Binding of retinoblastoma and normal sera to retinoblastoma-derived cultured cells

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We have observed increased binding of retinoblastoma patients' sera to a retinoblastoma-derived cultured cell line (Y-79). This reactivity was mediated by the serum IgG fraction and was directed toward different tumor or target cells (Y-79, Molt, Raji, and fibroblasts) cultured in media containing fetal calf serum. Normal pooled serum IgG fractions did not demonstrate any similar binding. When target cells were cultured in media containing human serum instead of fetal calf serum, a considerable reduction in retinoblastoma sera binding activity was observed. Reactivity against target retinoblastoma cells could be reduced but not entirely eliminated by quantitative absorption with non-retinoblastoma (Molt) cells grown in media with fetal calf serum. Retinoblastoma and normal sera binding to autologous fibroblasts, nonautologous fibroblasts, and cultured melanoma cells was also minimal. These findings suggest that residual binding activity in the sera tested may be directed against retinoblastoma tumor antigens. The fetal calf serum component responsible for reactivity with certain retinoblastoma sera was shown by immunoprecipitation, competitive inhibition, and gel electrophoresis to be bovine serum albumin.

Key words: retinoblastoma, Y-79 cells, bovine serum albumin, tumor-associated antigens, fetal calf serum

The importance of immunologic reactivity toward putative tumor-associated antigens in retinoblastoma is unclear. Recent studies have demonstrated that much of the immunologic reactivity observed in human malignancies may be toward differentiation and extraneous antigens and not toward tumor-associated antigens.1 We have previously demonstrated the presence of elevated levels of immune complexes in the sera of many retinoblastoma patients. The nature of the antigens to which antibodies in these complexes are directed have not been defined.

Retinoblastoma patients' sera demonstrate serologic (binding) reactivity with a cultured retinoblastoma cell line (Y-79).2, 3 In our current study, the nature of this reactivity was examined by extensive absorption procedures, by immunoprecipitation, and by competitive inhibition procedures. Significant reactivity was directed toward fetal calf serum (FCS) antigens. Some residual reactivity was probably directed toward retinoblastoma tumor-associated antigens; however, the biochemical and immunologic characteristics of these antigens is unclear.

Materials and methods

Cell culture. A retinoblastoma-derived tissue culture cell line (Y-79), human melanoma cell line (HT-144), Raji and Molt cells, and fibroblast explants from retinoblastoma patients were maintained in suspension cultures or monolayers.
ture media consisted of Dulbecco's modified Earles solution with either 10% FCS or pooled human (AB+) serum, penicillin-streptomycin and supplemented with glutamine. Cell cultures were kept in a CO₂-humidified incubator at 37°C.

**Serum collection.** Peripheral blood from retinoblastoma patients, nonretinoblastoma patients (seven uveitis, two choroidal nevus, and two glaucoma), choroidal melanoma (seven patients), and 30 age-matched normals were obtained by phlebotomy. The retinoblastoma patient group consisted of nine with bilateral retinoblastoma (four female, five male) and five with unilateral retinoblastoma (two female, three male). Ages of these patients ranged from 1 to 13 years. These retinoblastoma patients were selected on the basis of having elevated levels of immune complexes.

After clotting, the serum fraction was aliquoted into tubes (0.5 to 1.0 ml) and either used immediately or frozen in a Tecno freezer (−70°C). In our laboratory, serum samples and aggregate controls could be stored for several weeks without appreciable alterations in immune complex levels. 2, 3

**Binding assays.** Retinoblastoma-derived tissue culture cells (Y-79) were repeatedly washed and resuspended in Spinner's solution, and an aliquot of 2 × 10⁶ cells in 75 μl of solution was added to triplicate conical microcentrifuge tubes. These cells were incubated for 60 min at 37°C and then incubated for an additional 60 min at 4°C with 25 μl of test sera. Test sera consisted of the following: retinoblastoma sera (n = 14), nonretinoblastoma sera (150 μl, diluted 1:3 with PBS) were also absorbed overnight with increasing amounts of bovine serum albumin (BSA) (0 to 100 μl). The absorbed sera were corrected for dilution and assayed as described above using Y-79 target cells.

