, (TSTA), respectively. An aliquot of concentrated NP40-SA was applied on an agarose column (Bio-Gel A-1.5m) and gel filtrated. A typical profile is shown in Chart 3. Xenoantigenic activity of the TSSA distributed in a restricted molec-
Table 4
Recovery of TSSA and TSTA activities from membranes of sarcoma Meth-A

<table>
<thead>
<tr>
<th>Type of preparation</th>
<th>Protein (mg)</th>
<th>Units</th>
<th>Specific activity (units/mg)</th>
<th>Units</th>
<th>Specific activity (units/mg)</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude membrane</td>
<td>1,175</td>
<td>200,000</td>
<td>170</td>
<td>838,950</td>
<td>714</td>
<td>204,081</td>
</tr>
<tr>
<td>NP40-SA</td>
<td>535</td>
<td>172,484</td>
<td>322</td>
<td>381,990</td>
<td>714</td>
<td>243,181</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>46</td>
<td>92,304</td>
<td>2,006</td>
<td>6,578</td>
<td>0.14</td>
<td>143</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>1.8</td>
<td>17,110</td>
<td>9,506</td>
<td>1,499</td>
<td>0.17</td>
<td>833</td>
</tr>
</tbody>
</table>

a One unit corresponds to an amount of protein that induced 50% inhibition of the binding.
b One unit corresponds to an amount of protein that induced 50% inhibition of tumor growth compared with untreated control mice.
c Numbers in parentheses, percentage of activity.

Chart 3. Gel filtration of detergent-solubilized TSSA (Meth-A) on an agarose column. A 25-ml portion (125 mg) of Fraction NP40-SA was concentrated to 3 ml by membrane filtration (UM-20; Amicon Corp., Lexington, Mass.) and applied on an agarose (Bio-Gel A-1 0.5; Bio-Rad Laboratories, Richmond, Calif.), column (20 x 640 mm). The sample was eluted with Tris-HCl (0.05; pH 7.8) buffer containing 0.2% Brij 98 and 0.2 mM dithiothreitol. Arrows, elution position of the molecular-size glycoprotein marker proteins: ^125I-labeled IgM (m.w. 900,000), ^125I-labeled glycoprotein with a molecular weight of 70,000, and ^125I-labeled human β2-microglobulin (β2m) (m.w. 12,000). Antigenic (Ag) activities were assayed for every other fraction by radioimmunoassay.

Chart 4. Purification of TSSA by isotachophoresis (ITP) and preparative polyacrylamide gel electrophoresis (PAGE). A 1-ml portion (10 mg) of concentrated Fraction 1 was subjected to isotachophoresis. After isotachophoresis was carried out, the gel was sliced and antigens were eluted in buffer by shaking overnight at 4°C. TSSA activities were followed by radioimmunoassay. Sixty-μl aliquots (150 μg) of Fraction 2 were applied on 7 gels of polyacrylamide gel electrophoresis. After polyacrylamide gel electrophoresis was carried out, the gels were processed as described above and antigenic activities were determined. In both cases, fractions indicated were pooled and tested for TSTA activity. Results are shown in Chart 4 and Table 4. Anode is at the right.

TSSA xenoantigenic active fractions obtained from gel filtration (Fraction 1) were subjected to further purification by isotachophoresis and preparative polyacrylamide gel electrophoresis. A 1-ml portion (10 mg) of the concentrated Fraction 1 was applied to isotachophoresis (see "Materials and Methods"). After 5 hr of isotachophoresis, the gel was sliced and minced every 1 mm by an Autogel divider (Gilson Medical Electronics, Inc., Middleton, Wis.), and antigens were eluted in buffer by shaking overnight. TSSA activity was assayed for every other fraction. The high antigenic peak was obtained from a stacked band of the gel. Approximately 43% of the activity recovered was found in this peak. Some activities were found also in the preceding fractions. However, these activities should be attributable to unequilibrated antigens that result from a relatively short running time rather than to heterogeneity of the antigen because a small amount (200 to 300 μl) of the running sample, in a separate experiment, resulted in a single antigenic peak on isotachophoresis. The top 2 fractions (Fractions 46 and 47) were pooled (Fraction 2) (see Chart 4) and quantitatively assayed for TSTA activity. A linear-dose-dependent TSTA activity was observed (Chart 5). Approximately 1.2 μg were effective in inducing 50% inhibition of the tumor growth compared with untreated control mice. Approximately 22% of the TSTA activity found in Fraction 1 was recovered in this Fraction 2.

