Identification, Purification, and Radioimmunoassay of NB/70K, a Human Ovarian Tumor-associated Antigen

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ABSTRACT

NB/70K, a tumor-associated antigen of human ovarian epithelial tumor Fraction OCA, has been purified and identified as a glycoprotein which is stable in 0.6 M perchloric acid, binds to concanavalin A, and migrates electrophoretically with α-like mobility in barbital-buffered agarose at pH 8.6. NB/70K does not appear to contain normal serum, normal ovary, normal lung, or carcinoembryonic antigen-like cross-reacting antigenic determinants as measured by radioimmunoassay. NB/70K has been purified from ovarian antigen Fraction OCA by chromatography on γ-globulin coupled to Sepharose 4B and by elution from acrylamide gels. NB/70K migrates as a single band with an apparent molecular weight of 70,000 in sodium dodecyl sulfate:acrylamide gel electrophoresis.

A rabbit antibody raised against NB/70K was able to precipitate a polypeptide with a molecular weight of 70,000 as visualized by autoradiography of sodium dodecyl sulfate:acrylamide gels. A radioimmunoassay has been developed for measuring NB/70K activity, using Staphylococcus aureus protein A as a precipitating agent.

INTRODUCTION

Human tumor-associated antigen Fraction OCA was purified from surgical epithelial tumor specimens by perchloric acid extraction, affinity chromatography on concanavalin A-Sepharose 4B and antibody:Sepharose 4B, and gel filtration on Sephadex G-100. A radioimmunoassay for Fraction OCA was designed which was capable of detecting ng amounts of OCA activity in unextracted plasma. This assay appeared to measure a different antigen from that measured by the CEA3 Roche Test Kit, as indicated by a clinical trial with plasma samples from ovarian cancer patients. Immunoprecipitation experiments with anti-OCA and anti-CEA indicated considerable cross-reactivity between OCA and CEA. Fraction OCA contained more than one band in SDS:acrylamide gel electrophoresis, indicating heterogeneity. In a preliminary study to assess the specificity of the OCA assay, it was found that plasma OCA levels were elevated in more than 30% of patients with tumors other than ovarian tumors.

These data are consistent with current theory which proposes that an antigenic molecule may contain more than one antigenic determinant or family of determinants. Fraction OCA may contain molecules with only ovarian tumor determinants, molecules with only CEA-like determinants, and molecules with both types of determinants.

This paper reports the results of experiments designed to identify and purify the ovarian tumor component of Fraction OCA so that a radioimmunoassay specific for ovarian tumor-associated antigen may be developed.

MATERIALS AND METHODS

Immunochemical Reagents and Methods. Fractions OCA and OCC were prepared from human ovarian tumor tissue (19), and antibodies were prepared in New Zealand White rabbits as described previously (18). γ-Globulin was prepared from rabbit antibodies by 2 precipitations with 33% saturated ammonium sulfate.

By adding the perchloric acid extract of the absorbing protein to the antibody and incubating at 37° for 1 hr and 4° overnight, the antibodies were absorbed. The mixture was centrifuged at 15,000 × g at 4° for 30 min, and the supernatant was removed. If absorption of antibody with large amounts of perchloric acid extract (30 mg or more extract per ml antibody) was required, the antibody was diluted 1:10 in PBS, and the required amount of perchloric acid extract was added. The pellet, if required, was washed twice with ice-cold PBS and collected by centrifugation.

γ-Globulin:Sepharose 4B was prepared by coupling γ-globulin to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Montreal, Quebec, Canada) as described previously (18). Radioiodination. Protein was labeled with 125I by a modification of the chloramine-T method (12). Two to 5 µg of protein in 25 µl of PBS were added to 25 µl of 0.1 µ phosphate buffer, pH 7.0. One mCi of carrier-free 125I in 5 µl and 25 µg of chloramine-T in 10 µl of PBS were then added. After the mixture was gently shaken for 1 min, sodium metabisulfite (62.5 µg in 25 µl of PBS) was added to stop the reaction. Four drops of BSA (1% in PBS) were added, and the mixture was immediately applied to a BSA-coated Sephadex G-75 (1.6–x 30-cm) column or to a Sephacryl S-200 (1.6–x 70-cm) column, using PBS as the eluting buffer. In the case of separation on Sephadex G-75, all labeled protein eluted in a single peak at the void volume, and unbound 125I eluted at an effluent volume greater than the total volume of the column.

