Serum Levels and Biochemical Characteristics of Cancer-associated Antigen CA-549, a Circulating Breast Cancer Marker

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ABSTRACT

CA-549 is a circulating breast cancer-associated antigen that reacts with monoclonal antibody BC4E 549. Biochemical characterization of CA-549 revealed that it is an acidic (isoelectric point 5.2) glycoprotein that exhibits two bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions of apparent molecular weights of 400,000 and 512,000. Immunohistological staining of unfixed frozen tissue sections of human breast tumors and a variety of benign tissues with CA-549 revealed no preferential staining of tumor over benign breast tissue and cross-reactivity with a wide range of other benign tissues including kidney, liver, lung, colon, pancreas, ovary, and spleen.

Serum levels of CA-549 were initially tested by an enzyme-linked immunosorbent assay inhibition using BC4E 549. This assay showed that CA-549 concentrations were elevated in 19 of 27 sera from patients with advanced breast cancer compared to 0 of 22 and 0 of 129 sera from benign breast disease patients and healthy females, respectively. These preliminary data suggested that CA-549 was a useful breast tumor marker; thus BC4E 549 was adapted to a sandwich immunoradiometric assay format suitable for routine use in the clinical laboratory and its performance was evaluated on a panel of 668 serum samples. The test detected significant concentrations of CA-549 in the sera of 40 of 80 patients with advanced breast cancer, 1 of 30 with early breast cancer, 4 of 19 with advanced lung cancer, 2 of 40 with advanced colon cancer, and 5 of 29 with advanced prostate cancer. The test showed a high degree of specificity, producing false-positives in only 3 of 79 benign breast patients, 2 of 25 benign liver patients, 2 of 70 benign colon patients, 2 of 19 benign lung patients, 0 of 20 benign prostate patients, and 3 of 257 healthy individuals. These data represent an overall 50% sensitivity and 98% specificity as a test for advanced breast cancer. These data indicate that this immunoradiometric assay is a useful test for the detection of circulating CA-549 in advanced breast cancer patients and suggest that it may prove useful as a monitor in the management of that disease.

INTRODUCTION

The ability to diagnose and monitor various cancers by means of simple in vitro tests is potentially a powerful tool in the proper management of these conditions. One such test, that for carcinoembryonic antigen, has proven to be an effective monitor for colorectal cancer (1). Carcinoembryonic antigen is also commonly used to monitor breast cancer patients in spite of its relatively low sensitivity and specificity for that disease (2). Thus, there is currently a need for a more reliable breast cancer marker.

The advent of monoclonal antibody technology has provided investigators with new approaches in both the therapy and diagnosis of breast cancer (3). Monoclonal antibodies specific for breast tumor-associated antigens have been widely reported (4–8). One group of antigens of particular interest is the heterogeneous mixture of antigens isolated from human milk, termed milk fat globule membrane antigens (9–13). Antibodies to both breast tumor cell line membranes and human milk fat globule membranes were prepared and tested. Two of these antibodies, BC4E 549 and BC4N 154, were used together in a monoclonal immunoradiometric sandwich format to produce a very specific serum test for advanced breast cancer. The antigen recognized by this immunosassay is present in MFGM and tumor cytosol and has been partially characterized biochemically.

MATERIALS AND METHODS

Monoclonal Antibodies. Monoclonal antibodies were prepared by fusion of mouse myeloma cells (21) with spleen cells from immunized BALB/c mice according to a modification of the method of Kohler and Milstein (22). BC4E 549, a murine IgG1, was derived from a fusion using splenocytes from a mouse immunized with purified membranes from the T417 human breast tumor cell line grown in nude mice. BC4N 154, a murine IgM, was produced in a fusion using splenocytes from a mouse immunized with human milk fat globule membranes prepared as previously described (9). Antibodies were initially screened by ELISA for their ability to preferentially bind cytosol preparations of human breast tumors compared to normal breast tissue cytosol. Cytosols were prepared by homogenizing tumor in approximately 10 volumes of 100 mM Tris-2 mM CaCl₂, pH 7.2, buffer with 2 mM phenylmethylsulfonyl fluoride. Homogenate was first given a low speed 3000 RPM centrifugation for 5 min to remove large debris followed by a high speed spin at 100,000 × g for 1 h at 4°C to pellet cell membranes. The supernatant was collected for use as cytosol. The antibodies were then further screened by testing them for preferential inhibition of their ELISA signal by sera from patients with breast cancer compared to sera from patients with benign breast disease. Briefly, sera were diluted 1:3 in 0.16 M phosphate-0.15 M NaCl (PBS), pH 7.4, and preincubated for 1 h at room temperature with a equal volume of optimally diluted, antibody-containing culture supernatant. Seventy-five μl of this mixture were transferred to a microtiter well that had been precoated with optimally diluted breast tumor cytosol and incubated for 2 h at room temperature and assayed on an automated ELISA reader. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used in this paper are: MFGM, milk fat globule membrane; CA, cancer-associated antigen; ELISA, enzyme-linked immunosorbent assay; IRMA, immunoradiometric assay; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
The microtiter plate was then washed with water followed by the addition of an optimized amount of horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Tago, Burlingame, CA). After 1 h incubation at room temperature the plates were washed and the assay color developed by addition of 100 µl 0.03% H₂O₂ plus 0.1% o-phenylenediamine-2HCl (Sigma Chemical Co., St. Louis, MO) in 0.1 M phosphate-0.05 M citrate-0.15 M NaCl buffer, pH 5.0. The color was developed for 30 min at room temperature and then quenched with 50 µl of 4 N H₂SO₄. The ELISA signals were read spectrophotometrically at 490 nm. Results were expressed in terms of percentage of inhibition of ELISA signal.