**Immunoprecipitation.** Retinoblastoma-derived tissue culture cells, autologous fibroblasts (patients Mo and L) (2 × 10⁶ cells), and BSA (100 μg) were enzymatically iodinated with 2 mCi of iodine 125 (New England Nuclear, Boston, Mass.). After extensive washing, the cell pellets were lyed in 0.1% Nonidet P-40. The extracted, labeled cell proteins and labeled albumin were then separated from unbound iodine by chromatography on Sephadex G-25. Trichloroacetic acid–precipitable counts were determined for the eluted protein peaks. These extracts were then precleared with *Staphylococcus aureus* bacterial pellets. Fifty microliters of test sample (serum or IgG fractions) were incubated with the labeled precleared cell extracts (50 μl) (1.0 × 10⁶ cpm) or labeled albumin (10 μl) (1.0 × 10⁶ cpm) for 60 min at room temperature and overnight at 4°C. S. aureus (Pan sorbin; Calbiochem, La Jolla, Calif.) (100 μl of mild trypsinization (crystalline trypsin, 20 U/ml, in Earl's solution) for 10 min at 37°C and suspended in culture medium with 10% FCS, and approximately 2 × 10⁶ cells were pipetted into triplicate wells on a microtiter plate. After 48 hr of incubation to regenerate cell surface components, test sera or chromatographic fractions were added to the wells and incubated as described above. The cells were washed and ¹²⁵I-Protein A (1.0 × 10⁶ cpm) was added. The wells were rinsed with serum-free media and solubilized in 100 μl of 2M NaOH, and 50 μl of the supernatant were transferred to scintillation vials for counting. 4

**Quantitative absorption.** Retinoblastoma sera (n = 3) with elevated levels of reactivity toward Y-79 cells and normal sera (n = 3) were absorbed with Y-79 pellets of various sizes or with Molt cells (0.25 to 4 × 10⁶ total cells). Prior to absorption, the sera were diluted with phosphate-buffered saline (PBS) (1:3) and 150 μl aliquots were incubated with the cell pellets for 60 min at room temperature followed by overnight incubation at 4°C. After centrifugation, the absorbed serum was assayed as described, using 2 × 10⁶ target cells (Y-79 or Molt). Molt and Y-79 cells have comparable amounts of FCS proteins on their surfaces, as determined by crossed rocket immunoelectrophoresis of a fixed amount (5 μl) of FCS into intermediate agar gels containing rabbit anti-FCS (150 μl) absorbed with equal concentrations of either Y-79 or Molt cells (1.0 × 10⁷ cells). Test sera (150 μl, diluted 1:3 with PBS) were also absorbed overnight with increasing amounts of bovine serum albumin (BSA) (0 to 4 mg) or FCS (0 to 100 μl). The absorbed sera were corrected for dilution and assayed as described above using Y-79 target cells.
Fig. 1. Binding of retinoblastoma and non-retinoblastoma sera to cultured Y-79 retinoblastoma cells. Mean values are represented by horizontal bars. Significant differences were found between the two groups (p < 0.05). Shaded area, Normal sera (n = 30) mean range.

washed 10% stock suspension) was added, and after 30 min the bacteria were washed until the supernatants approached background level (250 cpm). The pellets were counted for radioactivity and some of these (five retinoblastoma with elevated binding and two normal sera as controls) were further analyzed by polyacrylamide gel electrophoresis and autoradiography (PAGE-A) after elution with sodium dodecyl sulfate-mercaptoethanol buffer.

Competitive inhibition. Unlabeled BSA (62 to 2000 μg (20 μl) was added to incubation mixtures containing 1.0 × 10⁶ cpm of [125I]-BSA and 50 μl of retinoblastoma or normal sera. After overnight incubation at 4°C, the samples were immunoprecipitated with S. aureus. The washed bacterial pellets were counted and eluted, and the supernatants were analyzed by PAGE-A.

Results

Seventy-one percent of the retinoblastoma patients (10/14) had binding values significantly (p < 0.05) above the mean values of either normal (n = 30) or nonretinoblastoma control sera (n = 11) incubated with a retinoblastoma-derived tissue culture cell line (Y-79) (Fig. 1). Four of the retinoblastoma sera could be serially diluted 1:32 to 1:64 before the binding activity approached normal serum levels. Sera from choroidal melanoma patients were also tested for binding activity against Y-79 cells and were found to have the same binding reactivity as normal control sera.

A similar discrimination between retinoblastoma sera (n = 9) and a random selection of normal sera (n = 15) was observed in immunoprecipitation studies (p < 0.01) (Fig. 2). Immunoprecipitation assays performed with labeled extracts from autologous fibroblast cells did not demonstrate any differences in binding reactivity for the retinoblastoma compared with the normal sera controls (data not included).

