Synthesis of Antigens, Cross-Reactive with Bovine Serum Albumin, by Cultured Neuroblastoma Cells


ABSTRACT

Antisera to bovine serum albumin (BSA) react with biosynthetic products of the LAN-1 neuroblastoma cell line. This immunological reaction was shown by analysis of immunoprecipitates prepared with anti-BSA and products of this cell line intrinsically labeled by the incorporation of $^3H$- or $^{35}S$-labeled amino acids. Moreover, antibodies in sera from two patients with neuroblastoma precipitate intrinsically labeled macromolecules produced by these tumor cells. Release of antigens cross-reactive with BSA by neuroblastomas may explain, in part, the high levels of antibody to BSA and circulating immune complexes containing hidden or “blocked” antibodies to BSA found in some patients with this tumor.

INTRODUCTION

In a previous study, we observed that the levels of antibody to BSA were inordinately high in sera of children with neuroblastoma, especially in females (2). Serum immune complex levels correlated significantly with the level of circulating anti-BSA in these patients. Moreover, sera that contained particularly high levels of immune complexes also contained hidden or blocked anti-BSA, suggesting that BSA, or antigens cross-reactive with BSA, accounted for a significant part of these immune complexes.

In the present report, we now show that one continuous line of neuroblastoma cells releases antigens cross-reactive with BSA, and we suggest that some of the stimulus for production of anti-BSA in children with neuroblastoma may emanate from the tumor.

MATERIALS AND METHODS

Cell Cultures. LAN-1, an established human neuroblastoma cell line (21) kindly provided by Dr. R. C. Seeger, UCLA School of Medicine, Los Angeles, Calif., was maintained in Waymouth’s medium (Microbiological Associates, Los Angeles, Calif.) with 20% heat-inactivated fetal calf serum, L-glutamine (50 mM), and gentamicin (50 μg/ml). Medium was changed every 3 to 4 days until cells became almost confluent, at which time they were transferred with trypsin:Versene (1 part 0.25% trypsin in Tris:0.15 M NaCl and 3 parts 0.02% Versene in Dulbecco’s PBS, pH 7.3). Subcultures of these cells were maintained in a chemically defined, serum-free medium (6). Supernatants were collected at weekly intervals; 200 KIU/ml aprotinin (Trasylol; FBA Pharmaceuticals, New York, N. Y.) and 2 mM PMSF, (Sigma Chemical Co., St. Louis, Mo.) were added to retard proteolysis, and the samples were stored at $-70^\circ$ until tested.

SK-N-SH, SK-N-LO, and SK-N-MC, 3 established human neuroblastoma cell lines (3), kindly provided by Dr. L. Helson, Sloan-Kettering Institute, New York, N. Y., were maintained in Eagle’s MEM (Grand Island Biological Co., Grand Island, N. Y.) with 15% heat-inactivated fetal calf serum, L-glutamine (100 mM), and gentamicin (50 μg/ml); the medium was changed every 7 days, and cells were transferred when almost confluent, as above.

Pulse Labeling with $^3H$- or $^{35}S$-labeled Amino Acids. Biosynthetic labeling of cell lines was carried out by adding: (a) high-specific-activity $^3H$-amino acid mixture (code TRK 550, Batch 11; Amersham Corp., Arlington Heights, III.) containing L-leucine, L-lysine monohydrochloride, L-phenylalanine, L-proline, and L-tyrosine; or (b) $^{35}S$.Methionine (1102.9 Ci/mmol; New England Nuclear, Boston, Mass.). The radioactive amino acids were added to well-washed cultured cells for 24 hr before harvesting supernatant and cells.

