Expression of Ductal Carcinoma Antigen in Breast Cancer Sera as Defined Using Monoclonal Antibody F36/22

Lawrence D. Papsidero,1 Takuma Nemoto, Gary A. Croghan, and T. Ming Chu

ABSTRACT

A quantitative immunoassay procedure has been constructed to evaluate levels of ductal carcinoma antigen recognized by murine McAb F36/22. Using this method, 3% of 64 apparently healthy individuals and 13% of 40 patients with benign breast disease expressed serum antigen levels above 70 units/ml. Greater than 50% of 116 patients with clinical evidence of breast cancer demonstrated circulating ductal carcinoma antigen levels above 70 units/ml. Patients with ductal carcinomas of other sites, including prostate and gastrointestinal tumors, also demonstrated elevated levels of antigen (11 and 27%, respectively). The incidence of elevated serum ductal carcinoma antigen levels correlated significantly with the incidence of intratumoral antigen expression. Lectin binding, molecular weight, and density measurements indicated that circulating antigen occurs as a high-molecular-weight glycoprotein with mucin-like characteristics.

INTRODUCTION

Murine hybridoma cell lines producing McAbs2 to human adenocarcinomas have been developed in this laboratory (21). These antibodies have been shown to produce specific tumoral effects upon passive administration to animals bearing progressively growing human heterotransplants (2, 3). One of these antibodies, F36/22, recognizes an antigen which is preserved in specimens of formalin-fixed and paraffin-embedded tissues, and a thorough analysis of antigen distribution has been performed (6, 7). Results indicated a relationship between tumorigenesis and antigen expression has been confirmed for some common organ sites of carcinoma development (6, 7). Tumors of mesenchymal derivation lacked antigen, the expression of which was generally restricted to carcinomas of ductal lineage. Presently, we extend these observations and evaluate human sera for antigen levels and physical characteristics of circulating antigen.

MATERIALS AND METHODS

McAb F36/22. Details concerning the immunization, fusion, cloning, and screening procedures are reported elsewhere (21). Briefly, murine hybridoma culture F36/22 secretes a subclass γ3-immunoglobulin which recognizes a ductal carcinoma antigen (6, 7, 21). Antibody was purified using Sepharose:Protein A chromatography (Pharmacia, Inc., Piscataway, NJ) (9) as used by us previously (3). Antibody was covalently conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) as described (20). Conjugates were stored at −20° in the presence of 50% glycerol at 1 mg of immunoglobulin/ml.

Papain Digests. Human primary breast cancer specimens were pooled and homogenized in 10 volumes of 10 mm Tris buffer, pH 7.4, containing 0.2% (w/v) sodium deoxycholate at 4°. The homogenate was quickly brought to 37°, and the following reagents (final concentration) were added under stirring: 1 mm cysteine (Sigma), 1 mm EDTA (Sigma), and papain (0.8 unit/ml) (Boehringer-Mannheim, Indianapolis, IN). After 5 min, digestion was stopped by the addition of 5 mm iodoacetamide (Sigma). The homogenate was centrifuged at 100,000 × g for 1 hr at 4°, then extensively dialyzed against 10 mm Tris/0.9% NaCl solution buffer, pH 7.4, containing phenylmethanesulfonfyl fluoride and aminocaproic acid, each at 10 mm. The homogenate was frozen in small aliquots at a concentration of 0.5 mg of protein/ml (17). Electrophoresis (15) revealed the presence of several major glycoproteins (10).

Enzyme Immunoassay Procedure. A sandwich-type assay procedure was developed using antibody-coated plates. Microtiter plates (Nunc I Immunoplates; Grand Island Biological Co., Grand Island, NY) were coated with McAbs (200 μg/ml) in 50 mm carbonate-bicarbonate buffer, pH 9.6, for 18 hr at 4°. After removal of the antibody solution, residual protein binding sites on the plastic were blocked by the addition of 200 μl of assay buffer (PBS containing 1% (v/v) murine serum and 1% (w/v) bovine albumin). After 1 hr of incubation at room temperature, the coated plates were used immediately for the assay procedure.