Fractions obtained by chromatography of pooled retinoblastoma and pooled normal sera were also studied in binding assays with the Y-79 retinoblastoma cell line, allogeneic and autologous fibroblasts, and a lymphoblastoid (Raji) line. A high molecular-weight (>900,000 daltons) and a low molecular-weight (160,000 daltons) protein peak from retinoblastoma sera were demonstrated to have increased binding activity with Raji cells (Fig. 3, middle). The high molecular-weight protein peak was absent in fractions obtained from normal sera; a low molecular-weight protein peak with minimal activity with Raji cells was observed. When retinoblastoma sera fractions were incubated with fibroblasts or Y-79 cells, only the low molecular-weight protein peak demonstrated any binding activity (Fig. 3, upper and lower). A known anti-BSA rabbit serum was also found to bind...
Fig. 3. Binding (cpm) of chromatographic fractions from pooled retinoblastoma sera (closed circles) (n = 3) and normal (open circles) (n = 3) human sera to Y-79 retinoblastoma cells (upper graph), Raji cells (middle graph), or fibroblasts (lower graph) detected by \(^{125}\)I-Protein A. Position of marker proteins (albumin [67,000 daltons], IgG [160,000 daltons], and IgM [900,000 daltons]) were determined by rocket immunoelectrophoresis of fractions.

to these target cells, whereas normal rabbit sera did not bind (except for binding to Raji cells, which have Fc receptors).

Characterization of eluted proteins from the S. aureus immunoadsorbents examined by PAGE-A indicated a protein band with electrophoretic migration similar to that of the BSA marker (64,000 daltons) in two of the retinoblastoma sera tested (Fig. 4, lanes C and F) and in none of the normal sera tested (the two normal sera assayed are shown in Fig. 4, lanes B and E). Surface-labeled proteins extracted from Y-79 cells demonstrated a complex protein profile upon characterization by PAGE-A (Fig. 4, lane D). Several of the protein bands eluting from immunoadsorbents, including the 64,000 dalton protein, could be discerned in the labeled Y-79 cell extracts.

Immune complex formation between \(^{125}\)I-BSA and certain retinoblastoma sera could be inhibited by the addition of unlabeled BSA to the reaction mixture. Decreasing amounts of labeled BSA appeared in the eluates from S. aureus immunoprecipitated mixtures, as shown by direct counting of radioactivity (data not shown) and by subsequent PAGE-A (Fig. 5, lanes C to F). The normal sera similarly treated did not have a 64,000 dalton band, indicating absence of reactivity with BSA antigen.

Retinoblastoma sera demonstrated reactivity for target tumor cells (Y-79) after being absorbed to an end-point with increasing quantities of nonretinoblastoma cells (Molt). The plateau level was attained after absorption of the retinoblastoma sera with \(2 \times 10^7\) Molt or Y-79 cells. Mean binding reactivity (cpm) at this plateau region (retinoblastoma sera) was at least twice the value obtained from normal sera similarly absorbed. Regardless of the absorbing cells (Y-79 or Molt cells cultured in media containing FCS), subsequent binding of normal sera for Y-79 target cells remained relatively unchanged. In addition, the plateau level (retinoblastoma sera absorbed with Molt cells) was significantly greater than the plateau level of retinoblastoma sera absorbed with Y-79 cells (see Table I for above data). Binding reactivity of retinoblastoma sera for Y-79 target cells was reduced from 10,025 (mean cpm) to a plateau of 6800 (mean cpm) when 2 mg of BSA were added to the test sera. Absorption with 65 \(\mu\)l of FCS reduced subsequent binding of test sera to a similar level (6622 mean cpm). Additional increments of FCS did not result in any further reduction in binding. Normal sera remained at a constant binding level (3175 mean cpm) regardless of absorption with FCS or BSA.

When Y-79 cells were short-term cultured...
Fig. 4. PAGE-A of eluates from *S. aureus* (Fam-sorbin), which were incubated with labeled retinoblastoma (Y-79) extracts and either normal sera (lanes B and E) or retinoblastoma patients' sera (lanes C and F). Note protein band (arrow, lanes C and F) with electrophoretic mobility similar to that of the albumin marker protein. Labeled surface proteins were extracted from Y-79 cells and used in this study (lane D). Labeled marker proteins (lane A) from top to bottom: albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), and trypsin inhibitor (20,000 daltons).

(7 days) in media containing pooled human sera (AB+), subsequent binding of test retinoblastoma sera was greatly diminished (compared with the binding reactivity obtained with Y-79 cells cultured in media containing FCS) (Table II, 11,690 vs 25,624 mean cpm binding reactivity, respectively). Binding of normal sera remained essentially unchanged (7890 vs 7848 mean cpm binding reactivity). Furthermore, when statistically analyzed, the differences in binding activity were still greater for the retinoblastoma sera (11,690 mean cpm) compared with those of the normal sera (7848 mean cpm) (p < 0.01). Y-79 cells required at least a 2 day incubation period in media containing human sera for a detectable reduction in FCS-mediated bind-

ing to occur. No difference in reactivity between the retinoblastoma sera tested and normal sera could be discerned when other target cells (Molt, autologous and nonautologous fibroblasts, and melanoma HT-144) were short-term cultured in media containing human AB+ sera.