Clinical Specimens. For preliminary ELISA studies a total of 271 sera from healthy females, advanced cancer patients, and benign disease patients were obtained from the University of California, San Diego Cancer Center. All the breast cancer patients had active metastatic disease (no site data available). The patients with other cancers were all stage 3 or stage 4 metastatic disease. The lung cancer patients comprised 11 cases of epidermoid cancer, 10 adenocarcinoma, and 6 small cell lung cancer. All the benign breast patients had fibrocystic breast disease. The benign lung patients were an approximately equal mixture of cystic fibrosis, pneumonia, asthma, tuberculosis, and lung inflammation. Benign colonic conditions included Crohn’s disease, colitis, appendicitis, colon polyp, and irritable bowel syndrome. The benign prostate patients all had benign prostatic hypertrophy. The normal population comprised 129 apparently healthy females.

For more detailed studies of the CA-549 IRMA, 668 sera were obtained from the Hybritech serum bank and from the National Cancer Center, Bethesda, MD, through a service contract with the Mayo Clinic. The advanced breast cancer patients comprised 80 patients, all with active metastatic breast carcinoma. This group included 39 with bone metastases and 41 with metastases to liver or other sites. The early breast cancer patients consisted of 28 with localized breast tumor and 2 with regional direct tumor extension but no lymph node involvement. Lung cancer patients included 11 cases of adenocarcinoma, 3 cases of large cell, and one case each of epidermoid, small cell, malignant histocytoma, and spindle cell sarcoma. Colon and prostate cancer patients all had advanced disease with metastases to distant sites. Benign breast disease patients comprised 65 cases of fibrocystic breast disease, 3 cases of fibrosclerosis, 2 cases each of breast abscess and hypothyroidism of the breast, and one case each of mammary duct ectasia, fibroadenosis, and cellulitis of the breast. Benign lung patients included 8 cases of postinflammatory fibrosis of the lung, one case each of chronic bronchitis, bronchiectasis, and pneumonia, and 8 cases of unclassified lung disease. The benign liver patients group comprised 7 cases of hepatitis, 5 cases of cirrhosis, and 13 cases of other liver diseases unrelated to alcohol. Benign colon conditions included 21 cases of benign polyposis, 18 cases of colitis, 9 cases of diverticulosis, 5 cases of irritable bowel syndrome, 3 cases of Crohn’s disease, and 14 cases of various other conditions. Benign prostate patients all had benign prostatic hypertrophy. The normal population consisted of 257 apparently healthy females ranging in age from 24 to 80 years.

Immunoradiometric Sandwich Assay. DEAE-chromatography purified antibody BC4E 549 was labeled with ¹²⁵I using solid-phase lactoperoxidase according to the method of David and Reisfeld (23). Purified BC4N 154 was covalently attached to 0.4×-in. diameter polystyrene beads (The Hoover Group, Sault Ste. Marie, MI) by the method previously described (24). Antibody coated beads were incubated with 100 µl serum plus 100 µl PBS for 2 h at room temperature with continuous shaking; the beads were then washed 4 times with a solution of 80 mm NaCl-0.2% Tween 20-0.01% NaN₃. A titrated amount (approximately 300,000 cpm) of radioiodinated CA4E 549, in 200 µl of tracer diluent (3) was added to each bead and incubated at room temperature for 2 h with shaking on a horizontal rotator. The beads were again washed 4 times with washing solution and counted in a gamma counter. Patients’ sera were assigned values of CA-549 activity by comparing tracer antibody binding in patients’ sera to binding produced by calibrators (prepared with T417 nude mouse tumor cytosol added to pooled normal human serum). CA-549 levels were expressed in terms of arbitrary assay units. A value of 10.0 units/ml or greater was considered to be “positive” for CA-549, based on the observed values of 25 apparently healthy individuals. The cutoff was set as the mean + 2 SD of the observed values and rounded off. Statistical significance of the variation in CA-549 levels observed between the various groups was determined by the Kruskal-Wallis test (24), a nonparametric single factor analysis of variance by ranks.

