

## Release into Culture Medium of Membrane-Associated, Tumor-Specific Antigen by B-16 Melanoma Cells<sup>1</sup> (39332)

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Soluble tumor antigen, in a free or antibody-complexed form, has been implicated in the blockade of cell-mediated tumor rejection (1-4). We have recently reported the deposition of soluble immune complexes consisting of soluble tumor antigen and antibody, in the kidneys of mice bearing a progressive B-16 melanoma (3). The intensity of renal deposition correlated directly with tumor size and the presence of metastases, and inversely with the degree of mononuclear leukocyte infiltration of the tumor *in vivo*. It was of interest, therefore, to determine by what means and in what form the soluble antigen was generated by the tumor cells.

Soluble materials which exhibit tumor-associated antigenicity have been described recently in the expended medium of cultured L-1117 mouse lymphoma cells (5) and B-16 melanoma cells (6). We have described the occurrence of such antigens in the serum of B-16 bearing mice (3), and the present study has confirmed the presence of soluble tumor antigens in expended B-16 culture fluids.

**Materials and methods.** *Preparation of soluble antigen from culture supernatant fluids.* Monolayers of B-16 cells were cultured until they reached maximum density in RPMI-1640 medium containing penicillin (100 units/ml)-streptomycin (100  $\mu$ g/ml), tylocine (anti-PPLO), and 15% heat-inactivated, virus-screened fetal calf serum (Grand Island Biological Company, Grand Island, New York) at 37° in a 5% CO<sub>2</sub> atmosphere. Monolayers were washed thoroughly with Hanks Balanced Salt Solution and cultured for 72-96 hr in serum-free RPMI-1640 medium as before. Supernatant medium was then decanted, paper-filtered, and concentrated 50-60-fold by negative

pressure dialysis. Concentration was carried out at 4° using 0.39-in. dialysis casing (Union Carbide). Concentrate was dialyzed against normal saline, centrifuged at 100,000 g for 1 hr, and the pellet was discarded prior to estimation of the total protein content of the supernatant by the Lowry method. The supernatant material retained its antigenic activity for at least 8 months at -20°.

For control purposes, similar concentrated fluids were prepared from cultures of an established, H-2 unrelated, mouse cell line (L-929) and from H-2 identical C57Bl/6J normal adult fibroblasts (C57F) recently established in this laboratory. Also, similar fluids were prepared subsequent to repeated freezing and thawing of B-16 cells in serum-free RPMI-1640 at the same cell number to medium volume ratio as used for live monolayer-derived supernates. One-half of this killed cell suspension was processed immediately and fluid from the other half was collected and concentrated after incubation at 37° for 72-96 hr in order to allow the killed cells to undergo autolysis. The various supernatant preparations were coded as follows: (i) B-16SA, from live B-16 cell monolayers; (ii) B-16SA<sub>k</sub>, from killed B-16 cells; (iii) B-16SA<sub>k37°</sub>, from autolysed B-16 cells; (iv) C57FSA, from live monolayers of C57F cells; (v) L-929SA, from live monolayers of L-929 cells.

**Preparation of antiserum.** Antiserum to B-16SA was prepared in rabbits by repeated weekly, and later, biweekly, sc injections of approx 1 mg total protein of B-16SA. Initially, the B-16SA was injected as an emulsion in complete Freund's adjuvant. Booster injections were given in saline without adjuvant. Sera obtained 9 days after each sc booster injection or 4 days after an iv boost were found to contain maximal levels of specific antibody beginning at about 2 months after the initial sensitization.

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**Precipitation in agar.** All supernatant preparations were tested by double diffusion in 1% Agar [Phosphate Buffered Saline (PBS), pH 7.2] and by immunoelectrophoresis (IEP) in 0.85% Agar (0.05 M barbital buffer, pH 8.2) against rabbit anti-B-16SA.

