Detection of a Breast Tissue-associated Antigen by Antiserum to Raji Cell-bound Circulating Immune Complexes of Human Breast Cancer

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ABSTRACT

Rabbits tolerant to human immunoglobulin G were used to raise antisera against the Raji cell-bound circulating immune complexes from human breast cancer sera. After solid-phase adsorption treatment with glutaraldehyde-cross-linked normal human plasma, acetone-extracted normal liver tissue powder, and glutaraldehyde-fixed Raji cells, one antiserum reacted specifically with breast tissue extracts but not with extracts of other tissues, as examined by a counterimmunoelectrophoresis technique. Immunological reactivity of the treated antiserum was removed by incubation with normal, primary, or metastatic breast tumor tissue extracts. Incubation with normal human serum or extracts derived from tissues other than the breast showed no neutralizing effect on the antibodies. This specific antiserum reagent was used in a modification of the Raji cell radioimmunoassay. Raji cells were incubated with sera from cancer patients or normal controls and then reacted with 125I-labeled F(ab')2 fraction of the treated antiserum reagent. The amount of 125I-F(ab')2 bound was then determined. Although all sera exhibited elevated circulating immune complexes by the conventional Raji cell radioimmunoassay, 14 of 18 breast carcinoma sera demonstrated a significant uptake when compared with the normal population group as opposed to five (three lung and two colon) of 29 other cancer sera examined (p < 0.001).

An immunologically reactive breast tissue-associated antigen, purified from malignant breast tumor or normal breast tissue extracts with the use of antiserum reagent, exhibited an apparent molecular weight of 85,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and a pl value of 4.9 ± 0.2. These results demonstrated that a breast tissue-associated antigen rather than a breast tumor-associated neoantigen, was involved in circulating immune complexes of breast cancer patients as detected by Raji cell immunoassay. It also implied the occurrence of disease-related autoimmunity in human breast cancer.

INTRODUCTION

Evidence suggests that tumor cells may elicit humoral and cellular immune responses (4, 14). The resultant antibody response may participate in the formation of antigen-antibody complexes which can play a role in blocking of cell-mediated tumor immunity (2). Such “blocking factors” have been variously identified as soluble CIC (3) of tumor antigen and anti-tumor antibody, tumor antigen alone, or a combination of these factors and have been demonstrated in the sera of cancer patients as well as in animals bearing tumors (3, 7, 12, 15, 23, 26). It has been shown that some breast cancer patients produce high levels of CIC, and these may represent the formation of antitumor antibodies with tumor-associated antigens (16). It would appear, therefore, that the isolation of CIC from cancer patients would yield information regarding the nature of these antigen(s). Furthermore, once antigen components of CIC are isolated and characterized, it would allow for the development of specific techniques that could be useful in the immunodiagnosis of cancer.

It was reported recently that Raji cells can be used to detect CIC as well as to isolate such complexes from the sera of cancer patients (29, 30). In this study, we describe the utilization of Raji cells to isolate antigen-antibody complexes from the serum of breast cancer patients and the subsequent production of heteroantisera against the Raji cell-bound CIC in rabbits tolerant to human IgG. We have used this specific antiserum to monitor the isolation of the immunoreactive antigen from breast tissue. Initial results obtained from a modification of the Raji cell radioimmunoassay using the raised heteroantisera indicate that these methodologies have the potential to generate reagents for development of antigen-specific immune complex assays.

MATERIALS AND METHODS

Serum and Tissue Specimens. Serum samples were obtained from patients who had histologically confirmed cancer of the breast, colon, prostate, and lung. Pleural effusions were obtained from breast cancer patients. Normal sera were obtained from healthy laboratory personnel. All sera were stored frozen (−80°) and thawed immediately before use. Normal tissue and primary as well as metastatic tumor tissues were collected from surgery or from necropsy within 12 hr of death. All tumor tissues obtained were identified histopathologically. Tissue extracts used in this study were prepared by homogenizing tissue in 4 volumes (w/v) of cold PB-NaCl buffer with a Sorvall Omnimixer followed by centrifugation at 10,000 X g for 30 min. The lipid surface layer and precipitate pellet were discarded, and the supernatant was dialyzed extensively against distilled water at 4° and lyophilized. All tissue extracts were then redissolved with PB-NaCl buffer to a protein concentration of 15 mg/ml.