Eagle’s MEM, deficient in the particular labeled amino acids, was added to the flask after thoroughly washing the tumor cells with MEM to dilute the fetal calf serum. Approximately 10$^7$ cells/flask were incubated with 1 μCi of labeling mixture at a final concentration of 0.033 μCi/ml in the absence of fetal calf serum. The procedure was repeated by all cell lines for 24 hr. Cells maintained normal morphology and birefringency. Passage numbers at the time of labeling were: LAN-1, 65 to 70; SK-N-SH, 186; SK-N-LO, 56; and SK-N-MC, 95. At the end of the 24 hr, however, all cultures contained increased numbers of floating cells compared to those under normal culture conditions. Spent medium was removed, centrifuged at 250 x g for 30 min, and dialyzed against 100 volumes of PBS containing 1 mM PMSF for 72 hr with 3 changes of buffer to remove all nontrichloroacetic acid-precipitable radioactivity. Cells were detached mechanically from the bottom of the flask, transferred to a plastic tube, and solubilized by addition of 4 ml of Tween 80 (5%) in PBS containing 1 mM PMSF. They were shaken overnight at 4$^\circ$ and then centrifuged at 163,000 x g for 60 min to remove cell debris, dialyzed as above versus PBS containing 1 mM PMSF, and centrifuged again at 2000 x g for 30 min. Proteins in the spent medium were concentrated by negative pressure applied to collodion bags (Schleicher and Schuell, Inc., Keene, N. H.) or by high-pressure ultrafiltration (Diaflo Ultrafilters; Amicon Corp., Lexington, Mass.). In [35S].Methionine labelings, aprotinin was also added to both spent medium and Tween 80 extracts at a final concentration of 100 KIU/ml.

Imune Complex Precipitation. Equal volumes of sera, diluted 1:5 in PBS, and the labeled tumor cell extract were mixed in test tubes precoated with 0.5% ovalbumin in PBS and incubated for 30 min at 37$^\circ$ and overnight at 4$^\circ$. PEG in borate-buffered saline was then added to a final concentration of 5%, and the mixture was incubated in an ice
bath at 0° for 60 min and then centrifuged at 1000 x g for 20 min. Precipitates were washed twice with 5% PEG; then dissolved by addition of 50 μl of 6 M urea in 4% SDS buffer, pH 7.4, with or without 2% 2-mercaptoethanol; and incubated at 63° for 60 min. Protein A (14) covalently coupled to CNBr-activated Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N. J.) and xenogeneic anti-immunoglobulin sera were also used as immunoprecipitants.

PAGE. Dissolved immunoprecipitates were analyzed by PAGE in the presence of SDS using 1.25-mm-thick 5% PAGE running gels and using an acrylamide:bisacrylamide ratio of 30:0.8 and 0.1% SDS (15, 23). Electrophoresis was performed at room temperature (22°) with a constant current of 25 ma/gel. After electrophoresis, gels were fixed in a 10% trichloroacetic acid solution, stained with 0.25% Coomassie blue, destained, and prepared for autoradiographic analysis (5). Autofluorographs were recorded on preflashed Kodak XRP-1 film (Eastman Kodak Co., Rochester, N. Y.). A highly sensitive silver staining method was used to identify protein bands in some PAGE slab gels (26). Note that this method, while highly sensitive to low concentrations of protein in the gel, paradoxically fails to stain proteins present in very high concentration. Thus, BSA in the gel shown in Fig. 1 is unstained and shows up as a white translucent band. The following molecular weight markers were used in the PAGE runs: ferritin, M, 220,000 (220K); phosphorylase, M, 94,000 (94K); BSA, M, 67,000 (67K); catalase, M, 60,000 (60K); ovalbumin, M, 43,000 (43K); and soybean trypsin inhibitor, M, 20,000 (20K).

Electrophoretic Transfer to Nitrocellulose Paper. Spent culture supernatants were applied to 3% polyacrylamide stacking gels and fractionated in 5% polyacrylamide running gels with 0.1% SDS using conditions described above. Molecular weight standards were applied at one end of the gel. After PAGE, the proteins in the gel were transferred to nitrocellulose paper by electrophoresis for 5 hr at 10 V/cm at 22° in 25 mM Tris:192 mM glycine:20% (v/v) methanol (24). Strips containing the molecular weight standards were cut out and stained with Amido black. The remainder of the sheet containing the transferred proteins from the spent culture medium was soaked in 5% ovalbumin or normal goat serum in 0.15 M NaCl with 10 mM Tris, pH 7.4, to block nonspecific binding of antisera. These were then incubated with affinity-purified rabbit anti-BSA diluted in 5% ovalbumin plus 0.05% Tween 20 for 60 min. The nitrocellulose paper was then washed 4 times in 0.15 M NaCl with 10 mM Tris, pH 7.4, with 0.05% Tween 20 and then incubated for 30 min with peroxidase-conjugated goat anti-rabbit IgG, diluted in 5% ovalbumin with 0.05% Tween 20 (Research Products, Miles Laboratories, Inc., Elkhart, Ind.). For the color reaction, sheets were soaked in 25 μg/ml O-dianisidine diluted in Tris:0.15 M NaCl containing 0.02% H₂O₂ for 20 min (24). All incubations were at room temperature (23°C).