Immunohistochemistry. Cryostat sections of snap-frozen, unfixed specimens or histopysms were cut, picked up on gelatin-coated microscope slides, and allowed to air dry at room temperature for 1 h. Sections were rehydrated in PBS, blocked with 5% normal goat serum in PBS, and incubated with primary antibody (murine ascites) diluted 1:1000 in 5% normal goat serum for 1 h at room temperature in a humid chamber. Following 3 PBS washes, horseradish peroxidase-conjugated polyclonal goat anti-mouse immunoglobulins (Bio-Rad) in 5% normal goat serum, 5% normal human serum, and 1% bovine serum albumin in PBS were incubated in the sections for 1 h and followed by 3 additional PBS washes. Slides were incubated for 5 min at room temperature with chromogenic substrate-0.6 mg/ml 3,3-diaminobenzidine (Sigma) in 0.01% hydrogen peroxide. Isotype and concentration matched murine monoclonals of irrelevant specificity, tumor-specific standard antibodies, and positive and negative control tissues served as reagent, procedure, and tissue positive and negative controls.

Isotope and Immunoblotting. SDS-PAGE gels were done by a modification of the method of Laemmli (25), using 3–15% acrylamide gradient gels under reducing conditions. Transfer of antigens to nitrocellulose paper was performed according to the method of Towbin et al. (26). Typical Western blot analysis was done with residual binding sites blocked by incubation of blotted nitrocellulose paper in BLOTTO (27) followed by staining with radiiodinated primary antibody or unlabeled primary antibody followed by radiiodinated secondary antibody. After washing and drying, blots were exposed to Kodak O-MAT AR film at −70°C.

Isoelectric Focusing Column. Approximately 7.0 mg of breast carcinoma cytosol prepared from T417 nude mouse tumors were focused using a water-jacketed 110-ml isolectric focusing column (LKB) in a 0–47% sucrose gradient containing ampholutes of the pH range 3.5–10. The column was prefocused at 600 V for 12 h at 4°C. The sample was injected into the middle of the gradient and focused an additional 30 h at 4°C. Fractions (1 ml) were collected from the column and the pH was immediately determined. Fractions were dialyzed overnight against 10 mM sodium phosphate, pH 7.0, and aliquots of each fraction were examined for reactivity with the antibody BC4E 549 by an ELISA assay as described above, except modified to operate with no patients’ sera added.

Lectin Inhibition of Antibody Binding. Individual wells of 96-well plates were coated with 1 µg of optimally diluted MFGM for use in the ELISA described above. The specific lectins were added at the concentrations (diluted in PBS, pH 7.4) indicated (50 µl/well) 1 h prior to primary antibody addition. Unbound lectin was washed away (3 times with PBS) and primary antibody was added and incubated for 1 h. Primary antibody binding was detected by subsequent additions of horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Tago) and o-phenylenediamine (Sigma) substrate with appropriate washing steps as described above. Wheat germ agglutinin and peanut agglutinin were obtained from Behring Diagnostics, San Diego, CA.

Neuraminidase Treatment. Approximately 25 µg of T417 breast carcinoma cytosol were adjusted to pH 5 with acetic acid and incubated for 1 h at 37°C with 10 millunits of Clostridium perfringens neuraminidase (Sigma) in 0.05 M sodium acetate buffer, pH 5. The sample was subsequently digested in SDS containing PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting as described above. Neuraminidase insensitivity, when observed, was confirmed by ELISA. Individual wells of 96-well plates were coated with 2.5 µg of milk fat globule membranes. Prior to addition of primary antibodies, the antigen-coated wells were each treated with 50 µl 0.05 M sodium acetate buffer, pH 4.5 which contained either 0, 0.1, 0.2, or 0.3 units/ml of neuraminidase. The digestions were allowed to proceed for 1, 2, or 4 h at 37°C with continuous shaking. The wells were then washed 6 times with PBS, pH 7.4, with 1% bovine serum albumin and 0.1% Tween 20. The remainder of the ELISA was performed as described above.
Protease Treatment. T417 breast carcinoma cytosol (25 μg) was incubated for 1 h at 37°C with 2 mg/ml (diluted in PBS, pH 7.4) final concentration of proteinase K (Sigma). The sample was subsequently diluted in SDS-containing PAGE sample buffer (25) and analyzed by SDS-PAGE and immunoblotting as described above.