Raji Cells. Raji cells, a human lymphoblastoid cell line derived from a patient with Burkitt’s lymphoma (24), were kindly provided by Dr. Jun Minowada of this Institute. The cells were cultured as stationary suspensions in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% heat inactivated (56°, 30 min) fetal bovine serum (Grand Island focusing; Con A, concanavalin A; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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1 This work was supported in part by Research Grant CA-25653, awarded by the National Cancer Institute, Department of Health, Education, and Welfare.  2 To whom requests for reprints should be addressed.  3 The abbreviations used are: CIC, circulating immune complexes(s); PB-NaCl, 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.2; NHS, normal human serum; IEF, immunoelectrophoresis; TTB, 0.08 M Tris:0.024 M tricine:0.025 M sodium barbital:0.3 mM calcium lactate:0.02% sodium azide, pH 8.8; CEA, carcinoembryonic antigen, MEM, minimal essential medium; IEF, isoelectric
Biological Co., Grand Island, N. Y.), penicillin, and streptomycin. Cell viability was assessed by trypan blue exclusion and was always greater than 95%. The receptor characteristics by which Raji cells selectively bind CIC have been described by Theofilopoulos et al. (29, 31). Raji cells were always used 72 hr after culture inoculation (32).

Induction of Tolerance. IgG was isolated from a pool of NHS by 33% saturated ammonium sulfate precipitation and a DEAE-Sephasel anion-exchange chromatography with elution of 0.02 m phosphate buffer, pH 7.2 (18). Purity was assessed by IEP against anti-human whole serum. The purified IgG solution was centrifuged at 105,000 × g for 90 min using a Beckman SW60 Ti rotor. The top two-thirds of the supernatant in each centrifuge tube were carefully withdrawn and considered ultracentrifugally homogeneous 7S γ-globulin (5). New Zealand White rabbits were rendered tolerant to IgG by i.v. injections with 1 ml (10 mg/ml) of 7S γ-globulin in 0.9% NaCl solution (5). The toleration procedure was repeated every 2 weeks for a period of 8 weeks. Seven days after the last injection, tolerance was confirmed when no immunoprecipitate was observed by staining after IEP analysis of the rabbit serum against NHS and human IgG.

Production of Antisera against Breast Tissue-associated CIC. The Raji cell radioimmunoassay, as described by Theofilopoulos et al. (32), was performed on serum specimens from 30 patients and 6 pleural effusions to detect elevated CIC levels (32). Sera or pleural effusions exhibiting CIC levels greater than 250 μg aggregated human IgG equivalent per ml were utilized for the production of heteroantisera. Raji cells, 5 × 10^5, were incubated at 37°C for 30 min with either 400 μl of serum from a breast cancer patient or 600 μl of pleural effusion from a breast cancer patient. Thereafter, cells were washed 8 times with 0.9% NaCl solution, resuspended in 1 ml 0.9% NaCl solution, and injected i.v. into mature rabbits previously made tolerant to human IgG. Ten mg of 7S γ-globulin were given i.v. at the time of immunization with Raji cell-bound CIC (30). The procedure was repeated every 2 weeks for a period of 10 weeks. Rabbits were bled 7 days after the last immunization.

Treatment of Antiserum. In order to remove antibodies to normal serum components and tissues other than the breast, antisera raised against breast tissue-associated CIC were sequentially treated with an equal volume of glutaraldehyde-cross-linked normal human plasma and mixed with acetone-extracted normal liver tissue powder and glutaraldehyde-fixed Raji cells (1). The mixture was allowed to incubate for 12 hr at 4°C followed by centrifugation and recovery of the supernatant. This procedure was repeated until immunological reactivity of the antisera to normal serum components and tissues other than breast tissue was removed as determined by counter immunoelectrophoresis.

The IgG fraction of the treated antisera was then obtained by 33% ammonium sulfate precipitation and DEAE anion-exchange chromatography as described previously (18). For the preparation of immunoglobulin fragments, a sample of IgG (prepared from Antiserum Reagent 58) was dissolved in 0.01 M sodium acetate buffer, pH 4.0. For 50 mg of IgG, 1 mg of crystallized pepsin (Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was incubated overnight at 37°C. F(ab')2 fragments were separated from unreacted IgG and from smaller peptides by gel filtration over Sephacryl S-300 medium (Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated in PBS-NaCl buffer. The column (2.5 x 70 cm) was calibrated by the chromatography of molecular weight standards, including IgG, bovine serum albumin, and egg albumin (Pharmacia). The solution of F(ab')2 fragments was concentrated by ultrafiltration over a PM-10 membrane (Amicon, Lexington, Mass.) with positive pressure. Trace labeling of F(ab')2 fragments with 125I (New England Nuclear, Boston, Mass.) was accomplished by the method of McConahey and Dixon (22). Unreacted iodide was separated from labeled protein by gel filtration over Sephadex G-25 (PD-10; Pharmacia). Labeled protein was completely precipitable with 25% trichloroacetic acid.