In control experiments, we demonstrated that rabbit anti-BSA, over a wide concentration range, did not bind to the nitrocellulose papers in the absence of transferred BSA. Similarly, goat anti-rabbit IgG did not react directly with proteins transferred into the nitrocellulose paper.

Antiserum. Polyclonal rabbit anti-BSA prepared in this laboratory was affinity purified by absorption over BSA bound to Sepharose 4B (Pharmacia Fine Chemicals). Commercially available rabbit anti-BSA (Lot 009601; Calbiochem-Behring Corp., La Jolla, Calif.) was also used, and normal rabbit serum (Grand Island Biological Co. Laboratories) served as control. Sera from children with neuroblastoma were collected as described previously (2).

Affinity Chromatography. BSA, coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) was used to isolate specific anti-BSA. Bound antibody, eluted with 0.2 M glycine-HCl, pH 2.8, was

Fig. 1. SDS-PAGE (10% polyacrylamide) of reduced immunoprecipitates prepared with anti-BSA and spent culture supernatants from the LAN-1 line grown in chemically defined, serum-free medium. Silver stain. Lane I, molecular weight markers; Lane II, immunoprecipitates of culture supernatants after 3 weeks in chemically defined, serum-free medium; Lane III, concentrated spent culture supernatants from LAN-1 after 3 weeks in chemically defined, serum-free medium; Lane IV, immunoprecipitates of fresh chemically defined, serum-free medium with anti-BSA; Lane V, 10-times-concentrated chemically defined, serum-free medium alone; Lane VI, immunoprecipitates with anti-BSA of culture supernatants from LAN-1 cells, collected between 3 and 4 weeks in chemically defined, serum-free medium; Lane VII, 10-times-concentrated culture supernatant from LAN-1 collected between the third and fourth weeks in chemically defined, serum-free medium. Lane VIII, 5-times-recrystallized BSA; Lane IX, affinity-purified rabbit anti-BSA. Note that for the first 3 weeks in chemically defined, serum-free medium LAN-1 culture supernatant still contains detectable BSA. Thereafter, BSA is not detectable in spent culture supernatants. Immunoprecipitates of culture supernatants with anti-BSA contain numerous bands not attributable to BSA or anti-BSA.
dialyzed versus PBS, pH 7.4, and conjugated to a new lot of CNBr-activated Sepharose 4B. The immobilized anti-BSA was used to adsorb biosynthetically labeled spent culture supernatants.

RESULTS

To evaluate whether neuroblastoma cells release antigens which might be recognized by antibodies to BSA, we tested culture supernatants from LAN-1 which had been adapted to and maintained in medium free of fetal calf serum for up to 5 weeks. Fig. 1 shows the proteins which were precipitated from these culture supernatants by anti-BSA. BSA could still be demonstrated for the first 3 weeks following transfer of the cells from media containing fetal calf serum (Fig. 1, Lanes II and III). However, after the third week, BSA (a M r 67,000 protein) was no longer detectable, even in culture supernatants which had been concentrated 10-fold (in Fig. 1, compare Lanes VII and VIII). Immunoprecipitates prepared with anti-BSA and spent culture supernatants collected after 3 weeks of growth in the chemically defined medium contained a band at 67 K. They also contained numerous proteins (Fig. 1, Lane VI) which were not found in BSA or anti-BSA (Fig. 1, Lane IX). These proteins were not evident in fresh chemically defined medium itself (Fig. 1, Lane V) or in fresh chemically defined medium mixed with anti-BSA (Fig. 1, Lane IV). Thus, it seemed likely that this neuroblastoma cell line releases proteins which cross-react with BSA.

