A Unique Tumor Rejection Antigen from the S91 Murine Malignant Melanoma

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ABSTRACT

We have identified and described the characteristics of a unique tumor rejection antigen (tumor-specific transplantation antigen) obtained from the murine malignant melanoma S91. This antigen is highly restricted to the autologous melanoma and provides striking inhibition of its growth. Previously, we described common or shared tumor-specific transplantation antigens on the murine malignant melanomas B16 F10, K1735, JB/RH, and JB/MS. No cross-reactivity was obtained in this study between S91 and those four other malignant melanomas. The common tumor-specific transplantation antigen resides on a glycoprotein molecule with a molecular weight of 65,000, termed B700, that shares homology with serum albumin as determined by N-terminal amino acid sequencing. B700, however, purified from S91 proved to be ineffective as an immunogen.

INTRODUCTION

This laboratory has been involved in characterizing tumor antigens, including those possessing tumor rejection antigen (TSTA) activity, that are produced by transformed melanocytes. Using a panel of malignant melanomas of different origins (spontaneous, carcinogen induced, ultraviolet induced) in different inbred strains of mice, we have reported that the major tumor antigens detected showed a cross-reactive pattern, so-called "common" TSTA; these are restricted to the murine melanomas (1, 2). Those antigens were detected by immunizing mice with irradiated cells, with spent tissue culture medium, or with purified antigen prepared from B16 melanoma cells, and by studying subsequent primary tumor growth or the growth of spontaneous and experimental metastases in immunized syngeneic and/or semisynzyngenic recipients (1, 2). The common antigen detected in four different melanomas, K1735 (ultraviolet induced), JB/RH and JB/MS (carcinogen induced), and the spontaneous B16 F10 melanoma, resides on a melanoma-specific glycoprotein (B700) that has a molecular weight of 65,000; this antigen has been purified to homogeneity and shows partial homology to serum albumin (3, 4).

We now report the characteristics of a TSTA expressed on the spontaneously arising S91 malignant melanoma. This TSTA is unrelated to the common antigen previously described, is specific for S91, and resembles the unique antigens so characteristic of carcinogen-induced sarcomas (see Ref. 5).

MATERIALS AND METHODS

Tumors and Mice. S91 arose as a spontaneous malignant melanoma in a DBA mouse (6). Early histopathological and transplantation characteristics of this tumor were recorded (7-9); however, more recently, S91 has been more extensively used for enzyme studies of mammalian pigmentation (see Refs. 10 to 12). The immunobiological characteristics of the S91 melanoma have not been reported. The S91 melanoma used in this study was obtained from the Jackson Laboratory, through the Division of Cancer Treatment Tumor Repository of the National Cancer Institute-Frederick Cancer Research Facility. This melanoma has been continuously passaged in DBA/2 strain mice. Progressive growth of this melanotic subline requires inoculation of at least 1 x 10⁶ cells. S91 has also been adapted to tissue culture and is passaged in medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, gentamicin (50 μg/ml), penicillin (1 unit/ml), and streptomycin (1 μg/ml). The population of S91 cells consists of round, spindle-shaped, and dendritic cells, many of which contain pigment granules; these cells, upon stimulation with α-melanocyte-stimulating hormone in vitro, become heavily pigmented. After a moderate number of passages in culture, S91 cells do not grow progressively when inoculated in vivo and were therefore used primarily in these studies for immunization of animals. The following malignant melanomas which bear cross-reacting melanoma-specific TSTA were also used: B16 F10; K1735; JB/RH; and JB/MS. The origin and maintenance of these tumors, in vivo and in vitro, have been previously described (see Refs. 1 and 2). In addition, the MCA105 sarcoma (13) and the T92497 sarcoma (14), both of C57BL/6 origin and induced by methylcholanthrene, and the GL26 glioma, of C57BL/6 origin (15), have been used as specificity controls. All tumor lines have been assayed and found negative for the following murine viruses: polyoma virus; adenovirus; hepatitis virus; minute virus; reovirus type 3; pneumonia virus; K virus; Theliger's virus; Sendai virus; lymphocyte choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus. All of these melanomas have been adapted to tissue culture using methods previously described (2). Pathogen-free DBA/2 and semisynzyngenic F₃, hybrid mice [C57BL/6N × DBA/2 F₃, (hereafter called B6D2F₃) and C57BL/6N × C3Hf/HeN F₃, (hereafter called B6C3F₃)] were used throughout these studies. They were 10- to 12- wk-old females obtained from the NIH Small Animal Section, Division of Research Services (see separate experiments for strains and F₃, hybrids used).