Since the active fraction recovered from isotachophoresis (Fraction 2) appeared to consist of 1 major band (Rv 0.66)
Solubilized Tumor Antigen

and some minor bands on analytical polyacrylamide gel electrophoresis (Fig. 1), an attempt was made to isolate the major band by preparative polyacrylamide gel electrophoresis. Each 60-μl aliquot (150 μg) of Fraction 2 was applied on 7 different gels, and polyacrylamide gel electrophoresis was carried out. After electrophoresis, gels were sliced and minced; then antigens were allowed to elute in buffer. Xenoantigenic activity was assayed for every other fraction of each gel. The fractions of high xenoantigenic activity were pooled (Fraction 3) and tested for TSTA activity. Because of the low protein recovery, we could not perform a quantitative tumor rejection assay. However, 2 injections of 300 μl (containing approximately 4 μg of protein or less) of Fraction 3 showed 80% inhibition of tumor growth compared with control mice. Injections of other gel fractions did not provide tumor growth inhibition (Table 5). Fraction 3 presented a faint band on analytical polyacrylamide gel electrophoresis corresponding to the position of the major band of Fractions 1 and 2 (Fig. 1).

DISCUSSION

Our results show that an antigen can be prepared from cell membranes of a chemically induced sarcoma that is recognized by a xenoantiserum rendered specific by in vivo absorption. The molecule was shown to have a molecular weight of approximately 60,000 and an α-globulin electrophoretic mobility migrating to a position of 0.66 Rf on 7% polyacrylamide gel electrophoresis.

The antigenic molecule, which we tentatively refer to as TSSA, was first found in the final product of partially purified TSTA of Meth-A cells (13). An important question can be raised, therefore, as to whether the TSSA antigen detected by in vivo-absorbed rabbit antiserum is related to TSTA of sarcoma Meth-A. The results reported suggest an association of the 2 different antigenic activities. The evidence includes: (a) the preparation of partially purified TSTA previously described (13) contained the TSSA anti-

![Chart 5. Quantitative in vivo assay for TSTA with the use of NP40-SA and chromatographed fractions. Varying amounts of NP40-SA (Δ), Fraction 1 (□), and Fraction 2 (○) were prepared in 0.2 ml of phosphate-buffered saline containing 0.1 mM dithiothreitol and 0.5% normal BALB/c mouse serum and immunized as described in "Materials and Methods." Each point represents a group of 8 mice. Mice were challenged with 10⁴ and 2 × 10⁴ Meth-A cells after the second immunization. Results were taken at 20 days after challenge.](chart5)

![Fig. 1. Analytical polyacrylamide gel electrophoresis of Fraction NP40-SA and chromatographed fractions of TSSA. A small amount (approximately 50 μg) of each sample was subjected to analytical polyacrylamide gel electrophoresis with the use of 5% stacking gel and 7% resolving gel in No. 2860 buffer system selected from the Multiphasic Buffer Systems Output (8). Anode is at the bottom. Samples are from left: Fraction NP40-SA, Fraction 1 from gel filtration, Fraction 2 isatophoresis (ITP), and Fraction 3 from preparative polyacrylamide gel electrophoresis. The band corresponding to a migration position of Rf 0.66 remained through the steps.](fig1)

![Table 5. TSTA activity of the TSSA preparation recovered from preparative polyacrylamide gel electrophoresis](table5)