Sodium Periodate Treatment. The sensitivity of the BC4E 549 epitope to mild periodate treatment was assessed by mixing IRMA capture beads with a sample containing approximately 50 units/ml of antigen for 2 h and washed as described above. The bead with captured antigen was then treated with 200 nM of 50 mM sodium acetate buffer, pH 4.5, that contained either 0, 0.25, 0.50, or 1.0 mM sodium periodate (Sigma) for 1 h with shaking in the dark at room temperature. The beads were then washed as above and treated with 200 μl of 0.01 M sodium borohydride plus 0.5 mM NaOH in PBS for 30 min with shaking at room temperature. Beads were then washed and tracer antibody added as described in the IRMA method.

RESULTS

Levels of CA-549 Antigen in Patients’ Sera. BC4E 549 was initially adapted to ELISA, and sera were tested for CA-549 levels by their ability to inhibit this ELISA signal as the result of preincubation with the BC4E 549 antibody. Fig. 1 shows the level of ELISA inhibition produced by a total of 271 sera from healthy volunteers, breast and other cancer patients, and benign disease patients. The results are expressed as a percentage of inhibition of the ELISA signal obtained with a normal human serum pool as a control. The cutoff between positive and negative was based on the observed levels of reactivities using sera from healthy individuals to establish the normal range of inhibition. Seventy % (19 of 27) of sera from patients with advanced breast cancer showed significant inhibition of ELISA signal compared to 0 of 22 benign breast disease sera and 0 of 129 sera from healthy individuals. A few sera from various other cancers showed some ELISA inhibition but not with the frequency seen in breast cancer.

The ELISA inhibition was useful for establishing that BC4E 549 reacted with a circulating antigen of interest. However, in this assay format, normal and benign disease sera produced a wide range (0–40%) of inhibition. Thus a substantial portion of the dynamic range of the test was consumed by CA-549 “negative” specimens. In addition, the data were expressed in terms of percentage of inhibition rather than the more useful numerical quantitation. Moreover, the ELISA was difficult to reproduce day to day (data not shown). Thus, in order to improve the performance of BC4E 549 as a diagnostic tool, it was adapted to a 2-site sandwich assay format using BC4N 154 as the capture antibody. BC4N 154 had previously been shown to work well with BC4E 549 in the sandwich assay (data not shown). Using this assay, arbitrary units of CA-549 activity were assigned to specimens by use of a standard curve (Fig. 2). The mean CA-549 level for 257 healthy individuals was observed to be 3.94 units/ml ± 2.83 (SD). The cutoff for the assay was the mean + 2 SD which equals 9.6. Thus a serum level of 10 units/ml or greater was considered positive for CA-549. In order to confirm that this 2-site IRMA gave results comparable to those observed for ELISA inhibition, a panel of 208 sera was tested by both methods and the results compared. Table 1 shows that the performances of the 2 tests were very similar. In this particular panel, ELISA had a slightly lower sensitivity for advanced breast cancer, 43% compared to 53% for IRMA. All of the patients positive by ELISA were also positive by IRMA.

To further evaluate the utility of the CA-549 IRMA for the detection of breast cancer, a larger panel consisting of 668 sera (which included the 208 sera assayed in the ELISA/IRMA comparison) was tested. The results are shown in Figs. 3 and 4. The mean CA-549 value for the advanced breast cancer patients was 63.0 ± 175, as compared to 3.71 ± 2.93 for benign breast patients (P < 0.0001) and 3.94 ± 2.83 for healthy individuals (P < 0.0001). The incidence of elevated CA-549 levels was not significantly different for patients with bone metastases compared to other sites. The mean for early breast cancer patients was 3.99 ± 2.94, not significantly different than normal or benign breast patients. These data indicate that CA-549 is a very useful marker for advanced breast cancer with a very low rate of false positives. However, in its current format the assay is not useful as a tool for early diagnosis of breast carcinoma. CA-549 levels are also moderately but not significantly elevated in lung cancer patients, 7.81 ± 9.4 compared to benign lung patients, 6.15 ± 6.29 (P < 0.59) and also in prostate cancer patients, 5.54 ± 5.37 compared to benign prostate patients, 2.99 ± 2.30 (P < 0.074). CA-549 levels are not significantly elevated in ovarian cancer compared to benign ovarian disease.