Acrylamide Gel Electrophoresis. SDS:acrylamide gel electrophoresis was carried out using the method of Laemmli (20). Routinely, a 3% stacking gel and a 9% separating gel in a 0.15 x 10 x 14 cm slab were used. In all gels, the ratio of acrylamide to bisacrylamide was 30:0.8. For non-denaturing and non-reducing gels, SDS and β-mercaptoethanol were omitted. Molec-
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ular weight standards were BSA (M.W. 68,000), egg albumin (M.W. 43,000), and phosphorylase a (M.W. 94,000). Gels were run with water jacket cooling at 60 V until the BPB tracking dye entered the separating gel and then at 150 V until the dye was 1 cm from the bottom edge of the slab gel. A typical run required 4 hr. Gels were stained with Coomassie blue by the method of Fairbanks et al. (11), but staining times were reduced to 30 min in each solution.

Stained acrylamide gels containing radiolabeled proteins were dried on Whatman No. 1 filter paper in a vacuum and were then autoradiographed on Kodak RPR Medical X-ray film.

Preparation of NB/70K by Affinity Chromatography. To obtain a population of normal and CEA-like antigens which binds to anti-OCA, an immune precipitate was prepared by adding 10 mg of each perchloric acid extract of normal human serum, normal ovary, normal lung, and adenocarcinoma of the colon to 5 ml of a 1:10 dilution of anti-OCA in PBS. This immune precipitate was injected into rabbits to produce antibody 701. ß-globulin was prepared from antibody 701 and was then covalently coupled to Sepharose 4B.

Five hundred µl of 125I-labeled OCA were passed through a 5-ml syringe column containing Antibody 701:Sepharose 4B. The unbound material was collected, and the column was thoroughly washed with PBS to remove all unbound protein. Bound material which contained CEA-, normal lung-, ovary-, or serum-like antigenic determinants was eluted with 1 M acetic acid. The eluted material was immediately neutralized with 1 N sodium hydroxide. Using this method, the recovery of applied radioactivity was routinely 80 to 95%, with approximately 40% of the applied radioactivity in the unbound fraction.

Preparation of NB/70K by Acrylamide Gel Electrophoresis. NB/70K was prepared from Fraction OCA by the following method. Tris buffer, 0.05 M (100 µl), pH 6.8, containing 0.001% BPS and sucrose (0.3 g/ml) was added to 1 ml of Fraction OCA. The sample was applied to 9 of the 10 wells preformed in an acrylamide slab (1.5 mm thick) with a 3% stacking gel and a 9% separating gel which had been prepared without SDS. Twenty µl of a 1-mg/ml solution of BSA in Tris buffer, pH 6.8, with BPB and sucrose was placed in the tenth well. The samples were electrophoresed until the BPB dye was 1 cm from the end of the slab. The gel was then removed from the sandwich. A vertical strip containing the BSA was cut from the gel and stained with Coomassie blue. The distance between the BSA and the BPB front was measured on the stained strip. A mark corresponding to the position of BSA above the tracking dye was placed on the unstained portion of the slab gel. A horizontal strip with an upper limit of 1.0 cm above the position of BSA and a lower limit of 1.0 cm below the position of BSA was then cut from the unstained gel. This 2.0-cm strip, which contained NB/70K, was then eluted in 10 ml of PBS at 4° with gentle rocking. After elution for 8 hr or overnight, the PBS was decanted from the gel. Second and third elutions with additional 10-ml aliquots of PBS were then carried out. The eluted NB/70K fractions were pooled.