**Discussion**

We have observed increased binding of the IgG fraction of some retinoblastoma patients' sera to retinoblastoma-derived tissue culture cells compared with the binding activity of nonretinoblastoma and normal control sera. This reactivity could be mediated by: (1) either Fc or C3b receptors present on cultured retinoblastoma cells,8 (2) immune complexes,2 or (3) antibodies to putative tumor-associated surface antigens. Reactivity
Table I. Quantitative absorption of retinoblastoma and normal sera with Y-79 and nonretinoblastoma cells*

<table>
<thead>
<tr>
<th>Absorbing cell concentration</th>
<th>Control sera1</th>
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<tbody>
<tr>
<td></td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td></td>
<td>4 × 10⁶</td>
</tr>
<tr>
<td>Y-79-absorbing cells</td>
<td>2726</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Molt-absorbing cells</td>
<td>2544</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td>6685</td>
</tr>
<tr>
<td>Normal</td>
<td>2836</td>
</tr>
</tbody>
</table>

*Values indicate mean (cpm) binding activity of normal (n = 3) and selected retinoblastoma sera (n = 3) on Y-79 target cells (2 × 10⁶) measured by ¹²⁵I-Protein A binding.

Table II. Binding of retinoblastoma and normal sera to Y-79 cells after short-term culture in media with human sera and FCS*

<table>
<thead>
<tr>
<th>Normal sera</th>
<th>Retinoblastoma sera</th>
</tr>
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<tbody>
<tr>
<td>FCS-cultured cells</td>
<td>5264 (±200)</td>
</tr>
<tr>
<td></td>
<td>7580 (±2104)</td>
</tr>
<tr>
<td>Human serum-cultured cells</td>
<td>8846 (±896)</td>
</tr>
<tr>
<td></td>
<td>8669 (±979)</td>
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</tbody>
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*Binding assay described in materials and methods and measured (radioactivity cpm) by ¹²⁵I-Protein A labeling of tumor cells after incubation with test sera.

Toward antigens on tumor-derived culture cells have been defined with a number of human malignancies, including melanoma. Three classes of antigens exist: antigens restricted to autologous tumor cells (class 1), antigens shared by some allogeneic tumors of the same histologic type (class 2), and antigens widely distributed on cultured cells (class 3).1

Fc receptors did not play a significant role in the binding observed; IgG levels in the test and control samples were in the same normal range (8 to 17 mg/ml), yet an obvious difference in binding was observed. C3b receptors on Y-79 cells and immune complexes in retinoblastoma sera are also unlikely to be responsible for the binding observed, since binding activity was confined to the IgG fraction. Immune complex binding mediated by Fc or C3b receptors usually involves proteins with a heavier chromatographic distribution (>160,000 daltons).9 Moreover, if immune complexes were involved, the binding activity profile of serum fractions and Y-79 cells would look more like that obtained with Raji cells (which have C3b receptors mediating immune complex binding), and this was not found.

The apparent discrepancy between the immunoprecipitation data from fibroblast extracts and test sera (which indicated no reactivity) and the binding data obtained with the chromatographic fractions and fibroblast cells (demonstrating a low molecular-weight protein peak with measurable reactivity) can be attributed in the latter case to the presence of FCS contaminants reincorporated into the membrane structure. In the former case, the extracts were prepared immediately after trypsinization and conceivably FCS contaminants were removed by enzymatic digestion.

Antibodies to FCS components (class 3 antigens) are not uncommon in human sera.10 FCS antigens can adsorb and become integrated into the membrane structure of cells grown in FCS.11, 12 Sera containing such an-
Antibodies would then have reactivity with target cells grown in FCS. Antibodies to FCS components and related reactivity to target cells can be eliminated by absorption of test sera with FCS or by absorption with other nonretinoblastoma cells grown in media containing FCS; in this study we could also eliminate binding by culturing target cells in media containing human sera instead of FCS. The FCS component on cultured tumor cells was characterized by PAGE-A after immunoprecipitation with reactive sera and labeled cell extracts; its molecular weight and electrophoretic mobility was identical to that of BSA. No other FCS proteins could be identified by immunoprecipitation and PAGE-A. FCS antigens are present on cultured cells (indirectly demonstrated by immunoelectrophoretic analysis of rabbit antisera prepared against trypsin-digested, exhaustively washed, cultured Y-79 and Molt cells); these are apparently less significant than BSA in serologic testing of patients’ sera. Purified unlabeled BSA could also inhibit reactivity between patients’ sera and labeled BSA, further substantiating the nature of this antigen.

The retinoblastoma tumor cell surface contains numerous potential antigens, as indicated by the complex protein profile after surface labeling and detergent extraction and by the production of specific antisera in rabbits immunized with 10% human serum. This residual reactivity observed was probably directed against tumor-associated antigens (class 2 antigens). Since antibodies to class 3 antigens were predominant, these must be removed either by absorption (as used in this study) or by other immunochemical protocols before further characterization of class 1 or class 2 antigens can be achieved.

REFERENCES