To determine whether the proteins reactive with anti-BSA were biosynthetic products of LAN-1, we added 3H-labeled amino acids to the media in which LAN-1 cells were growing. Radiolabeled proteins reactive with anti-BSA were precipitated from these culture supernatants by adding anti-BSA and then 5% PEG to bring down soluble immune complexes. In Fig. 2, Lanes II and III demonstrate that affinity-purified anti-BSA as well as another antiserum to BSA from a commercial supplier precipitated 3 high-molecular-weight proteins (Mr >300,000) from the 3H-labeled culture supernatants. The molecular weights of these proteins (Fig. 2, Lanes II and III) could only be estimated since they were larger than the biggest standard available. Precipitation of these proteins did not occur if the 3H-labeled culture supernatants were incubated with normal rabbit serum or PBS before adding PEG (Fig. 2, Lanes IV and V). Passage over anti-BSA Sepharose selectively depleted the high-molecular-weight components which were otherwise found when these LAN-1 culture supernatants were reacted with anti-BSA and precipitated with 5% PEG before electrophoresis (in Fig. 2, compare Lanes II and III with Lanes VIII and IX). We could not demonstrate 3H-labeled antigens in the eluates from these anti-BSA columns, however (Fig. 2, Lane VII).

Radiolabeled proteins, precipitable by anti-BSA, were demonstrated only in spent medium from the LAN-1 line, but not in immunoprecipitates of spent medium from the SK-N-SH, SK-N-LO, or SK-N-MC lines under these conditions. The proteins cross-reactive with BSA were best demonstrated using 5.0% PEG to precipitate immune complexes formed with anti-BSA. Staphylococcal protein A and a goat anti-rabbit serum both caused nonspecific precipitation of radiolabeled components present in the culture supernatants of LAN-1 cells.

Additional experiments were set up using 35S)methionine to label the biosynthetic products of LAN-1 cells. Immunoprecipitates prepared with anti-BSA and supernatants of [35S]methionine-labeled LAN-1 cells demonstrated a single high-molecular-weight component which did not leave the 5% polyacrylamide stacking gel (Fig. 3, Lanes II and IV). Precipitation of this 35S-labeled component was blocked by adding excess unlabeled BSA to the culture supernatant before adding the anti-BSA (Fig. 3, Lanes III and V). The larger size of the product precipitated by anti-BSA from the 35S-labeled culture supernatants may reflect the fact that these cultures were protected with both PMSF and aprotinin against proteolytic enzyme cleavage, whereas only PMSF had been added to the 3H-labeled culture supernatants.

Immunoprecipitates prepared with sera from patients with neuroblastoma and the 35S-labeled culture supernatant also contained a high-molecular-weight radioactive component of approximately the same size as was brought down by anti-BSA (Fig. 3, Lanes VII to X). Three additional biosynthetically labeled components were precipitated by these patients' sera. Precipitation of these proteins by the antibodies in the patients' sera was not prevented by addition of unlabeled BSA (Fig. 3, Lanes VIII and X), suggesting that the patients' antibodies recognized epitopes on these proteins which are not present in BSA.

Anti-BSA and the patients' sera precipitated these biosynthetically labeled components from spent culture supernatants but not from Tween 80 extracts of [35S]methionine-labeled tumor cells.

To evaluate whether the high-molecular-weight, intrinsically labeled component identified in these experiments could have been brought down as a result of a nonspecific complex formed...
Fig. 3. SDS:PAGE. Results of immunoprecipitates with anti-BSA or patient serum in the presence of 5% PEG, using $^{35}\text{S}$ methionine-labeled, concentrated spent medium of LAN-1 as the source of antigen. Immunoprecipitates were dissolved in 6 M urea with 4% SDS under nonreducing conditions. Exposure of autoradiograph, 430 hr. Arrows, proteins of interest. Lane I, spent medium alone; Lane II, spent medium precipitated with affinity-purified rabbit anti-BSA; Lane III, Same as Lane II, but with 0.1%-unlabeled BSA added to the anti-BSA; Lane IV, spent medium precipitated with antibodies in commercial anti-BSA; Lane V, same as Lane IV, but with 0.1%-unlabeled BSA added; Lane VI, 14C-labeled standard proteins; Lane VII, 35S-labeled spent medium precipitated with serum of NBL patient; no unlabeled BSA added; Lane VIII, same as Lane VII, but with 0.1%-unlabeled BSA added; Lane IX, labeled spent medium incubated with serum of another NBL patient; no additional BSA added; Lane X, same as Lane IX, but with 0.1%-unlabeled BSA.