Immunization Studies. Mice were immunized by s.c. and i.p. inoculations of irradiated, tissue culture-passaged, or dissociated in vivo passaged cells in phosphate-buffered saline. Cells were irradiated at 10,000 R in a J. L. Shepherd Model 68 ¹³⁷Cs γ-irradiator. Either two or three immunizations were carried out, the former at 10-day intervals and the latter at 7-day intervals. Challenges were done s.c. 7 days after the last immunization and were usually carried out in the TLD₉₀-₁₀₀. Palpable tumor growths were measured at regular intervals, and the MTD reported was measured at 20 days after challenge, or as noted in the figure and table legends. The MTD represents the sum of the diameters of all tumors in each group divided by the total number of challenge sites. Statistical analyses were carried out using Student's r test. Similar immunization and challenge schedules were used for studies on the efficacy of tumor growth inhibition of purified B700 antigens obtained from S91 melanoma cells. The glycosylated B700 antigen was isolated and purified from in vivo passaged S91, using an identical method for obtaining the immunogenic and cross-reacting B700 from B16 F10 melanoma cells (2). Briefly, the procedure includes homogenization of melanoma tumor and isolation of native antigen by density gradient centrifugation, gel filtration chromatography, and preparative gel electrophoresis. The purity of the antigen was characterized by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and isoelectric focusing.

Flow Cytometric Analysis. Melanoma cells in exponential growth phase in culture were washed twice in HBSS and then harvested with 0.02% EDTA in HBSS. After removal, the cells were washed one time in HBSS containing 1% FBS and 0.02% sodium azide (HBSS:FBS). For cytoplasmic staining, the cells were permeabilized with 70% ethanol for 30 min at 4°C and then washed in HBSS:FBS an additional 2 times. Approximately 2 x 10⁶ cells were incubated for 30 min at 4°C in 40 μl of a 1:10 dilution of anti-B700 serum (3) in HBSS:FBS and then

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1 To whom requests for reprints should be addressed, at the National Cancer Institute, Bldg. 37, Rm. IB22, Bethesda, MD 20892.

2 The abbreviations used are: TSTA, tumor-specific transplantation antigens; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; TLD₉₀-₁₀₀, tumor lethal dose range of 80 to 100% (TLD₉₀_,₁₀₀).

3 The abbreviations used are: TSTA, tumor-specific transplantation antigens; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; TLD₉₀-₁₀₀, tumor lethal dose range of 80 to 100% (TLD₉₀_,₁₀₀).

4 The abbreviations used are: TSTA, tumor-specific transplantation antigens; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; TLD₉₀-₁₀₀, tumor lethal dose range of 80 to 100% (TLD₉₀_,₁₀₀).
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washed twice with HBSS:FBS. The cells were then stained for 30 min at 4°C in 40 µl of a 1:10 dilution of fluorescein isothiocyanate-conjugated anti-rabbit IgG, then again washed twice with HBSS:FBS, and analyzed by a FACS II flow cytometer.

RESULTS

Protective immunity with greater than 95% inhibition of primary growth was induced through immunization with irradiated S91 melanoma cells as shown in three separate experiments in Fig. 1. Tissue culture-grown cells were used in these experiments for immunization. To rule out any possible artifacts caused by tissue culture medium, tumor challenges were also made with in vivo grown S91 melanoma cells. The immunity induced was stable; immunized mice which had successfully rejected an initial challenge were able to resist subsequent challenges with S91 (data not shown). In further in vivo tumor rejection assays, two immunizations with irradiated cells proved nearly as effective against later S91 challenge as were three immunizations; even a single immunization was effective (see Table 1).

Cross-protection specificity studies against other murine melanomas were performed using immunization of mice with S91 melanoma cells and challenge with those malignant melanomas shown previously to bear "common" but melanoma-specific TSTA. No evidence of cross-protection was observed (Fig. 2). Several experiments using reciprocal cross-protection (i.e., immunizing with K1735 or JB/RH and challenging with S91) yielded similar results; that is, no significant protection was observed after challenge with S91.