Immunoprecipitation Methods. Counterimmunoelectrophoresis was carried out on cellulose acetate membranes (BioWare, Inc., Wichita, Kans.) using 1% agarose (high electroendoosmosis; Bio-Rad) in TTB buffer as described previously (8). Briefly, 10-μl samples of the IgG fraction of treated Antiserum Reagent 58 (3 mg IgG per ml, w/v) and various tissue extracts and proteins were applied to circular wells (3 mm) and electrophoresed at 40 mA constant current until the tracking dye (0.1% bromophenol blue) migrated 3 cm anodally. Gels were then washed thoroughly in 0.9% NaCl solution and distilled water, press dried, and then stained for protein with Coomassie Blue R-250 (8). A reaction was considered positive when, after staining, an immunoprecipitate was observed between the wells (sensitivity, 0.5 μg of antigen per ml). Each tissue extract and sample examined was electrophoresed against treated Antiserum Reagent 58 (IgG fraction) and against rabbit normal serum and PB-NaCl buffer controls. Purified casein was purchased from Calbiochem-Behring Corp. (La Jolla, Calif.), and hemoglobin, fibrinogen, lactalbumin, transferrin, and α1-antitrypsin were purchased from Sigma.

Reactivity of treated Antiserum Reagent 58 (IgG) to CEA was determined by incubation of Reagent 58 (IgG) with 125I-CEA (Hoffman-La Roche CEA kit) and then precipitation with Panisorbin (Calbiochem). The mixture was washed twice with PB-NaCl buffer, and radioactivity uptake was then determined in a Packard Auto-Gamma scintillation spectrometer.

Rocket IEP, using treated Antiserum Reagent 58, was used to monitor the purification of the reactive breast tissue antigen. Rocket IEP was performed on cellulose acetate membranes (BioWare) using 0.83% agarose (low electroendoosmosis; Sigma) in TTB buffer. Treated Antiserum Reagent 58 at a final concentration of 5% was incorporated into the agarose at 56°C prior to plating (33). Samples were applied to circular wells (5 mm) and electrophoresed at 165 V for 2 hr at 4°C using TTB as running buffer (sensitivity, 0.5 μg of antigen per ml). Gels were then washed extensively in 0.9% NaCl solution and distilled water, press dried, and then stained for protein with Coomassie Blue R-250 (8).

The effect of various absorption treatments on the immunological reactivity of Antiserum 58 was determined in the following manner. Aliquots of treated Antiserum 58 (100 μl) were incubated at 37°C for 1 hr with 5 mg of each of the following lyophilized tissue extracts: normal breast; fibrocystic breast; primary breast tumor; metastatic breast tumor; normal lung; lung tumor; normal prostate; prostate tumor; normal pancreas; and pancreas tumor. Following incubation, the mixtures were centrifuged and then tested for reactivity by rocket IEP against normal breast tissue and breast tumor extracts as described above.

Antisera against human albumin, α1-antichymotrypsin, α-2-
acid glycoprotein, &alpha;-antitrypsin, Gc-globulin, C-reactive protein, C1s inactivator, C3 activator, C4, and plasminogen were obtained from Calbiochem-Behring Corp.

Modification of Raji Cell Radioimmunoassay. To further characterize the specificity of treated Antiserum Reagent 58 (IgG), a modification of the Raji cell radioimmunoassay was developed to determine the circulating antigen(s) complexed with antibody bound on Raji cells. Raji cells (4 x 10^5), in triplicate, were incubated at 37° for 45 min with 50 &mu;l of the test serum diluted 1:4 in 0.9% NaCl solution. After 3 washes in MEM, 50 &mu;l of 125I-labeled Fab fragment preparation from the treated Antiserum Reagent 58 diluted 1:2 in MEM containing 1% human serum albumin were added to all tubes, and incubation was continued with gentle shaking at 4° for 30 min. The cell pellets were then washed 3 times with MEM containing 1% human serum albumin, and the radioactivity in the cell pellet was determined in an Auto-Gamma spectrometer (Packard). The amount of Raji cell-bound 125I-F(ab')2 was determined. The uptake of 125I-F(ab')2 by Raji cells incubated with serum specimens from the breast cancer group was compared with that of the other cancer groups as well as the normal control group. The serum of each patient that was used in the study previously had been shown to contain CIC, 40 &mu;g aggregated human IgG equivalent per ml or greater, as determined by the Raji cell radioimmunoassay (32) and by the polyethylene glycol:C1q immunodiffusion test described by Grangeot-Keros et al. (11). Controls included cells incubated in normal rabbit serum, NHS, or medium (MEM) alone.