between residual BSA in the culture medium and components of the tumor cells, the $^{35}$S-labeled proteins present in spent medium from this experiment were reduced with 2-mercaptoethanol, fractionated by SDS:PAGE, and transferred electrophoretically to nitrocellulose paper. The strips were incubated with affinity-purified rabbit anti-BSA, washed repeatedly, and then incubated with peroxidase-conjugated antibodies specific for rabbit IgG. In 3 of 3 experiments, both BSA (Mr 67,000) and a macromolecule (Mr >300,000) were recognized in these transfers (Fig. 4). Thus, it seems likely that these culture supernatants contained a macromolecule, larger than native BSA, which bound antibodies to BSA.

To further evaluate whether these tumor cells could have released components which can form a nonspecific complex with BSA or BSA fragments, we incubated LAN-1 cells for up to 72 hr with medium containing BSA, previously labeled with $^{125}$I. As a control, the same quantity of $^{125}$I-labeled BSA was added to tissue culture flasks containing the same medium but no tumor cells. In 4 experiments using cells maintained with medium containing fetal calf serum as well as cells supported by the chemically defined medium, we could not demonstrate that the LAN-1 cells released substances which retarded the passage of $^{125}$I-labeled BSA through polyacrylamide gels (Chart 1).

**DISCUSSION**

Media in which LAN-1 cells have grown contain proteins precipitable by antibodies to BSA. These antigenic proteins can be detected in supernatants of cells cultured in chemically defined, serum-free medium that contains no albumin of human or animal origin (6). Indeed, the only proteins in this medium were human transferrin and bovine insulin. Transferrin is the only protein visible when 10-times-concentrated, chemically defined medium is fractionated by PAGE (Fig. 1, Lane V); it is still the dominant protein in medium that has supported the growth of LAN-1 cells for 1 week (Fig. 1, Lane VII). Transferrin is not evident in immunoprecipitates of the spent culture medium and anti-BSA (Fig. 1, Lane VI). Thus, it is unlikely that antigenic homologies between subunits of transferrin and BSA account for the many proteins precipitated from the spent culture supernatant by the anti-BSA, unless these subunits display hidden antigenic determinants not evident in the native molecule.
Intrinsically labeled macromolecules synthesized by LAN-1 also contain antigenic determinants that were recognized by antibodies in sera from patients with neuroblastoma; 2 patients' sera, chosen because they contained high titers of antibody to BSA, precipitated 4 intrinsically labeled components from supernatants of cells pulsed with \(^{35}S\)methionine. Precipitation of these proteins was not blocked by adding excess native BSA (Fig. 3, Lanes VII and X).

Experiments with supernatants of cells, pulsed with \(^{3}H\) and \(^{35}S\)-labeled amino acids, verified that LAN-1 cells synthesize at least one macromolecule which shares antigens found in BSA. Comparison of the results shown in Figs. 2 and 3 suggested the possibility that one or more of the lower-molecular-weight proteins seen in Fig. 2 may have been a degradation product of the large protein demonstrated in Fig. 3.

We recognized that the idea that LAN-1 produces one or more proteins that cross-react with BSA would be invalid if we could show that the intrinsically labeled proteins brought down by anti-BSA (Figs. 2 and 3) or by antibodies in patients' sera were, in fact, precipitated because they had become nonspecifically associated with residual BSA, introduced into these culture supernatants as a component of fetal calf serum. To test this possibility, immunoprecipitates of the \(^{35}S\)-methionine-labeled culture supernatant and anti-BSA were fractionated by PAGE under reducing conditions. The proteins in the gel were electrophoretically transferred to nitrocellulose paper where they could be detected immunologically (24). Both BSA and a high-molecular-weight protein, reactive with anti-BSA, were demonstrated in these electroblots (Fig. 4), indicating that the macromolecule, precipitated by anti-BSA from these culture supernatants, was able to bind anti-BSA even when separated from BSA itself. The experiment, shown in Fig. 5, also argues against the notion that BSA formed nonspecific complexes with products of the LAN-1 cell.