<table>
<thead>
<tr>
<th>Type of preparation</th>
<th>Immunization schedule (ml × 2)</th>
<th>No. of mice with tumor/total no. of mice</th>
<th>Mean tumor volume (μm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 3</td>
<td>0.3</td>
<td>3/5</td>
<td>39.0 (19.9)</td>
</tr>
<tr>
<td>Control fraction</td>
<td>0.3</td>
<td>5/5</td>
<td>250.1 (127.9)</td>
</tr>
<tr>
<td>None (control)</td>
<td>7/9</td>
<td></td>
<td>195.5 (100)</td>
</tr>
</tbody>
</table>

a Fractions of high xenoantigenic activity were pooled as indicated in Chart 5 (Fraction 3), and 0.3-ml aliquots were injected, s.c., into female BALB/c mice.

b Numbers in parentheses, percentage of control tumor volume.

c Fraction 30 was collected from each gel and used as a control fraction.

gen; (b) the most highly purified TSSA antigen obtained by a sequence of procedures including gel filtration, isatophoresis, and preparative polyacrylamide gel electrophoresis retained immunogenicity in syngeneic BALB/c mice against Meth-A tumor cells; and (c) physicochemical properties of TSTA and TSSA are similar. However, the specific activities of both TSSA and TSTA do not suggest copurification. The values obtained (Table 4) do show a specific 50-fold increase for TSSA and less than a 1.2-fold increase for TSTA. Taken as such these activities appear to suggest the existence of 2 different molecules. The association of 2 antigenic activities has also been observed in the histocompatibility antigen system (HL-A) where distinctive xenoanti-
genic determinants are associated with a large component (the alloantigenic molecule) and with the small component (β2-microglobulin), respectively (18). In this context, TSTA, which is known to be individually specific, could represent alloantigenic specificities, while the xenoantigenic activity or TSSA might represent a common antigenic feature of the molecule as in the case of histocompatibility antigens. This finding also indicates that xenoantigenic markers may be useful tools for the isolation and purification of antigens when other reacting reagents are not available (3).

It has been reported recently that TSTA(s) of chemically induced tumors in the mouse represent H-2-modified antigens or antigens of alien specificities on the basis of indirect but suggestive evidence (5-7, 12). In the studies presented here and in our previous paper, it would appear that TSTA or TSSA of Meth-A are molecules distinct from major histocompatibility antigens (H-2). In our previous work (13), the final product of TSTA was obtained from a material that did not bind to the LcH (Lens culinaris) affinity column. In the present experiments, H-2 activities were again well separated from TSSA activities on gel filtration (Chart 3). H-2.4 activities were not detected in the isotachophoresis fraction (Fraction 2) (Table 4). Furthermore, immunologically purified TSSA did not bind to rabbit anti-H-2 antiserum, which is capable of binding with H-2 antigens regardless of the specificities carried, while it did bind well with in vivo-absorbed rabbit anti-TSSA antiserum (Table 3), which indicates distinctive xenoantigenic characteristics of TSSA. These findings suggest that the TSSA is not H-2 or an H-2-modified antigen. It is very unlikely, therefore, that TSSA or TSTA activities detected here are derived from foreign H-2 specificities on Meth-A cells as recently reported by Garrido et al. (5).

We cannot exclude the possibility that TSSA is one of unknown non-H-2 alloantigens of the mouse as is reported by Invernizzi et al. (6), as in the case of TL antigen. Although the strain distribution of TSSA has not yet been established, some preliminary data show that normal tissues and other tumor cell lines of syngeneic and allogeneic origin have xenoantigenic activities similar to those of TSSA although quantitatively the amount was very low (Table 2; unpublished data). An interesting issue that can be raised is whether the activities found on other tumor cells represent individually specific TSTA that share a common portion. Purification of these proteins from normal and other tumor cells and comparing them with TSSA will hopefully shed some light on the nature and origin of TSTA.

REFERENCES


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Immunoochemical Evidence of a Tumor-specific Surface Antigen Obtained by Detergent Solubilization of the Membranes of a Chemically Induced Sarcoma, Meth-A

Takashi Natori, Lloyd W. Law and Ettore Appella


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