Isolation of Breast Antigen. Rocket IEP with the treated antiserum 58 was used to monitor the presence of the immunologically reactive breast antigen throughout the purification procedure. Pooled normal or primary and metastatic breast tissue, 100 g, was extracted with PB-NaCI buffer (4°) as described previously in “Materials and Methods.” Ammonium sulfate fractionation was carried out in 4 successive steps (0 to 20, 20 to 40, 40 to 60, and 60 to 80% saturations) by the addition of the required amount of solid salt and then stirred at 4° for 1 hr. The resultant precipitate in each step obtained by centrifugation at 10,000 x g was dissolved in 0.02 M Tris-HCl buffer, pH 8.0, and residual ammonium sulfate was removed by thorough dialysis at 4° against Tris-HCl buffer. The dialyzed solution was clarified by centrifugation at 36,000 x g for 30 min to remove any precipitate formed during dialysis.

Preparative flat-bed IEF was then carried out on the dialyzed solution that fractionated at 60 to 80% saturated ammonium sulfate. IEF was performed according to the manufacturer’s recommendation, as described previously (20). Briefly, 5 ml of Ampholyte (pH 3 to 10; Bio-Rad, Richmond, Calif.) plus 30 ml of dialyzed solution were mixed with 65 ml of water. To this solution, 5 g of IEF Sephadex (Pharmacia) were added. The mixture was allowed to swell for 30 min, stirred, and then degassed. The gel suspension was then poured into the template (11 x 24 cm). IEF was performed using a Buchler Model 1500 power unit set at 2 watts, constant power, for 40 hr at 4°. A 30-zone fractionating grid was pressed into the gel bed at the cessation of the run. The pH gradient was determined using Bio-Lyte Pro-Philer (Bio-Rad) and a Fisher Accumet Model 140 pH meter. Each zone of isofocused materials was collected and separated from the gel media by filtration through Whatman No. 1 filter paper by applying 20 ml of Tris-HCl buffer to the gel. Each fraction obtained (20 ml) was then dialyzed extensively at 4° against 0.1 m acetate: 0.5 m NaCl buffer, pH 6.0, containing 0.1 m each of MgCl2, MnCl2, and CaCl2 (acetate buffer). Immunologically reactive protein(s) as determined by rocket IEP were found to be contained in the isofo- cused fractions exhibiting pi values from 4.7 to 5.2.

The fractions which contained the immunologically reactive protein(s) were pooled and concentrated by ultrafiltration over a PM-10 membrane (Amicon) using positive pressure and applied to a Con A-Sepharose 4B (Pharmacia) column (2 x 13 cm) equilibrated with acetate buffer. Con A nonbinding proteins were washed through the column with acetate buffer until the monitored absorbance at 280 nm reached base line. Con A-binding material containing the reactive antigen(s) was then eluted with 0.1 m &alpha;-methyl-D-mannoside (Sigma) in acetate buffer. After dialysis, the concentrated solution was further purified by application to a column (2 x 13 cm) packed with DEAE-Sephadex (Pharmacia) and equilibrated in Tris-HCl buffer. Nonbinding proteins were washed through the column until the absorbance at 280 nm of the fractions reached base line. DEAE-bound proteins were eluted in a step-wise fashion with 0.1 to 0.5 m NaCl in Tris-HCl buffer. Immunologically reactive protein(s) were located in the fractions which eluted with 0.2 m NaCl in Tris-HCl buffer.

Further purification of the immunoreactive protein(s) was accomplished by the use of hydrophobic interaction chromatography. The sample was applied to a column (2 x 13 cm) packed with Phenyl-Sepharose CL-4B (Pharmacia) equilibrated with start buffer (0.02 m phosphate buffer, pH 6.8, containing 25% saturated ammonium sulfate). Elution was con- tinued with start buffer until the absorbance at 280 nm reached base line. The column was then eluted stepwise with start buffer containing decreasing concentrations of ammonium sulfate while simultaneously increasing the concentration of ethylene glycol. The final concentrations, expressed as percentages of ammonium sulfate and ethylene glycol in start buffer, used for each step elution were as follows: 20:10; 15:20; 10: 30; and 5:40. The fractions obtained between each elution step were pooled, concentrated, and dialyzed extensively at 4° against Tris-HCl buffer prior to further purification by an affinity chromatography of anti-human transferrin antibodies.