Molecular interactions have been demonstrated, nevertheless, between polymerized albumin and hepatitis B particles (11, 12, 17). It has been suggested that the complex of hepatitis B virus with albumin may be responsible for the antibodies to polymerized albumin that are found in sera of some patients with hepatitis (16).

Our experiments were originally undertaken to determine why some children with neuroblastoma have abnormally high levels of antibody to BSA. The demonstration that one neuroblastoma cell line synthesizes a protein or proteins cross-reactive with albumin does not prove that these tumors regularly stimulate antibodies to the protein. Indeed, we studied 3 other neuroblastoma lines that did not release proteins precipitable by anti-BSA. Nevertheless, the demonstration of BSA-like antigens in products of one neuroblastoma cell line suggests that activation of genes which code for polypeptides cross-reactive with epitopes present in BSA occasionally occurs as a part of the events associated with the development of neuroblastomas. Demonstration of amino acid sequence homology between BSA and products of LAN-1 or other neuroblastoma cell lines may help to support this hypothesis.

These experiments are also noteworthy because they suggest that a human tumor can synthesize and release proteins that cross-react immunologically with a protein that is a major constituent in the diet, especially that of children. Small amounts of BSA and other dietary proteins cross the gastrointestinal barrier, even in normal individuals. When they enter the systemic circulation, they can form complexes with circulating antibodies (4, 7, 8). Thus, antigens from milk could have the effect of neutralizing some of the circulating antibodies formed in response to tumors in children with neuroblastoma.

It is interesting to consider (a) whether the development of these antibodies to antigens cross-reactive with BSA could...
have an impact on the growth of the tumor and (b) whether the presence of BSA in the diet might influence the host response to the tumor. In a previous report, we have shown that sera from children with disseminated neuroblastoma frequently have higher levels of anti-BSA than are found in sera of age-matched normal controls (2). These children often had circulating immune complexes as well. Indeed, there was a statistically significant correlation between the levels of immune complexes and the quantity of antibodies to BSA in their sera (2). We proposed that these immune complexes may result from the interaction of antibodies to BSA both with tumor cell products that are antigenically cross-reactive with BSA and with fragments of ingested BSA that cross the gastrointestinal tract.

By itself, interaction of anti-BSA with BSA from the diet would probably not directly impair the humoral immune response to the tumor, even if antibodies cross-reactive with BSA were prominently displayed on the tumor cell surface. In fact, they are probably not a prominent feature of the cell surface, since they cannot be demonstrated in immunoprecipitates prepared with anti-BSA and Tween 80 extracts of the tumor cells. Moreover, as shown in Fig. 3, binding of patients’ serum antibodies to the biosynthetic products of LAN-1 are not blocked by addition of excess unlabeled BSA, indicating that the sera of these children contain antibodies to other epitopes on the tumor-associated macromolecules.

However, it is well established that the presence of immune complexes, regardless of their composition, in proximity to the cells of the immune system will suppress a variety of immune responses (9, 20, 22). By contributing to the quantity of immune complexes in the circulation, interactions of dietary BSA with antibodies to BSA in the blood could have a significantly adverse effect on the host response to the tumor.

In this regard, it is noteworthy that neuroblastoma is relatively rare in geographical areas where infant feeding with cow’s milk rarely occurs (10, 18). The low incidence of neuroblastoma in these areas is probably not the result of underdiagnosis (18) nor indicative of strong genetic differences in susceptibility to neuroblastoma. Neuroblastoma is almost as common in black as it is in white children in North America. Its frequency is 6-fold less in children from Nigeria than in ethnologically related children from North America (19). Conceivably, in children who react vigorously to BSA-like antigens released by nascent neuroblastoma cells, absence of BSA in the diet may reduce the quantity and/or frequency of circulating immune complexes that would otherwise nonspecifically suppress the host response of these children to their tumors.

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