**Characterization of B-16 specific antigen. Discontinuous gel electrophoresis.** Seven percent polyacrylamide analytical gels were prepared according to the method of Davis (7). Two-hundred microliters of B-16SA containing 80 mg of sucrose and having a total protein content of 250  $\mu$ g were layered on top of a 3% stacking gel. A loading current of 2 mA/gel was applied for approx 60 min to complete the electrophoretic separation. Longitudinal sections of the gels were fixed and stained for protein with Aniline blue-black or Buffalo black. Unfixed longitudinal sections were assayed for antigen location by double diffusion in agar. Comparable gels were run with glycerol substituted for sucrose and were stained for glycoprotein with Alcian blue or for carbohydrates with Periodic Acid Schiff stain.

**Sephadex column chromatography.** Three milliliters of B-16SA containing 0.3 g of dextrose was applied to a  $2.5 \times 45$ -cm column of Sephadex G-150 and eluted at 4° with PBS pH 7.4. The effluent was collected in 3-ml columns at a flow rate of 10 ml/hr and screened at 216 and 280 nm on a Gilford 240 spectrophotometer. Serial 15-ml pools were concentrated 10-fold by negative pressure dialysis and screened for B-16 specific antigen by IEP and double diffusion.

**Immunogenicity of B-16SA in C57Bl/6J mice.** C57Bl/6J male mice (18–20 g) obtained from the Jackson Labs (Bar Harbor, Maine) were injected at weekly intervals both sc and ip with either (i) 50  $\mu$ g B-16SA, (ii) 50  $\mu$ g C57FSA, (iii) 50  $\mu$ g L-929SA, (iv) polyacrylamide isolated B-16 specific fraction of B-16SA, or (v) whole frozen-killed B-16 cells ( $1 \times 10^6$ ). The antibody titers were determined by mixed hemadsorption (3) 1 week after the last immunizing injection (approx 2 months after initial injection) using pooled serum.

**Results.** Rabbit anti-B-16SA showed several precipitin lines in IEP with B-16SA (Fig. 1A). Some components with similar mobilities were also present in the L-929SA

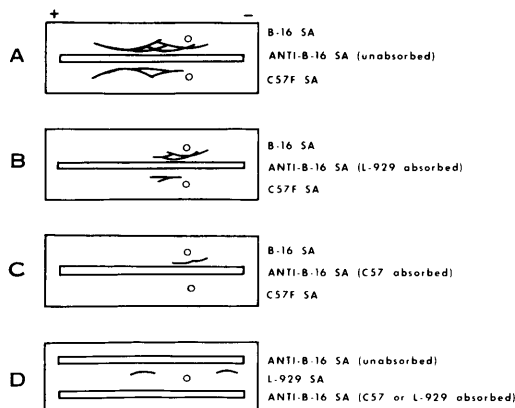


FIG. 1. Immunoelectrophoresis patterns of B-16SA, C57FSA, and L-929SA run against unabsorbed and absorbed rabbit anti-B-16SA. The B-16 specific antigens (C) could no longer be detected after the antiserum was absorbed once with B-16 lyophilized powder or with B-16SA.

and/or the C57FSA. Repeated ( $3 \times$ ) absorption of the antiserum for a minimum of 2 hr each at 4°, RT and 37° with a saline-insoluble lyophilized powder of L-929, or a single absorption for 1 hr at 37° with L-929SA, removed all reactivity against L-929SA and removed reactivity against the most anodally migrating component of C57FSA and B-16SA (Fig. 1B and D). Absorption with C57 tissue ( $5 \times$ ) powder or C57FSA ( $1 \times$ ) removed all reactivity against both L-929SA and C57FSA. Two antigenically identical components with slightly different beta mobilities could still be detected in the B-16SA after extensive absorption of the anti-B-16SA serum with C57 tissue powder or C57FSA (Fig. 1C). The B-16 specific reactivity could be completely removed, however, by a single absorption with B-16SA or with a saline-insoluble lyophilized powder of B-16 tumor tissue derived from sc growth in C57Bl/6J mice.