Table 1 Comparison of BC4E 549 ELISA inhibition to CA-549 IRMA

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of sera</th>
<th>ELISA (% positive)</th>
<th>IRMA (% positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced breast cancer</td>
<td>30</td>
<td>13 (43)</td>
<td>16 (53)</td>
</tr>
<tr>
<td>Early breast cancer</td>
<td>30</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Advanced lung cancer</td>
<td>19</td>
<td>4 (21)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Advanced colon cancer</td>
<td>20</td>
<td>0 (0)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Benign breast</td>
<td>30</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Benign colon</td>
<td>20</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Benign lung</td>
<td>19</td>
<td>2 (10.5)</td>
<td>2 (10.5)</td>
</tr>
<tr>
<td>Healthy</td>
<td>40</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Fig. 2. CA-549 sandwich IRMA standard curve. Calibrators were prepared by adding partially purified CA-549 to pooled human serum. Sera that contained ≥10.0 units/ml of CA-549 activity were considered positive based on preliminary studies with sera from healthy individuals to establish a normal range of activity.

Fig. 1. Inhibition of BC4E 549 ELISA original by sera from patients with various cancers and benign diseases as well as healthy females. Positive/negative cutoff was determined by preliminary tests of sera from healthy females to establish a normal range of inhibition (data not shown).
significantly elevated in colon cancer patients, 4.5 ± 3.40 compared to benign colon patients, 3.92 ± 3.1. Moreover, neither benign colon nor benign liver disease patients (5.31 ± 3.61) CA-549 levels are significantly elevated compared to healthy individuals.

The CA-549 IRMA produces a 50% sensitivity and 98% specificity as a test for advanced breast cancer. Sensitivity is defined as the number of true-positives divided by the true-positives plus false-negatives. Specificity is defined as the number of true-negatives divided by the true-negatives plus false-positives.

Immunohistochemistry. Results of a preliminary immunohistochemical study of the specificity of BC4E 549 and BC4N 154 are presented in Table 2. Antibody BC4E 549 stained all breast carcinomas tested with no apparent selectivity for either lobular or infiltrating ductal types. The intensity of tumor cell staining was equivalent to that of normal breast epithelium in 12 of 13 specimens examined and did not appear to be limited to membrane staining alone. Other normal tissues stained by BC4E 549 included goblet cells and mucus of normal colon with variable staining of the surface epithelium. BC4E 549 also stained kidney tubule epithelium, bile duct epithelium, bronchial and alveolar epithelium in the lung, the exocrine, endocrine, and ductal epithelia of the pancreas, germinal epithelium of one of 3 ovaries tested, and minor staining of the white pulp of one of 2 spleens tested.

Antibody BC4N 154 (Table 2) was similar to BC4E 549 in tumor staining intensity and specificity but was more cross-reactive with normal tissues, generally staining more tissue types and with greater intensity. In addition to normal kidney, liver, lung, pancreas, and spleen, BC4N 154 stained normal small bowel epithelium and nuclei of Sertoli cells of the testis, but failed to stain germinal epithelium of the ovaries tested.

Biochemical Characterization of CA-549. Fig. 5 shows Western blot analysis of reducing PAGE of various antigen preparations stained with BC4E 549. Using cytosol from human breast tumor, this antibody stained 2 major bands of apparent molecular weights of approximately 400,000 and 512,000. Human serum from breast cancer patients displayed the same 2 bands but the 400,000-band stained relatively darker, compared to the 512,000-band, than it did in tumor cytosol. Whether this represents a difference between the serum and tumor form of the antigen or variability between individual patients is unclear. No staining was produced by a radiolabeled isotype-matched antibody of irrelevant specificity (data not shown).

Fig. 6 shows the elution profile of CA-549 activity from an isoelectric focusing column run in a sucrose stabilized pH

Table 2 Immunohistological screening evaluation of murine monoclonal antibodies to human breast carcinoma, BC4E 549 and BC4N 154