Anti-human transferrin antiserum (IgG fraction) and Affi-Gel 10 were purchased from Bio-Rad. Conjugation of the IgG to Affi-Gel was performed according to the manufacturer’s procedure. Anti-human transferrin IgG conjugated to Affi-Gel 10 was then packed into a column (0.7 x 10 cm) equilibrated with running buffer (0.5 m phosphate buffer: 0.5 m NaCl, pH 7.2). Sample was applied, and the flow-through fractions were collected, pooled, and concentrated for further analysis. The bound protein was eluted with 3.5 m KSCN, dialyzed immediately against water, and concentrated also.

SDS-PAGE. SDS-PAGE was performed under reducing conditions as described previously (20). In addition, SDS-PAGE was performed under nonreducing conditions. Briefly, 25 &mu;g of purified antigen preparation were electrophoresed using a constant current of 4 mA/tube until the tracking dye (bromphenol blue) migrated to within 5 mm of the end of the gels. Marker proteins used in these experiments and their molecular weights were: phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; and &alpha;-lactalbumin, 14,400 (Pharmacia). All gels were stained with Coomassie Brilliant Blue G as de-
scribed previously (8).

**Protein Determination.** Determination of total protein was performed using the Bio-Rad no. 500-001 protein assay.

**Statistical Analysis.** Comparisons between patient groups were computed using Student’s t test; if a p value was less than 0.05, the difference in values between groups was assessed as statistically significant.

**RESULTS**

**Production of Antiserum against Antigen(s) in CIC of a Breast Cancer Patient.** Nine rabbits were successfully rendered tolerant to 7S γ-globulin as determined by IEP analysis of the rabbit sera against NHS and human IgG. These rabbits received injections of intact Raji cells bearing CIC from serum of a breast cancer patient (4 rabbits) or pleural effusion (4 rabbits). One control rabbit was given an injection of Raji cells incubated in MEM alone. Subsequent to several immunizations, antibodies to NHS components and various tissue extracts were found to be present in the serum from 6 of 9 rabbits, while Antisera 57, 58, 60, 61, and 64, from 5 rabbits, demonstrated immunological reactivity to breast tissue extracts, as shown by counterimmunoelectrophoresis (Table 1). These 5 antisera were sequentially treated with glutaraldehyde-cross-linked normal human plasma, acetone-precipitated normal liver extracts, and glutaraldehyde-fixed Raji cells to remove antibodies directed against normal serum components and other proteins not related to breast tissue. After these treatments, only Antiserum Reagent 58 retained immunological reactivity with breast tissue extracts, including breast tumor tissue extract.

**Table 1**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>NHS</th>
<th>Pleural effusion</th>
<th>Breast carcinoma</th>
<th>Breast</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
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<tbody>
<tr>
<td>57</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>58</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td>NR</td>
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</tr>
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<td>+</td>
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</table>

* Pleural effusion from the same breast cancer patient was used for the production of Antiserum 61 to 65.

**Modification of Raji Cell Radioimmunoassay.** Sera containing CIC from patients with carcinoma of the breast, lung, colon, and prostate were used to further demonstrate the specificity of treated Antiserum 58. F(ab')2 fragments prepared from the treated antiserum were used in these experiments. Raji cells were incubated with sera which had been shown to contain elevated levels of CIC from 47 patients and from 12 apparently normal healthy individuals, and the cells then reacted with 125I-labeled F(ab')2 of Antiserum Reagent 58. Levels of 125I-F(ab')2 bound to the Raji cells were determined. When compared with the normal group (Chart 1, mean + 2 S.D.), 14 of 18 (77%) of the breast cancer group and 5 of 29 (3 male patients with lung cancer and 2 female patients with colon cancer) of the other cancer group demonstrated an increased uptake. The difference between the means of radioactivity uptake from the breast cancer group and the other cancer group was significant at the p < 0.001 level as determined by Student's t test.