To perform the assay, 200-μl samples, diluted in assay buffer, were applied for 1.5 hr at 37°. After 3 washes using assay buffer, 200 μl of enzyme-conjugated McAb F36/22 was applied for 1.5 hr at 37°. The conjugate was diluted to a concentration of 0.5 μg of immunoglobulin per ml of PBS containing 10% (v/v) murine serum. Following a wash procedure as above, 200 μl of substrate per well were applied for 0.5 hr at room temperature. Substrate solution contained 0.4 mg of o-phenylenediamine per ml of pH 5.0 citrate buffer and 0.003% hydrogen peroxide. The reaction was stopped by addition of 50 μl of 2 N sulfuric acid, and absorbance was monitored at 488 nm using an enzyme assay plate reader (Fisher Scientific Co., Pittsburgh, PA).

The percentage of bound enzyme conjugate was calculated by the formula:

\[
\frac{(B - B_0)(B_i - B_0)}{B_i} \times 100
\]

where \(B\) = absorbance of the sample, \(B_i\) = maximal absorbance, and \(B_0\) = absorbance of the blank. Each assay was performed in triplicate using a standard papain digest and 25-fold diluted serum samples diluted in assay buffer. Samples producing an absorbance corresponding to greater than 50% bound were serially diluted and reasayed. Samples showing a standard deviation above 10% were also reassayed. An interassay coefficient of variation of 12% was observed for 14 consecutive assays.

Subjects. Serum samples from apparently healthy young laboratory personnel were obtained as well as specimens from age-matched controls (mean age, 61 years) under the auspices of the Saving Army (Buffalo, NY). Serum samples from patients with cancer were obtained from a frozen serum bank maintained at Roswell Park Memorial Institute with the cooperation of Dr. C. S. Killian. Clinical parameters, such as extent of disease, were provided by staff clinicians for all serum samples analyzed.

Biochemical Evaluation of Sera. Serum samples (0.5 ml) from breast
cancer patients were filtered over a column (1.6 x 90 cm) packed with Sephacryl S-400 (Pharmacia). Column fractions eluted with PBS were diluted in enzyme assay buffer and evaluated for antigen activity as above. The column was precalibrated using protein standards of known molecular weight, including bovine thyroglobulin, aldolase, catalase, and albumin (Sigma).

Column fractions exhibiting peak antigen activity were further fractionated under equilibrium density gradient ultracentrifugation. Samples in PBS were brought to a density of 1.45 g of cesium chloride per ml. After centrifugation (1.5 x 10^6 x g for 72 hr at 10°), fractions were assayed for antigen activity as above and for density using an analytical balance.

Solid-Phase Adsorption Treatments. Serum specimens obtained from patients with breast cancer were incubated with the following solid-phase adsorbents (Sigma): (a) concanavalin A-Sepharose; (b) wheat germ-Sepharose; (c) peanut agglutinin-Sepharose; (d) lentil lectin-Sepharose; and (e) Protein A-Sepharose. Sepharose adsorbents derivatized with McAbs F36/22 and F5 (22) were prepared in this laboratory using cyanogen bromide-activated Sepharose according to recommendations from the manufacturer (Pharmacia).

For adsorption studies, serum dilutions were incubated with adsorbents (50%, v/v) at a volume ratio of 1:40 for 18 hr at 4°. All tests were performed in triplicate. After centrifugation (500 x g), the supernatant was assayed for antigen activity as described above. Negative binding controls consisted of identical treatments using nonderivatized Sepharose (Pharmacia).

Immunoperoxidase Studies. Histologically diagnosed tumors obtained from Pathology Department files were stained with McAb F36/22 for antigen activity as above and for density using an analytical balance. The column was precalibrated using protein standards of known molecular weight, including bovine thyroglobulin, aldolase, catalase, and albumin (Sigma).

Column fractions exhibiting peak antigen activity were further fractionated under equilibrium density gradient ultracentrifugation. Samples in PBS were brought to a density of 1.45 g of cesium chloride per ml. After centrifugation (1.5 x 10^6 x g for 72 hr at 10°), fractions were assayed for antigen activity as above and for density using an analytical balance.

RESULTS

Enzyme Immunoassay of DCA. Based upon our observations using immunoprecipitation analyses which indicated the occurrence of multiple antibody combining sites on DCA (to be presented elsewhere), a sandwich-type of enzyme immunoassay has been constructed. To develop the procedure, antigen standards comprised a papain digest of breast tumor specimens shown previously by immunoperoxidase staining to contain the antigen. The dose-response curve generated, as shown in Chart 1, demonstrated linearity (r = 0.98, linear regression analysis) between antigen input of 0.625 to 10 units/ml. For serum analysis, the range was 16.25 to 260 units/ml, since these samples were diluted 26-fold prior to assay. The values of antigen content were arbitrarily assigned in the absence of purified antigen during development of the assay.