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. of specimens stained/total tested</th>
<th>Staining intensity</th>
<th>Major component stained</th>
<th>No. of specimens stained/total tested</th>
<th>Staining intensity</th>
<th>Major component stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC4E 549</td>
<td></td>
<td></td>
<td></td>
<td>BC4N 154</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td>17/17</td>
<td>2+</td>
<td>e</td>
<td>10/11</td>
<td>2+</td>
<td>e</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>1/1</td>
<td>3+</td>
<td>e</td>
<td>1/1</td>
<td>3+</td>
<td>e</td>
</tr>
<tr>
<td>Benign Breast</td>
<td>12/13</td>
<td>2+</td>
<td>e</td>
<td>10/10</td>
<td>2+</td>
<td>e</td>
</tr>
<tr>
<td>Colon</td>
<td>2/3</td>
<td>2+</td>
<td>e</td>
<td>2/2</td>
<td>3+</td>
<td>e</td>
</tr>
<tr>
<td>Duodenum</td>
<td>0/2</td>
<td>2+</td>
<td>e</td>
<td>1/1</td>
<td>3+</td>
<td>e</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0/3</td>
<td>2+</td>
<td>e</td>
<td>1/1</td>
<td>3+</td>
<td>e</td>
</tr>
<tr>
<td>Kidney</td>
<td>2/2</td>
<td>2+</td>
<td>t</td>
<td>2/2</td>
<td>3+</td>
<td>t</td>
</tr>
<tr>
<td>Liver</td>
<td>2/2</td>
<td>2+</td>
<td>b</td>
<td>1/2</td>
<td>3+</td>
<td>b</td>
</tr>
<tr>
<td>Lung</td>
<td>3/3</td>
<td>2+</td>
<td>a, B</td>
<td>2/2</td>
<td>3+</td>
<td>a, B</td>
</tr>
<tr>
<td>Ovary</td>
<td>1/3</td>
<td>2+</td>
<td>G</td>
<td>0/2</td>
<td>3+</td>
<td>G</td>
</tr>
<tr>
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<td>x, n, d</td>
<td>2/2</td>
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<td>x, n, d</td>
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<tr>
<td>Spleen</td>
<td>1/2</td>
<td>1+</td>
<td>w</td>
<td>1/2</td>
<td>1+</td>
<td>w</td>
</tr>
<tr>
<td>Testes</td>
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<td></td>
<td></td>
<td>2/2</td>
<td>2+</td>
<td>S</td>
</tr>
</tbody>
</table>

* Specimens from unrelated donors.
* Mean staining intensity on a scale of 0-4+.
* e, epithelium; a, alveolar epithelium; B, bronchial epithelium; G, germinal epithelium; t, kidney tubules; x, exocrine pancreas; b, bile duct epithelium; d, pancreatic ducts; n, endocrine pancreas; S, seminiferous tubules; w, white pulp of the spleen.
Fig. 5. SDS-PAGE Western blot analysis of CA-549 in human serum and partially purified cytosol from T417 human breast tumor grown in nude mice. Staining was produced by 125I-labeled BC4E 549 and visualized by autoradiography. Lanes 1-7, sera from different breast cancer patients with active disease; Lane 8, pooled sera from healthy individuals; Lane 9, CA-549 partially purified from T417 human breast tumor cytosol by Sephacryl S-400 gel filtration. K, thousands.

Fig. 6. Elution profile of CA-549 activity from an isoelectric focusing column run in a sucrose-stabilized pH gradient of pH 3.5–10. One-mL fractions were collected and dialyzed in PBS prior to being run in a BC4E 549 ELISA absorbance.

Fig. 7. Lectin blocking of BC4E 549 binding to milk fat globule antigen. Varying concentrations of wheat germ agglutinin (•) and peanut lectin (○) were preincubated with milk fat globule antigen immobilized on microtiter plates. Binding of BC4E 549 to treated and untreated antigen was compared by ELISA assay.

Fig. 8. Western blot analysis of neuraminidase and protease treated T417 human breast tumor cytosol followed by immunostaining with BC4E 549. Lanes 4 and 5, untreated antigen stained with BC4E 549 (Lane 4) and an isotype-matched control antibody of irrelevant specificity (Lane 5); Lane 2, cytosol that was pretreated with proteinase K. The effect of neuraminidase treatment is shown in Lane 3 compared to Lane 1 which contains control antigen treated only with pH 4.5 buffer used in the neuraminidase digestions.

The antigenic activity eluted sharply at pH 5.2, which is thus the apparent isoelectric point for this antigen. Evidence that BC4E 549 reacts with a glycoprotein antigen is derived from 3 different experiments. Lectin inhibition experiments (Fig. 7) demonstrate that BC4E 549 binding to the MFGM antigen can be completely inhibited by wheat germ agglutinin at 20 µg/ml and partially inhibited (up to 70%) by peanut agglutinin at 600 µg/ml. These results indicate that the BC4E 549 antigen contains carbohydrate residues that interact with these lectins. Further characterization of the antigen was performed by glycosidase and protease digestion (Fig. 8). The results show that the detection of the antigen demonstrates that BC4E 549 binding to the MFGM antigen is not sensitive to treatment with neuraminidase; however, the migration of the antigen into the gel decreased after this treatment, indicating that CA-549 contains sialic acid residues. In order to confirm this apparent neuraminidase insensitivity, MFGM
were coated onto microtiter plates and treated with increasing amounts of neuraminidase and for increasing times of digestion, and then assayed by ELISA. Fig. 9 shows that the binding of neither BC4E 549 nor BC4N 154 was decreased by treatment with increasing amounts of neuraminidase up to 0.3 units/ml. Control antibody ZBC 049, an antibody with specificity for an MFGM epitope known to be neuraminidase sensitive, was inhibited approximately 60% in this same assay. The data in Fig. 9 represent treatment with neuraminidase for 1 h; longer treatments of up to 4 h also had no effect on BC4N 154 and BC4E 549 binding (data not shown). These experiments as a whole suggest that the antigen is a glycosylated protein containing sialic acid but that sialic acid is not necessary for antibody binding. Characterization of the antigen using mild periodate oxidation at acidic pH was used to determine if BC4E 549 was binding to a protein or carbohydrate epitope on the glycoprotein. The antigen was bound on an IRMA capture bead and treated with increasing concentrations of sodium periodate and subsequently assayed for the ability to bind radiiodinated BC4E 549 in an IRMA assay. The results indicate that BC4E 549 binding was inhibited (approximately 50% inhibition) by periodate oxidation using 0.25–1.0 mM NaIO₄ (Fig. 10). Since mild periodate oxidation at acidic pH has been shown to cleave carbohydrate vicinal hydroxyl groups without altering the structure of the polypeptide chains (28) these results suggest that BC4E 549 binds to a carbohydrate determinant on the glycoprotein antigen.