**Purification of the Immunoreactive Protein.** Rocket IEP with the use of Antiserum Reagent 58 was performed to monitor the purification of the immunologically reactive protein from either pooled malignant breast or normal breast tissue extracts. Upon ammonium sulfate fractionation of phosphate-buffered saline tissue extract, the precipitate fractionated at 60 to 80% saturation was found to react with Antiserum Reagent 58. This fraction was then applied to a preparative IEF using a pH gradient of 4.7 to 5.2 were found to contain the immunoreactive protein(s). A Con A-Sepharose affinity chromatography (Chart 2A) was then used to separate the immunoreactive substance from other proteins, as an initial experiment indicated its binding to Con A. The Con A-bound material, after elution, was applied to a DEAE-Sephadex anion-exchange column for additional purification. As shown in Chart 2B, the eluate with 0.2 M NaCl reacted with Antiserum 58. This fraction was further purified by application to a hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B (Chart 2C) which resulted in the separation of 5 protein fractions. The fraction which eluted with 0.02 M phosphate buffer, pH 6.8, containing 15% ammonium sulfate and 20% ethylene glycol, was shown to react with Antiserum 58. SDS-PAGE of this fraction revealed, with and without the nonreducing agent, the presence of 2 protein bands which was derived from the same patient whose serum CIC were used for the production of this antiserum (Table 1).

In order to demonstrate the immunological specificity of treated Antiserum Reagent 58, a panel of extracts of various normal and cancerous tissues was examined by counterimmunoelectrophoresis (Table 2). Only breast tissue extracts reacted with this antiserum, including: 5 of 8 normal; 2 of 3 fibrocystic; 13 of 18 primary breast carcinomas; 3 of 4 breast carcinoma metastases to the axilla; and 2 of 3 metastases to the liver. None of the other normal tissue extracts or none of the extracts derived from malignant prostate, lung, colon, pancreas, and lymphoma tissue was found to react (Table 2) at the sensitivity level (0.5 μg/ml) of this counterimmunoelectrophoresis described. Immunological reactivity of the treated Antiserum 58 to breast tissue extracts was completely removed by incubation with extracts derived from normal breast or cancerous breast tissue. Incubation with tissue extracts derived from nonbreast origin or with a pool of NHS showed no neutralizing effect on the immunological reactivity to breast tissue extracts by Antiserum 58.

**Table 2**

<table>
<thead>
<tr>
<th>Tissue extracts</th>
<th>Antiserum 57</th>
<th>Antiserum 58</th>
<th>Antiserum 60</th>
<th>Antiserum 61</th>
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<td>Cancer tissue</td>
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<td>++</td>
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<td>Liver</td>
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<td>NR</td>
<td>NR</td>
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<tr>
<td>Spleen</td>
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<tr>
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</tbody>
</table>

* Breast carcinoma tissue extracts

**Statistical Analysis.** Comparisons between patient groups were computed using Student’s t test; if a p value was less than 0.05, the difference in values between groups was assessed as statistically significant.
Table 2

<table>
<thead>
<tr>
<th>Tissue extract (normal)</th>
<th>Reactivity</th>
<th>Tissue extract (cancerous)</th>
<th>Reactivity</th>
<th>Other proteins and body fluids</th>
<th>Reactivity</th>
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<tbody>
<tr>
<td>Breast</td>
<td>5/8</td>
<td>Breast</td>
<td>13/18</td>
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<tr>
<td>Breast (fibrocystic)</td>
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<td>2/3</td>
<td>Amniotic fluid</td>
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<td>Breast (metastatic axilla)</td>
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<td>Transferrin</td>
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a All lyophilized tissue extracts and proteins listed were adjusted to a final concentration of 15 mg protein per ml for testing.

b Same criteria as in Table 1.

c Others included one each of the following: kidney; intestine; bladder; urethra; heart; brain; testes; pancreas; spinal cord; and muscle.

d Radioimmunoprecipitation as defined under "Materials and Methods."

with molecular weights of 85,000 and 76,000, respectively (Fig. 1). Rocket IEP using antisera to whole human serum indicated coisolation of a single normal serum component in the fraction. After an extensive analysis with antisera to individual normal serum proteins, the contaminant protein was identified to be transferrin, since only anti-human transferrin antisera showed an immunological reaction with the fraction. None of the following antisera, among others, examined by rocket IEP yielded a positive reaction: human serum albumin; α1-antitrypsin; Gc-globulin; C-reactive proteins; complement components C1s, C3, and C4; and plasminogen. Transferrin, a normal serum component, has a molecular weight of 76,500 (21). This contaminant protein in the fraction which eluted with 15% ammonium sulfate:20% ethylene glycol was then removed by an antibody affinity chromatography step using anti-human transferrin IgG coupled to Affi-Gel 10. Examination of this