Specificity of the immunoassay was examined by substituting various antibody reagents at the solid phase. As seen in Chart 2, assay plates coated with McAb F5 (prostate antigen specific) (22), McAb M7/105 (distinct tumor antigen specific) (21), or nonimmune murine immunoglobulin failed to bind detectable levels of DCA. In contrast, antibody F36/22 was effective at high dilutions. Also, enzyme conjugates prepared from antibody M7/105 failed to react with DCA previously immobilized onto F36/22 antibody-coated plates (not shown).

Serum DCA in Normal Controls. Sera obtained from 64 apparently healthy individuals exhibited a mean value of approximately 28 units of DCA/ml (Chart 3). Only 3% of the samples expressed serum antigen at 70 units/ml or above, and this value was arbitrarily chosen as cutoff for elevated serum levels. This group, in addition to laboratory personnel, contained 32 samples obtained from age-matched controls (Table 1). No statistically significant difference was observed between these groups regarding circulating DCA levels. Furthermore, long-term (1 year or greater) storage of sera or 3 cycles of freeze-thaw failed to significantly alter DCA values. Similar values were obtained using freshly drawn and unfrozen sera from 10 volunteers (mean value, 29 units/ml). All sera to be evaluated were diluted 26-fold prior to assay, as based upon antigen recovery experiments. At dilutions below 20-fold, the recovery of antigenic activity was less than 100% and a source of experimental underestimate of DCA levels.

Patients with Benign and Malignant Breast Disease. Sera from patients with benign disease of the breast (most with fibrocystic disease) exhibited a mean DCA value of 41.5 units/ml (Chart 3 and Table 1). The incidence of values above 70 units/ml was 13% (5 of 40 specimens). Patients with breast cancer (with evidence of disease) exhibited a wide range of circulating levels of DCA (Chart 3), with a mean value above 700 units/ml. The incidence of elevated values for this group was approxi-
Relationship between Circulating Levels of DCA and Intratumoral Expression of Antigen. Retrospective assessment of antigen expression by human tumors was performed previously using immunoperoxidase staining (6). Results were statistically evaluated for their relationship with serum levels of DCA at the corresponding disease histotypes. As shown in Chart 4, a high correlation (p < 0.01) was found between incidence of elevated serum antigen levels and incidence of DCA expression by tumors.

Biochemical Characteristics of Circulating DCA. Sera obtained from 5 patients with breast cancer were subjected to size fractionation on columns of Sephacryl S-400. Fractions thus obtained were evaluated for immunoreactivity using the enzyme immunoassay procedure. Results, as seen in Chart 5, demonstrated the presence of antigen activity eluting in high-molecular-weight fractions. Results were similar among the specimens examined. The density of serum antigen was evaluated using equilibrium ultracentrifugation and, as shown in Chart 6, it exhibited a peak value of approximately 1.45 g/ml, although a broad range of reactivity was observed.

The ability of serum antigen to interact with various solid-phase adsorbents was also studied. As seen in Chart 7, a significant (p < 0.01) amount of antigen was bound by immobilized wheat germ lectin, indicating the presence of available β-N-

approximately 53% (61 out of 116 specimens). Patients with early stage disease or no clinical evidence of disease demonstrated a decreased incidence of elevated serum antigen values (Table 1). Approximately 30% of these specimens contained levels of DCA above 70 units/ml.

Serum samples were also obtained from 12 patients prior to and 4 days after mastectomy in order to evaluate the response of serum antigen levels to surgery. Of these 12 patients, 4 showed pretreatment levels greater than 70 units/ml. After surgery, each patient in this latter group demonstrated a decrease in serum DCA level to the normal range (Table 2).

Patients with Non-Breast Cancer. Sera obtained from 65 patients with prostatic cancer and 112 patients with gastrointestinal cancer (advanced disease) were evaluated (Chart 3 and Table 1). The incidence of elevated DCA values was 11 and 27%, respectively. Mean serum values from the group with gastrointestinal cancer (60 units/ml) were significantly higher than control levels.