Summarized, these data indicate that CA-549 is an acidic (isoelectric point 5.2) glycoprotein that consists of 2 bands, under reducing conditions, of apparent molecular weights of 400,000 and 512,000.

**DISCUSSION**

Monoclonal antibody BC4E 549 was raised by immunizing mice with a partially purified membrane preparation from T417 human breast tumor grown in nude mice. The antibody binds to a high molecular weight glycoprotein antigen found in many breast tumor preparations as well as in the sera of some breast cancer patients. The occurrence of this antigen in patient sera has permitted the preparation of an immunoradiometric sandwich assay using BC4E 549 that affords the opportunity to conveniently detect advanced breast cancer.

The results of the assays of clinical specimens in this study indicate that the circulating antigen (CA-549) is elevated in the sera of approximately 50% of patients with advanced breast cancer as well as a few patients with other cancers. A major feature of this assay is its high degree of specificity (98%). BC4E 549 shows a variable reactivity to normal or benign sera within a certain normal range (0–10 units/ml), but false-positives in the clinically significant range (>10 units/ml) are rare. This is particularly true of normal, benign breast, and benign colon patients which produced 3 of 256, 3 of 79, and 2 of 70 CA-549 false positives, respectively. In its current format the assay is not useful for widespread diagnostic screening, since the antigen was not elevated in the sera of patients with early breast cancer. The data presented in this study clearly indicate a significant elevation of CA-549 in the sera of metastatic breast cancer patients; however, the exact clinical utility of this test cannot be determined from these data. Extensive and detailed clinical studies will be required to determine the exact limits of the clinical utility of this assay. Further studies are currently ongoing to correlate the serum CA-549 levels to a disease state. Additional studies will be directed at finding other antigens that are preferentially expressed in the sera of patients with active disease that do not express the BC4E 549 epitope.

The BC4E 549 antigen (CA-549) is a high molecular weight, acidic glycoprotein that consists of 2 species of approximately 400,000 and 512,000 molecular weight. The 2 species are expressed roughly equally in breast tumor cytosol preparations but the lower molecular weight species sometimes predominates in serum. It is clear from the neuraminidase digestion experiments that sialic acid is not required for BC4E 549 binding. However, pretreatment of the antigen with neuraminidase reduces the electrophoretic mobility of CA-549 on SDS-PAGE. This curious phenomenon has been observed for other proteins including glycophorin (29) and TAG-72, a high molecular weight tumor-associated glycoprotein (30). This reduced mobility is thought to be associated with highly sialylated glycoproteins where the high levels of sialic acid produce a significant negative charge on the SDS:glycoprotein complex. This charge is abolished by desialylation, with resultant reduction in electrophoretic mobility. The reduced antibody binding upon periodate oxidation suggests that the epitope itself involves carbohydrate. Since periodate oxidation of the antigen is able to reduce antibody binding by only 50%, a contribution by the protein backbone to the epitope cannot be ruled out.

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**Fig. 9.** Effect of neuraminidase treatment on the binding of BC4E 549 (○) and BC4N 154 (●) to milk fat globule antigen. Antigen immobilized on polystyrene microtiter plates was treated with increasing concentrations of neuraminidase for 1 h. Binding of antibodies to treated and untreated samples was compared by ELISA. ZBC 049 (▲), an antibody with a neuraminidase sensitive MFGM epitope, was run as a control.