Chart 1. A modified Raji cell radioimmunoassay using the F(\(\text{ab}'\))\(_2\) of treated Antiserum 58. Binding of \(^{125}\text{I}-\text{F(\(\text{ab}'\))}\(_2\) fragments to Raji cell-bound components obtained from sera of various cancer patients and normal controls. The other cancer (CA) group comprised of sera from 12 lung, 6 colon, and 11 prostate cancer patients. Points, mean; bars, S.D. in each group. , 2 S.D.'s above the mean of the normal group. Comparisons between patient groups were computed using Student's \(t\) test (breast cancer versus other cancer, \(p < 0.001\); breast cancer versus control, \(p < 0.001\); breast cancer versus other cancer, \(p < 0.001\); breast cancer versus control, \(p < 0.001\)).

Chart 2. Protein profiles of sequential chromatographic steps in the purification of immunoreactive breast antigen. A, Con A affinity chromatography of the sample obtained from a preparative flat-bed IEF (see "Materials and Methods"). Sample was applied to a column (2 x 13 cm) of Con A-Sepharose and eluted stepwise with 0.0 to 0.1 M α-methyl-D-mannoside in acetate buffer, pH 6.0, as indicated by the arrow. Eluted fractions were monitored for protein by absorbance at 280 nm. Fractions containing the immunoreactive proteins, as indicated by the shaded protein peak, were pooled, concentrated, and used for subsequent purification by DEAE-Sepharose and Phenyl-Sepharose CL-4B. Each fraction collected contained a volume of 3.5 ml. B, DEAE-Sepharose anion-exchange chromatography. Sample was applied to a column (2 x 13 cm) of DEAE-Sepharose and eluted stepwise with 0.0 to 0.2 M NaCl in Tris-HCl buffer as indicated by the arrow. Each fraction collected contained a volume of 3.5 ml. C, Phenyl-Sepharose CL-4B hydrophobic interaction chromatography. Sample was applied in start buffer (0.02 M phosphate buffer, pH 6.8, containing 25% saturated ammonium sulfate) and then eluted in stepwise fashion with start buffer (0.02 M phosphate buffer, pH 6.8) containing different final concentrations of ammonium sulfate and ethylene glycol (EG) as indicated. Total volume in each fraction collected was 2.0 ml.
affinity-purified material (flow-through fraction) on SDS-PAGE demonstrated a single protein band with a molecular weight of 85,000 (Fig. 1) and on rocket IEP revealed its reaction with Antiserum 58. The final yield of purified breast tissue antigen preparation was approximately 100 and 150 µg from 100 g each of normal and benign tumor breast tissues, respectively. This protein did not react with anti-human transferrin antiserum, but the bound and eluted contaminant from the affinity column was shown to be immunoreactive on rocket IEP with anti-transferrin antiserum and not immunoreactive with Antiserum 58.

DISCUSSION

In searching for tumor-associated antigens, various biochemical approaches to the isolation of antigens have been reported. Conventionally, tissue is initially homogenized in PB-NaCl, 3 mM KCl, or perchloric acid in order to isolate the antigen(s) (17, 19). However, until recently, not much attention has been focused on isolating tumor-associated antigen(s) from CIC. Raji cells have been established successfully to detect complement-fixing CIC in a variety of malignant diseases (32). Apart from detection of CIC, Raji cells also have been utilized to isolate CIC in model systems, with subsequent isolation of the constituent antigens and antibodies (30). In the presence of an ideal milieu used in the model system, production of heteroantiserum to bovine serum albumin:antialbumin complexes bound via complement receptors to Raji cells has been reported (30). In this study with biological fluid, we describe the production of heteroantiserum raised to soluble CIC of breast cancer patients as bound to Raji cell surface and the isolation of a protein immunologically reactive with the antiserum.

Although the receptor characteristics of Raji cells which selectively bind the complement-fixing CIC have been described thoroughly (29, 31), a recent report by Galbraith et al. (10) demonstrates the presence of transferrin and albumin receptors on Raji cells. The presence of these receptors and receptors for possibly other yet unknown normal tissue antigens may explain the production of antibody to NHS or other normal tissue constituents (Table 1), since both transferrin and albumin are found in sera and tissues. The observation that transferrin is detected in the same fraction as the purified breast tissue antigen represents just a consequence of co-isolation physicochemically of these 2 proteins, as Antiserum 58 has been treated with normal serum and tissue extract. Of particular interest in this study is the observation that, after an exhaustive solid phase absorption treatment with various tissue extracts and NHS in order to remove antibodies to proteins not associated with breast tissue, Antiserum 58 still possesses an immunological reactivity, at the sensitivity level of techniques used, with tissue extracts of breast origin including normal breast tissue, primary breast carcinoma, and secondary metastases to the nodes and the liver (Table 2).