Radioimmunoassays. The OCA-RIA method has been described previously (19).Briefly, a primary incubation of rabbit anti-OCA (20 µl of a 1:4,000 dilution) and 125I-labeled OCA (20 µl of a 1:125 dilution) was carried out at 37° for 16 to 20 hr. A secondary incubation with donkey anti-rabbit globulin (Burroughs-Wellcome, Research Triangle Park, N. C.; 100 µl of a 1:25 dilution in PBS containing 0.01 M EDTA and 0.5% normal rabbit serum) was carried out at 37° for 1 hr. The pellets were collected by centrifugation at 1100 x g for 30 min at 4°. Binding of 125I-labeled OCA in the absence of anti-OCA (nonspecific binding) was routinely 5%, and binding of labeled OCA to antibody in the absence of unlabeled antigen was routinely 30% of the total counts added.

A second radioimmunoassay procedure, SA assay, is based on the Staphylococcus aureus protein A-binding method of Cullen and Schwartz (10) and Kessler (15). For antibody titration curves, 20 µl of the appropriate dilution of antibody in PBS were added to 10- x 75-mm glass tubes. Twenty µl of 125I-labeled protein containing approximately 50,000 cpm were then added to each tube. The tubes were incubated at 37° for 1 hr and were then placed in an ice bath. One-tenth ml of a 10% suspension of Pansorbin (Calbiochem-Behring, La Jolla, Calif.) in PBS was added to each tube. The tubes were mixed and allowed to stand for 15 min. One ml of ice-cold PBS was added to each tube, and the tubes were centrifuged at 4° for 30 min at 1100 x g. The supernatant was removed by decantation, and the pellets were counted for 1 min in a gamma counter.

If a competitive inhibition of perchloric acid extracts was to be measured, 20 µl of an appropriate dilution of the extract in PBS were added to each tube prior to the addition of antibody.

Binding experiments with various radiolabeled antigens and CEA antiserum were carried out at the McGill Cancer Centre, Montreal, Quebec, Canada. The CEA preparation used in these experiments ran as a single diffuse band with an apparent molecular weight of 180,000 in SDS:acrylamide gel electrophoresis, and iodinated CEA exhibited an activity in radioimmunoassay equivalent to other standard CEA preparations. Anti-CEA antiserum was raised against purified CEA in a horse and was absorbed with lyophilized aqueous extracts of normal human liver, lung, bowel, and serum (10 mg each per ml of antiserum). Absorbed anti-CEA showed no detectable interaction with normal tissue extracts in immunodiffusion. In addition, absorbed anti-CEA did not bind the radiolabeled NCA, nor was the binding of labeled CEA to anti-CEA inhibited by NCA. The diluent for all assay components was 0.05 M boric acid:borax buffer, pH 8.5, containing 0.5% (v/v) normal horse serum. Each tube contained 500 µl of diluted horse anti-CEA (or only normal horse serum in the case of blank tubes) and about 20,000 cpm of 125I-labeled CEA in 100 µl. The tubes were incubated for 2 hr at 37°, followed by the addition of 150 µl of undiluted sheep anti-horse IgG (determined to be in excess by pretitration). After 1 hr at 37° and 1 hr in an ice bath, precipitates were collected by centrifugation at 9000 x g for 25 min and counted.

Collection and Solubilization of Immune Precipitates. To visualize the reactive components in the OCA-RIA and SA assay, 10 replicate samples were prepared for each type of immune precipitate to be analyzed. After collection of the pellets by the normal double-antibody or SA assay method, 0.1 ml of ice-cold PBS was added to each tube, the tubes were vortexed, and the suspended precipitates were pooled. The tubes were washed twice more with 0.1-ml aliquots of PBS. The pooled precipitates were centrifuged at 15,000 x g for 10 min at 4°, and the supernatant was discarded.

* M. Krantz, personal communication.
Immune precipitates collected by the OCA-RIA were resuspended in 110 µl, and those collected by the SA assay were resuspended in 50 µl of 0.05 M Tris buffer, pH 8.6, containing 5% (w/v) sucrose, 0.02% β-mercaptoethanol, and 0.05% BPB. The precipitates were solubilized in a boiling water bath for 15 min. The solubilized precipitates obtained by the SA assay were then centrifuged at 15,000 x g for 10 min. The supernatants were removed and analyzed.

RESULTS

Demonstration of Ovarian Tumor Components of Fraction OCA. A first series of experiments was designed to demonstrate that Fraction OCA contains a population of ovarian tumor molecules.