Double diffusion in agar (Fig. 2) with unabsorbed anti-B-16SA showed an antigenic component common to B-16SA, C57FSA, and L-929SA. A second B-16SA antigen was absent in L-929SA but displayed partial identity with a C57FSA component. A third antigen was detectable only in the B-16SA. The antigen common to all three supernates and the antigen limited to the B-16SA and C57FSA could no longer be detected when the antiserum was ab-

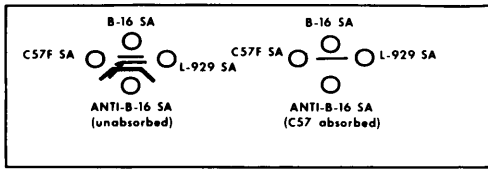


FIG. 2. Immunodiffusion patterns of B-16SA, C57FSA, and L-929SA against rabbit anti-B-16SA. The component which is common to all three supernates and the component which shows partial identity in the B-16 and C57 supernates are both removed by absorption with C57. The spur on the B-16SA is not detectable after absorption. However, the component which is present only in the B-16SA remains after C57 absorption and can only be removed by absorption with B-16.

sorbed with C57FSA or C57 powder. After extensive absorption with C57, a single line of reactivity remained against the B-16SA only. The B-16 specific reactivity could be removed only by absorption with B-16. An antigen identical to the B-16SA specific component could be detected in autolysed B-16 supernatant but not in non-autolysed killed cell supernatants (Fig. 3).

Rabbit anti-B-16SA also showed specific activity against B-16 cell membranes in the indirect immunofluorescence assay. Again, this activity could be removed by absorption with B-16SA or B-16 powder but not with similar preparations from either C57 or L-929 cells (Fig. 4).

Fourteen–sixteen protein staining components could be resolved in the B-16SA by discontinuous polyacrylamide gel electrophoresis. A line drawing representation of double diffusion in agar of one longitudinal section of B-16SA gel against anti-B-16SA is shown in Fig. 5. Two B-16 specific components were detected; the extreme anodal migration of the more prominent component contrasts with its slight anodal migration in IEP suggesting that the extreme migration in gel is a function of small size rather than large net negative charge. Carbohydrates were not demonstrable in areas of the gel in which B-16 specific antigen appeared.

The elution profile of the B-16SA on G-150 Sephadex is shown in Fig. 6. The antigen common to B-16SA, C57FSA, and L-929SA eluted in the first peak after the void volume. The location of the peak suggests a

mol wt range of 70,000–150,000. The G-150 isolated B-16 specific antigens were reapplied to a calibrated G-75 column and their molecular sizes were determined. The major antigen appeared to have a mol wt of approx 25,000 and the minor antigen of a mol wt of 20,000.

Mice immunized with either unfractionated B-16SA or with the polyacrylamide gel derived fraction developed a serum antibody response to the B-16 cells, as measured by mixed hemadsorption, which was comparable to that developed by animals immunized with whole B-16 cells. Mice immunized with L-929SA or C57FSA did not develop and anti-B-16 antibody (Table I).

*Discussion.* At least one, and possibly two, specific membrane-associated antigens can be detected in the supernatants of cultured B-16 cells. The fact that these antigens remained in solution after centrifugation at 100,000 g suggests that they are not large membrane fragments. Furthermore,

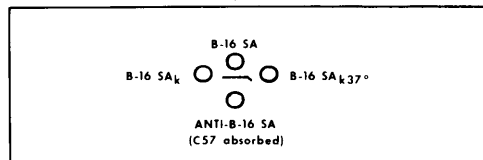


FIG. 3. B-16 specific antigens in supernates of live cells, killed cells, and autolysed cells: The B-16 specific component appeared only in the supernate from live cells (B-16SA) and in that from autolysed cells (B-16SA<sub>k37°</sub>). The concentration of specific antigen relative to the total protein concentration appeared to be greater in the supernate from live cells.

TABLE I. ANTIBODY RESPONSE IN SOLUBLE ANTIGEN-SENSITIZED C57BI/6J MICE.