Table 1 Influence of number of immunizations upon inhibition of S91 melanoma primary growth

<table>
<thead>
<tr>
<th>Immunization</th>
<th>No. of immunizations</th>
<th>No. with tumors/no. challenged</th>
<th>MTD</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>S91</td>
<td>3</td>
<td>2/8</td>
<td>1.3 ± 1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>&quot;</td>
<td>2</td>
<td>3/8</td>
<td>2.5 ± 1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>&quot;</td>
<td>1</td>
<td>10/16</td>
<td>4.1 ± 1.5</td>
<td>0.07</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>7/8</td>
<td>8.3 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

* Tissue culture-propagated cells (4 × 10^6) were irradiated at 10,000 R. Randomized groups of 8 B6D2F1 recipients were s.c. immunized 3 times at 7-day intervals with 3 to 5 × 10^6 irradiated S91 melanoma cells in PBS, or with PBS only. Challenge with S91 cells was done 7 days after the last immunization as follows. In one experiment (left), mice were challenged with 7.5 × 10^5 S91 cells propagated in vitro; in the other experiments (middle and right), mice were challenged with 5 × 10^6 and 2 × 10^6 S91 cells, respectively, propagated in vivo. P values between immunized and control groups ranged from <0.01 to <0.001. Points, mean; bars, SE.

Fig. 1. Effect of immunization on primary growth of S91 melanoma. Eight B6D2F1, recipients were s.c. immunized 3 times at 7-day intervals with 3 to 5 × 10^6 irradiated S91 melanoma cells in PBS, or with PBS only. Challenge with S91 cells was done 7 days after the last immunization as follows. In one experiment (left), mice were challenged with 7.5 × 10^5 S91 cells propagated in vitro; in the other experiments (middle and right), mice were challenged with 5 × 10^6 and 2 × 10^6 S91 cells, respectively, propagated in vivo, P values between immunized and control groups ranged from <0.01 to <0.001. Points, mean; bars, SE.

Fig. 2. Results of cross-immunization studies. Immunizations were accomplished with 4 × 10^6 irradiated S91 melanoma cells (4 × 10^6 cells) twice at a 10-day interval. Challenges were s.c. 10 days after the last immunization and were as follows: B16, 5 × 10^6; K1735, 1 × 10^6; JB/RH, 5 × 10^6; and S91, 2 × 10^6. All challenges were at a TLD of 100. a, immunized mice; b, control mice. Groups of eight B6D2F1 mice were used except in the K1735 challenge experiment where B6C3F1 mice were the recipients. The final MTD was recorded at Day 20. Columns, mean; bars, SE. P values between experimental and control groups were not significant except in the S91 challenge group where P < 0.01.

Fig. 3. Specificity assays of S91 immunization against primary tumor growth of nonrelated tumors (the MCA-105 and T92497 sarcomas and the GL 26 glioma) are all methylcholanthrene induced and of C57BL/6 origin (see text). Groups of 8 B6D2F1 mice were immunized with 4 × 10^6 irradiated S91 cells propagated in vitro, or with PBS alone. Two immunizations were done with tumor cell challenges s.c. 10 days following the last immunization. TLDs, tumor cell challenges were: MCA-105, 1 × 10^6 cells; T92497, 3 × 10^6 cells; GL 26, 5 × 10^6 cells; and S91, 2 × 10^6 cells. Inhibition of growth was found only with the S91 challenge where P < 0.01.
Table 2 Effect of soluble S91 antigen (B700) on tumor rejection