Rabbit antibody against Fraction OCA was absorbed with the perchloric acid extract of normal ovary, pooled normal human serum (including serum of all major blood groups), normal lung, adenocarcinoma of the ovary, or adenocarcinoma of the colon at a concentration of 200 mg of extract per ml of undiluted antibody. In these experiments, adenocarcinoma of the colon extract was a source of molecules with CEA-like antigenic determinants. Adenocarcinoma of the colon extract as well as the extracts from normal lung and serum were sources of nonspecific antigens which cross-react with CEA, including NCA as described by von Kleist et al. (23) and NCA as described by Burtin et al. (5). Absorbed antibodies were titrated with 125I-labeled OCA (60,000 cpm; approximately 0.2 ng OCA) using the OCA-RIA (Chart 1). Antibody absorbed with normal ovary or normal serum gave essentially the same titration curve as that for unabsorbed antibody (not shown). Although absorption with normal lung appeared to have little effect on the titration curve, absorption with adenocarcinoma of the ovary or colon significantly reduced the binding of 125I-labeled OCA to antibody. Chart 2 demonstrates the competitive inhibition of binding of labeled OCA to rabbit anti-OCA (1:4000 dilution) by perchloric acid extracts of normal lung, adenocarcinoma of the ovary, and adenocarcinoma of the colon. As much as 3 µg of normal lung extract per assay tube did not significantly decrease 125I-labeled OCA binding to anti-OCA. Similarly, extracts of normal serum and normal ovary did not decrease binding (data not shown). In absorption studies with extracts of adenocarcinoma of the colon and ovary, there were maxima of approximately 70 and 90% reductions, respectively, in 125I-labeled OCA binding. When the experiment was repeated using anti-OCA which had been previously absorbed with normal ovary, normal serum, and normal lung extracts (1:200 dilution of absorbed antibody used for the assay), the maximum reduction in binding caused by adenocarcinoma of the colon and ovary remained approximately 70 and 90%. These data suggest that there is a population of molecules which contain only ovarian tumor antigenic determinants.

Removal of CEA-like antigens from Fraction OCA. 125I-labeled Fraction OCA was chromatographed on Antibody 701: Sepharose 4B. The unbound fraction, eluted fraction, and original Fraction OCA were tested in antibody titration experiments using both anti-OCA and antibody 701 (Chart 3). No residual binding of the unbound fraction to antibody 701 occurred, indicating that the capacity of the column had not been exceeded and that all of the antigen which was capable of binding to antibody 701 had been removed by the coupled Sepharose.

There was residual binding of the unbound fraction to anti-OCA, indicating that there was still a population of molecules which appeared to contain only ovarian tumor-associated antigen. The eluted material appeared to have been damaged by its exposure to acid; evidence for this was the very high nonspecific binding (45% of the total counts) when compared to that of the unbound material and the original OCA preparation (5% of the total counts) as well as the very low binding of the eluted material to antibody 701.

Radiolabeled unbound fraction, eluted fraction, and original OCA were examined by autoradiography of SDS:acrylamide gels (Fig. 1). The original OCA contained 4 bands, the eluted fraction contained 3 bands, and the unbound fraction contained a single band with an apparent molecular weight of 60,000 to 70,000 as compared to molecular weight marker proteins. The binding of radiolabeled OCA, the unbound fraction, and the eluted fraction to an absorbed antibody against a highly purified CEA preparation was examined (Chart 3). The original preparation of OCA, the eluted fraction, and 125I-labeled CEA bound to absorbed anti-CEA, while the unbound fraction had
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Chart 3. Binding of $^{125}$I-labeled Fraction OCA and the unbound (NB) and eluted (EL) fractions obtained by affinity chromatography of Fraction OCA on antibody 701-Sepharose 4B. a, antibody (Ab) 701; b, anti-OCA; c, absorbed anti-CEA with $^{125}$I-labeled CEA as a control. A total of 30,000 cpm was added to each tube. The OCA-RIA was used. Nonspecific binding for labeled OCA, CEA, and the unbound fraction was 5% of the total cpm added. Nonspecific binding for the eluted fraction was 45% of the total cpm added in a and b and 16% of the total cpm in c. Nonspecific binding was subtracted before graphing results.

little, if any, binding to absorbed anti-CEA. This unbound fraction has been named NB/70K; it binds to anti-OCA, does not bind to antibody 701 or anti-CEA (according to the Montreal experiments), and gives a single band of labeled material with an apparent molecular weight of 60,000 to 70,000 on SDS: acrylamide gels.