Sensitizing antigen <sup>b</sup>	Antibody titer <sup>a</sup>	
	vs B-16 cells	vs L-929 cells
C57FSA	<1:10	<1:10
L-929SA	<1:10	1:640
B-16SA <sup>c</sup>	1:320	<1:10
Killed whole B-16 cells	1:512	ND <sup>d</sup>

<sup>a</sup> As measured by mixed hemadsorption using pooled serum.

<sup>b</sup> All antigen preparations were given both sc and ip in multiple doses over a period of 2 months.

<sup>c</sup> Polyacrylamide gel isolated B-16 specific antigen.

<sup>d</sup> Not done.

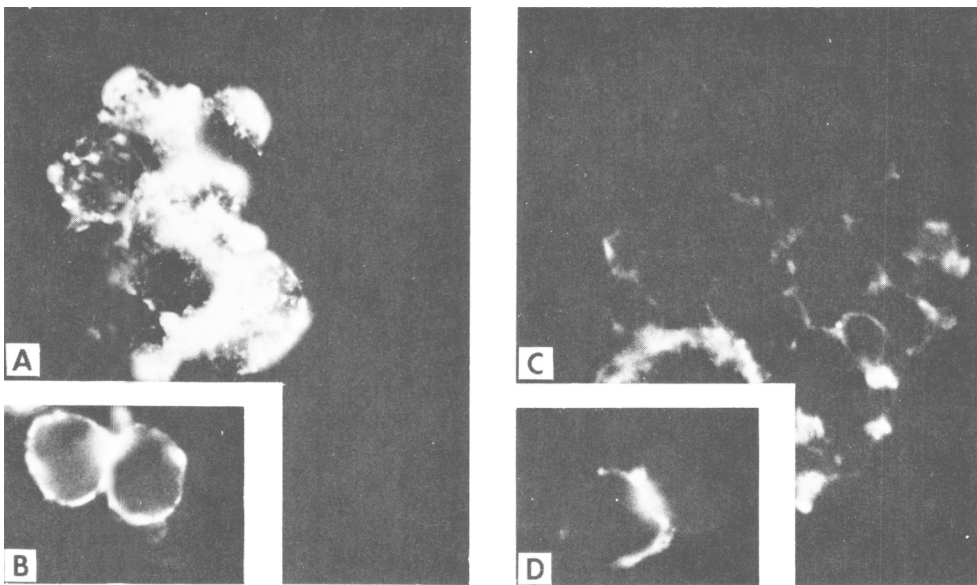


FIG. 4. Indirect immunofluorescence staining of B-16 cell membranes by rabbit anti-B-16SA and anti-rabbit IgG-FITC: Anti-B-16SA was absorbed (A) with C57 lyophilized saline insoluble tissue powder (B) with C57FSA (C) with B-16 lyophilized saline insoluble tissue powder (D) with B-16SA. Magnification  $\times 400$ .

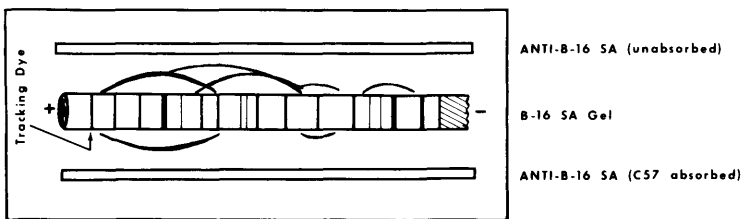


FIG. 5. Immunodiffusion pattern of B-16SA 7% polyacrylamide gel. The approximate positions of protein-staining bands in the gel were derived by staining one longitudinal section of the same gel which was tested in double diffusion. There appear to be two components, migrating at 10–13 and 30–34 mm, respectively, from the tracking dye which are B-16 specific. Absorption of the antiserum with B-16 removed this specific reactivity.