**Fig. 10.** Effect of sodium periodate treatment on BC4E 549 binding to CA-549 captured on a polystyrene bead by means of antibody BC4N 154. Antigen immobilized on the antibody-coated beads was treated with increasing concentrations of sodium periodate and then reduced by treatment with 0.01 M sodium borohydride to prevent artificial cross-linking of antigen and antibody. The binding of 125I-labeled BC4E 549 to treated and untreated samples was compared by IRMA.
In addition to being expressed in tumors and sera of some breast cancer patients, immunohistochemical staining with BC4E 549 reveals that CA-549 is fairly widespread in normal tissue, including breast, colon, and kidney, as well as in both breast and colon carcinoma, with no immunohistological specificity of tumor over normal breast or colonic epithelium in frozen, unfixed tissue specimens. This finding suggests that the antigen is a normal tissue antigen that is anomalously shed or secreted into the circulation of advanced breast cancer patients rather than fetal or tumor-specific antigen not normally found in healthy individuals. Finally, BC4E 549 was tested against frozen tissues of rhesus, African green, and cynomolgous monkeys, and baboon and chimpanzee and found to stain only chimpanzee tissues in a pattern similar to that seen in human tissues (data not shown), suggesting that antigen recognized by this antibody is not highly conserved.

Other investigators have described monoclonal antibodies/antigens that show some utility as serum breast cancer markers. For example, Goodall et al. (31) reported the development of a monoclonal-based IRMA that detected an antigen elevated in breast cancer patients' sera; however, the assay failed to discriminate between benign disease and breast cancer patients. Others have described as assay using the antibody SP-2 which reacts with $M_r$ 90,000 antigen that is elevated in some breast cancer patients (32). In addition, immunization with MFGM has produced several antibodies of interest. The antibody BLMLR-HMFG-MC3 is directed against a $M_r$ 46,000 antigen that is present in the sera of patients with metastatic, but not primary, breast carcinoma (17). These relatively low molecular weight antigens are unlikely to be related to the larger ($M_r$ 400,000–500,000) CA-549 antigen reported here. Other antibodies raised against MFGM by several investigators have reacted with high molecular weight antigens but these are variable in their performance in tumor detection. For example, the antibody HMFG-1 has a low false-positive response but detected only 30% of advanced breast cancer patients; conversely, HMFG-2 was roughly comparable to BC4E 549 in its ability to detect breast cancer (53%) but it had a high false-positive rate of 16% (14). The antibody NCRC-11 identified a high molecular weight antigen present in MFGM and in tumors but its presence in serum has not been established (33). Antigens detected by the antibodies DF3 (15) and 115D8 (16) are reported to be elevated in about 70% of patients with advanced or metastatic breast cancer. They resemble CA-549 in that they are high molecular weight antigens and present in MFGM. The CA-549 epitope is probably a part of this same complex antigen. However, the precise epitopic reactivities are likely to be different. For example, BC4E 549 binding is not inhibited by treatment of the antigen with neuraminidase, but antibody binding is significantly inhibited by peanut agglutinin. This is the opposite of the pattern reported for DF3 which was not inhibited by peanut agglutinin, while its binding was abolished by neuraminidase treatment (19). Another difference between antibody DF3 and the antibodies examined in this study is their immunohistochemical reactivities with breast tumor and benign breast tissue. Neither BC4E 549 nor BC4N 154 can distinguish between breast tumor and benign breast tissue by immunohistological staining, but DF3 is reported to display a cytoplasmic staining pattern in breast tumor sections in contrast to an apical pattern in benign breast tissue (34). Antibody DF3 and 115D8 have been used together in an immunoradiometric assay called CA15-3. This assay detects about 70% of advanced cancer patients and have been shown to be useful in monitoring disease progression (20). However, this test is also reported to be positive in 9.4% of healthy women as well as 20% of benign breast and 44% of benign liver patients. Curiously, this relatively high rate of false-positives became evident only when the 2 antibodies were used together. When DF3 antibody was used alone in an ELISA format low numbers of false-positives were reported (20). This is in contrast to BC4E 549 which showed good specificity not only in ELISA but also when used with capture antibody BC4N 154 in the CA-549 IRMA.

It is clear from this study and studies done by several groups that the circulating high molecular weight mucin-like antigens are useful for advanced breast cancer detection and monitoring (14–16, 20, 35). It is also apparent that there are many epitopes present on these large mucin-like molecules, and antibodies directed against them have many different and overlapping specificities (36). It is likely that even small differences in epitopic specificities may play a significant role in immunoassay performance and clinical utility, as is shown in the assay system described in this paper. The differences in epitope specificity between the CA-549 assay and other previously described assays for this antigen system result in an assay with a lower rate of false-positives. As immunoassays detecting the most clinically useful of the various epitopes become routinely available, the clinicians' ability to manage the treatment of breast cancer is likely to be significantly enhanced.

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Serum Levels and Biochemical Characteristics of Cancer-associated Antigen CA-549, a Circulating Breast Cancer Marker

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