Several investigators have used CIC assays in an attempt to correlate the levels of CIC with the activity of neoplastic disease as reviewed recently by Theofilopoulos and Dixon (28). Using an assay based upon the binding of 125I-C1q to CIC, Rossen et al. (25) have found high C1q binding values most frequently in cancer patients with evidence of disease prior to therapy as compared to patients without evidence of disease. It was shown previously by Theofilopoulos et al. (27) that an increase in tumor mass and metastatic disease was associated with high levels of CIC in cancer patients as determined by the Raji cell radioimmunoassay. However, because CIC assays rely on the detection of altered physicochemical and biological properties of antigen-complexed immunoglobulin, they are antigen non-specific (28). In this report, we describe how Raji cells incubated with serum exhibiting CIC of a breast cancer patient can be utilized to raise a heteroantiserum, which contains an antibody fraction specific for a breast tissue component. Furthermore, data are presented to demonstrate the potential usefulness of the heteroantiserum in the development of a possible antigen-specific CIC assay. By using a modification of the Raji cell radioimmunoassay, an increased uptake of 125I-F(ab')2 fragments of the antiserum has been detected in a significant number, 14 of 18, of breast carcinoma sera in comparison with 5 of 29 of the other cancer sera examined (Chart 1). These data suggest that the antiserum reagent is reacting with a complexed antigen(s) bound to the Raji cells.

In order to identify the immunologically reactive breast antigen(s), the specific Antiserum 58 has been utilized to monitor the purification of the antigen from breast tissue extracts. A
protein with a molecular weight of 85,000, identified as the immunoreactive antigen, with no apparent subunit structure and exhibiting a pl value of 4.9 ± 0.2 has been isolated. Although this antigen is breast tissue specific as determined at the sensitivity level of techniques utilized, it cannot be ruled out at present that the antigen is found in significantly higher levels in breast tissues relative to other tissues examined. Also, it should be noted that the antigen recognized by specific Antiserum 58 appears not to be a breast tumor-associated neoantigen and that its presence may be related to disease-associated autoimmunity. In short, observation of a tumor-associated formation of autoantibodies has been presented. This finding is not surprising, since in other tumor systems, such as in patients with oat cell carcinoma of the lung, immune complexes have been found to contain an adenocorticotropin hormone-like protein, and no tumor-associated antigens could be demonstrated (13).

In both humans and experimental animals, the existence of antigens associated with breast cancer has been reported. However, the approaches taken to identify these antigens are much different from our own. Leung et al. (19) identified a candidate breast tumor-specific antigen in the perchoric acid-soluble glycoprotein fraction of human ductal carcinoma of the breast after removal of known glycoproteins by affinity chromatography. This breast tumor-specific glycoprotein has an apparent molecular weight of 19,500 and a pl value of 5.35. Kamiyama et al. (17) have isolated a breast tumor antigen from detergent and hypertonic salt extractions of breast adenocarcinoma. The presence of this glycoprotein (M.W. 67,000) antigen was monitored by polyacrylamide gel electrophoresis and found only in adenocarcinoma of the breast. Other investigators have examined human milk for the presence of breast antigens. Dion et al. (9) have demonstrated a human milk glycoprotein related to the major envelope protein of murine mammary tumor virus which has a molecular weight of 55,000. Human milk also has been utilized as the source for surface differentiation antigens of human mammary epithelial cells carried on the human milk fat globule as reported by Ceriani et al. (6). Rabbit antibodies raised against components of the human milk fat globule were shown by immunofluorescence studies to be specific for human breast epithelial cells; however, the antigen(s) responsible for eliciting the antibody production have yet to be isolated and fully characterized (6). On the basis of this information, the breast tissue antigen described presently appears to be distinct from other reported breast tumor-associated antigens. Further characterization of this antigen and application of the specific antiserum in immunodiagnosis and treatment of breast cancer are under way.

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References

Breast Tissue-associated Antigen


Detection of a Breast Tissue-associated Antigen by Antiserum to Raji Cell-bound Circulating Immune Complexes of Human Breast Cancer