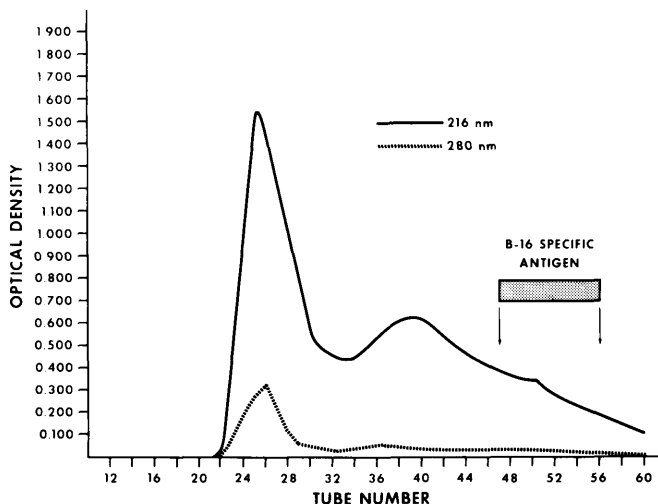


FIG. 6. Elution profile of B-16SA: The major B-16 specific antigen eluted in tubes 47–51 and the minor specific antigen in tubes 47–56 on G-150 Sephadex. The antigen common to B-16SA and C57FSA eluted in tubes 37–46. The antigen which appeared in L-929SA as well as B-16SA and C57FSA eluted in tubes 32–46. No antigenic activity was associated with the first peak (tubes 22–32).

the antigens were found to be soluble in distilled water and we believe that they may represent spontaneously shed, soluble, membrane-derived tumor-specific antigens. The B-16SA antigen which also appeared in L-929SA and C57FSA may be species-specific, and the antigen which was common to B-16SA and C57FSA but not present in L-929SA may be an H-2 antigen. This was not confirmed directly, however.

The fact that isolated B-16 supernatant antigens can induce an anti-B-16 cell membrane antibody in C57Bl/6J mice as well as in rabbits clearly demonstrates that the supernatant antigen is membrane associated. We have reported previously (3) that the serum and renal immune complex deposits from mice bearing a progressive B-16 tumor contain a material which is antigenically identical to the specific supernatant derived material and is smaller than 50,000 mol wt.

The relatively small size of the supernatant fluid derived B-16 specific antigen(s) in this study contrasts with the mol wt estimate of 150,000–200,000 for culture medium derived B-16-associated glycoprotein antigens reported by Bystryn *et al.* (6). Their material was not entirely B-16 specific, however, as it also appeared to some extent in the medium of C57 fibroblasts. This material may correspond to the larger, common antigens in our supernatant preparations. Furthermore, we were unable to detect carbohydrate associated with the B-16 specific antigen in PAS and Alcian blue-stained polyacrylamide gels. The relatively low sensitivity of these stains does not allow us to say that our material was definitely not a glycoprotein, however.

*In vivo* release of a human melanoma antigen into tumor cyst fluid and urine has been reported (8). The material was found to be mitogenic for lymphocytes from several melanoma patients but not for normal human lymphocytes. The soluble human melanoma antigen was similar to the supernatant-derived B-16 specific antigen in that it showed a beta mobility in IEP and its

presence in urine indicated a mol wt of under 40,000.

We believe that the release of B-16 specific antigen may occur as a normal part of cell membrane turnover during which other, nontumor specific membrane antigens are also released. The release of soluble tumor antigen *in vitro* by living cells and by autolysis of dead cells may be paralleled *in vivo*; such *in vivo* release may further be related to the failure of demonstrably sensitized hosts to reject their tumors (3).

*Summary.* Serum-free supernatant fluids from monolayer cultures of B-16 mouse melanoma cells were found to contain a soluble membrane associated tumor-specific antigen. The 100,000 g supernatant of the culture fluid induced an antibody response to the B-16 cells both in rabbits and in the mouse strain of origin (C57Bl/6J).

Similar supernatant fluids derived from an unrelated cell line (L-929) or from normal C57Bl/6 fibroblasts did not contain the B-16 specific material. Preliminary results indicate that the B-16 specific material is a protein of low molecular weight which is released into the culture fluid chiefly by living cells and, to a lesser extent, by autolysing cells.

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