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization*</th>
<th>Challenge</th>
<th>No. positive/ no. chal-</th>
<th>MTD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a B16 (B700), 100 µg</td>
<td>JB/RH (TC) (5 x 10⁵ cells)</td>
<td>3/10</td>
<td>1.7 ± 0.9 *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b B16 (B700), 10 µg</td>
<td>JB/RH (TC) (5 x 10⁵ cells)</td>
<td>7/10</td>
<td>5.8 ± 1.6 &lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c None</td>
<td>JB/RH (TC) (5 x 10⁵ cells)</td>
<td>9/10</td>
<td>8.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>a B16 (B700), 100 µg</td>
<td>S91 (S) (3.5 x 10⁶ cells)</td>
<td>8/8</td>
<td>3.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b B16 (B700), 10 µg</td>
<td>S91 (S) (3.5 x 10⁶ cells)</td>
<td>7/8</td>
<td>10.6 ± 1.2 NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c None</td>
<td>S91 (S) (3.5 x 10⁶ cells)</td>
<td>8/8</td>
<td>11.9 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>a S91 (B700), 100 µg</td>
<td>JB/RH (S) (5 x 10⁶ cells)</td>
<td>7/8</td>
<td>12.3 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b None</td>
<td>JB/RH (S) (5 x 10⁶ cells)</td>
<td>5/8</td>
<td>7.4 ± 2.8 NS</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>a S91 (B700), 100 µg</td>
<td>B16 F10 (TC) (2 x 10⁶ cells)</td>
<td>5/8</td>
<td>11.3 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b None</td>
<td>B16 F10 (TC) (2 x 10⁶ cells)</td>
<td>3/7</td>
<td>5.2 ± 2.3 NS</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>a S91 (B700), 100 µg</td>
<td>S91 (S) (5 x 10⁶ cells)</td>
<td>7/8</td>
<td>10.9 ± 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b None</td>
<td>S91 (S) (5 x 10⁶ cells)</td>
<td>6/8</td>
<td>11.3 ± 1.3 NS</td>
<td></td>
</tr>
</tbody>
</table>

*Groups were immunized with B700 (prepared either from B16 or S91 melanomas) in PBS or with PBS alone in the controls, 3 times at weekly intervals with tumor cell challenges 7 days after the last immunization. TC, tissue culture-passaged tumor cells; S, dissociated cells obtained from solid tumor growths passaged in vivo; NS, not significant. *Mean ± SE.

The results in Fig. 3 further demonstrate the specificity of the S91 TSTAs. S91 immunization did not affect the primary growth of three nonmelanoma tumors of C57BL mice; MCA-105 and T92497, both chemically induced sarcomas; and G1 26, a glioma, each of which has its own TSTA (Refs. 13 to 15; Footnote 3). None of these tumor cell lines showed any reduction in growth rate following immunization with S91 cells.

It was shown previously (2) that immunization with a soluble melanoma-specific antigen, termed B700, isolated and purified from B16 F10 melanoma cells, protected against B16 challenge and also provided significant protection against the other malignant melanomas studied, K1735 and JB/RH. More recently, protection has also been observed against the primary tumor growth of the chemically induced JB/MS melanoma (16) following immunization with the B700 antigen. The highly immunogenic nature of B700 in this study is also seen in Table 2, Experiment 1, against JB/RH challenge; this again shows the TSTA cross-reactivity observed in our previous studies using irradiated cells, butanol-derived soluble antigens, or purified B700 antigens. In contrast, S91 primary growth was not affected by B700 immunization (Group 2). However, immunization with B700 isolated and purified from S91 melanoma cells did not inhibit growth of JB/RH (Group 3) or B16 F10 (Group 4) nor did it influence the primary growth of the S91 melanoma (Group 5). Thus, although B700 is extractable from S91 cells and, as shown previously (17), seems identical to the B700 antigen of B16 by electrophoretic and immunological techniques, it is observed here not to be immunogenic by tumor rejection criteria.

We examined the expression of B700 on these melanoma cell lines by flow cytometry; representative histograms of the surface staining are presented in Fig. 4, and the data for surface and cytoplasmic expression of this antigen are reported in Table 3. Although B700 was expressed both in the cytoplasm and on the cell surface by B16 F10, K1735, JB/RH, and JB/MS melanoma cells, it was only demonstrable in the cytoplasm of the S91 cells and was not detectable on the cell surface.

**DISCUSSION**

Results of the present study show that the S91 malignant melanoma is highly immunogenic as demonstrated by immu-
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Table 3 Flow cytometric analysis of B700 expression by melanoma cells

<table>
<thead>
<tr>
<th>Expression</th>
<th>B16 F10</th>
<th>JB/MS</th>
<th>S91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell surface</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>7.9</td>
<td>22.5</td>
<td>7.8</td>
</tr>
</tbody>
</table>

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References

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