Preparation of anti-NB/70K for Use in a Radioimmunoassay. Only small amounts of purified NB/70K could be produced by passage through antibody 701-Sepharose 4B. Therefore, NB/70K was prepared by elution from nonreducing and non-denaturing acrylamide gels as described in "Materials and Methods" (Fig. 2). Eluted NB/70K was used to produce a rabbit antibody against NB/70K. Eluted NB/70K was iodinated with $^{125}$I, and a double-antibody radioimmunoassay was developed. The nonspecific binding in this assay was about 50% of the total cpm added, while the binding of labeled NB/70K to a 1:100 dilution of anti-NB/70K was about 65% of the total cpm added. Attempts were unsuccessful to reduce the nonspecific binding or to increase the binding of labeled NB/70K to antibody by changing the assay conditions, by adding additional BSA to decrease nonspecific adsorption of labeled material, or by using the SA assay instead of the double-antibody assay.

Separation of $^{125}$I-labeled NB/70K from $^{125}$I-labeled Fraction OCA. Since Fraction OCA contained both high-molecular-weight CEA-like antigens and NB/70K, separation of these 2 components by gel filtration of Fraction OCA would appear feasible. This would eliminate the necessity for affinity chromatography on Antibody 701-Sepharose 4B, saving both labor and material.

Separation of the chloramine-T iodination mixture of Fraction OCA by gel filtration on Sephacryl S-200 resulted in 2 major peaks of iodinated OCA material and one peak of free $^{125}$I (Chart 4). When the peaks were examined by autoradiography of SDS:acrylamide gels, Peak 1 contained material in 2 bands with CEA-like mobility (also similar to material eluted from antibody 701-Sepharose 4B), while Peak 2 contained material in a single band with NB/70K-like mobility. Analysis of immune precipitates (collected using the SA assay method) of Peaks 1 and 2 with antibody 701 and anti-NB/70K by autoradiography of SDS:acrylamide gels confirmed that Peak 1 contained only CEA-like material and Peak 2 contained only NB/70K. There was essentially no binding of Peak 1 to anti-NB/70K or of

Chart 4. Sephacryl S-200 elution profile of the iodination mixture of Fraction OCA. The column was 1.6 x 70 cm. Peak 1 was at the void volume, and Peak 2 was at 1.2 times the void volume.
Peak 2 to antibody 701; these immune precipitates contained very little radioactive material, and no bands could be detected on autoradiograms.

Examination of Fraction OCC and Anti-OCC. Fractions OCA and OCC are prepared by Sephadex G-100 chromatography of a partially purified ovarian tumor extract (Table 1). Fraction OCA contains predominantly high-molecular-weight antigens, and the yield of NB/70K from this fraction is therefore not large. On the other hand, Fraction OCC contains predominantly lower-molecular-weight material and would theoretically contain a higher proportion of NB/70K material than does Fraction OCA. Therefore, Fraction OCC was investigated as a possible source of NB/70K.

Iodinated Fraction OCC eluted from Sephacryl S-200 in one large peak, corresponding to Peak 2 obtained with iodinated Fraction OCA. 125I-labeled Fraction OCC and the immune precipitates prepared by the SA method from labeled Fraction OCC and antibody 701, anti-NB/70K, and anti-OCC were examined by autoradiography of SDS:acrylamide gels (Fig. 3). Fraction OCC contained 3 bands of radiolabeled material. All 3 bands had apparent molecular weights less than that of ovalbumin. Only one of these 3 bands was present in immune precipitates prepared using anti-NB/70K. This band runs in the same position as does the band seen in the immune precipitate of OCA Peak 2 with anti-NB/70K; on this basis, it seems to be identical to NB/70K. In addition, this radioactive band migrated just ahead of the leading edge of BSA, as did the unbound fraction obtained by affinity chromatography of 125I-labeled OCA on Antibody 701:

<table>
<thead>
<tr>
<th>Table 1 Preparation of Fractions OCA and OCC from surgical ovarian epithelial tumor tissue</th>
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<tbody>
<tr>
<td>1. Homogenize tissue in 0.9% NaCl solution.</td>
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<tr>
<td>2. Extract with perchloric acid.</td>
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<tr>
<td>3. Chromatograph on concanavalin A-Sepharose 4B. Elute bound material with D-mannose.</td>
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<tr>
<td>4. Chromatograph on Sepharose 4B conjugated to γ-globulin from antibodies against normal ovary and normal serum to remove normal contaminants.</td>
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<tr>
<td>5. Chromatograph on Sephadex G-100. Peak eluting at void volume = Fraction OCA. Peak eluting at 1.3 times the void volume = Fraction OCC.</td>
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Duplication cpm values are typically within 5% of the mean. Binding of 125I-labeled Fraction 2 OCC to anti-NB/70K is approximately 35 to 40%, and nonspecific binding is approximately 8 to 10% of the total cpm added. When data from the standard curve of a typical assay are analyzed using a log-logit plot (Chart 5), a straight line results, with a correlation coefficient of more than 0.98 and S.E. of the estimate of approximately 0.2 unit.

**DISCUSSION**

In the WHO Collaborative Study on Ovarian Antigens, it was demonstrated that Fraction OCA is different from ovarian antigens OvC-1 and OvC-2 (14, 21), OCAA and OCAA-1 (1–3), and TA (6–8) at the limit of detection of rocket immunoelectrophoresis. In this same study, it was shown that Fraction OCA contains CEA-like antigen. This is confirmed by our SDS:gel electrophoresis experiments, which indicate that Fraction OCA contains 2 high-molecular-weight bands of CEA-like material. In addition, Fraction OCA contains 2 other bands. One of these bands has an apparent molecular weight considerably less than that of BSA and binds to Antibody 701: Sepharose 4B but has not been further characterized. The fourth band of Fraction OCA appears to contain only ovarian tumor antigen and has been named NB/70K.

Two other ovarian antigens which were not included in the WHO study may be similar to NB/70K. The unnamed antigen described by Stolbach et al. (22) was purified from malignant effusion fluids of ovarian cancer patients, has an apparent molecular weight of 64,000, and appears to be different from CEA, human chorionic gonadotropin, Regan isoenzyme, and histamine. A second ovarian tumor antigen described by Hollenshead (13) was purified from cancer tissue and is a protein with an apparent molecular weight of 78,000. This antigen does not show cross-reactivity with breast cancer, colon cancer, CEA, normal tissue, various cultured cell lines, or fetal tissue antigens.
NB/70K resembles antigen A which has previously been described and characterized by this laboratory (16, 17). Both NB/70K and antigen A have similar relative mobilities in acrylamide gel, both elute just before BSA on gel filtration, and both have α-like mobility in electrophoresis in barbital-buffered agarose, pH 8.6 (data not presented). Antigen A was found to be present in all of the ovarian epithelial tumor extracts tested by gel filtration and SDS electrophoresis. If NB/70K is indeed antigen A, then NB/70K would be a tumor marker common to serous cystadenocarcinoma, mucinous cystadenocarcinoma, and endometrioid carcinoma. A radioimmunoassay capable of detecting such an antigen in plasma might be useful for early detection of ovarian carcinoma. Further development of the NB/70K assay for use in detecting NB/70K in plasma is currently in progress. When implemented, this assay may be useful not only for the early detection of ovarian cancer but also for the monitoring of treatment of ovarian cancer patients.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 3. Analysis of immune precipitates (SA method) of $^{125}$I-labeled OCC with anti-NB/70K (aNB/70K) and anti-OCC (aOCC) by SDS-acrylamide gel electrophoresis. Molecular weight standards were phosphorylase a (Phos a), BSA, and egg albumin (OA). NSB, nonspecific binding in the absence of antibody. a, Coomassie blue-stained gel. Radioactive ink marks the BPB dye front and the leading edge of the stained bands. b, autoradiograph of the gel shown in a.
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