Table 1
Detection of ductal carcinoma antigen in serum

<table>
<thead>
<tr>
<th>Group</th>
<th>No. tested</th>
<th>Mean (units/ml) ± S.D.</th>
<th>&gt;70 units/ml (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparently healthy controls</td>
<td>32</td>
<td>28.9 ± 18.2 ± S.D.</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Lab personnel</td>
<td>32</td>
<td>27.9 ± 16.3 ± S.D.</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Age-matched</td>
<td>40</td>
<td>41.5 ± 43.2 ± S.D.</td>
<td>5 (13)</td>
</tr>
<tr>
<td>Patients with benign breast disease</td>
<td>65</td>
<td>28.3 ± 24.8 ± S.D.</td>
<td>7 (11)</td>
</tr>
<tr>
<td>Patients with non-breast cancer</td>
<td>112</td>
<td>60.2 ± 50.4 ± S.D.</td>
<td>30 (27)</td>
</tr>
<tr>
<td>Prostate</td>
<td>15</td>
<td>49.9 ± 23.7 ± S.D.</td>
<td>5 (33)</td>
</tr>
<tr>
<td>Miscellaneous gastrointestinal</td>
<td>101</td>
<td>719.1 ± 3446 ± S.D.</td>
<td>56 (56)</td>
</tr>
<tr>
<td>Patients with breast cancer</td>
<td>15</td>
<td>52.9 ± 22.8 ± S.D.</td>
<td>4 (27)</td>
</tr>
</tbody>
</table>

* p < 0.05 as compared to control population (Student's t test analysis).  
* ED, patients with clinically detectable evidence of disease; NED, no evidence of clinically detectable disease.
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**Volume of bovine thyroglobulin; V₀, void volume; Vₚ, total volume.**

**Chart 5.** Gel filtration chromatography of serum from breast cancer patients. Sieving chromatography, using Sephacryl S-400, was used to size-fractionate serum components. Enzyme immunoassay results (absorbance at 488 nm) indicated the presence of peak DCA at high-molecular-weight regions. 669 KD, elution volume of bovine thyroglobulin; V₀, void volume; Vₚ, total volume.

**Chart 6.** Equilibrium density gradient ultracentrifugation analysis of serum antigen. Antigen obtained from gel filtration was subjected to isopyknic ultracentrifugation in the presence of cesium chloride. Fractions obtained were assayed for antigen activity (absorbance at 488 nm) and density as described in "Materials and Methods."

**Solid Phase Adsorbent**

- Concanavalin A
- Wheat Germ Lectin
- Lentil Lectin
- Peanut Agglutinin
- Protein A
- McAb F5
- McAb F36/22

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>% Bound Serum Antigen (± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 20 30 40 50 60 70 80 90 100</td>
</tr>
</tbody>
</table>

**Chart 7.** Solid-phase adsorption of serum antigen. Specimens of breast cancer sera (N = 6) were allowed to react with various solid-phase adsorbents as described in "Materials and Methods." Nonderivatized Sepharose served as a negative control (i.e., 0% bound antigen). After incubation, each supernatant was assayed for antigen activity (triplicate measurements), and this value was used to calculate the percentage of bound antigen. McAb F36/22-Sepharose was used as a positive control for these experiments. Binding specificities: concanavalin A, α-D-mannosyl groups; wheat germ lectin, β-N-acetylgalactosaminyl groups; peanut agglutinin, β-N-acetylgalactosaminyl groups; Protein A, Fc⁺, immunoglobulin domain. McAb F5 reacts with an antigen specific for prostate tissue (22) Statistical evaluations (Student's t test) indicated that the solid-phase adsorbents prepared from wheat germ lectin and McAb F36/22 bound statistically significant (p < 0.01) amounts of serum antigen as compared to control Sepharose.

Acetylgalactosaminyl groups. The specificity of this interaction was confirmed by competitive inhibition using β-N-acetylgalactosamine (100 mg/ml). Negligible binding to Protein A-Sepharose suggested the absence of circulating immune complexes. Significant amounts of antigen were not observed to react with other lectins, including concanavalin A, lentil lectin, and peanut agglutinin.

**DISCUSSION**

The cell-surface component(s) recognized by McAb F36/22 are associated with a lineage of epithelial tumors from ductal origin (6, 7, 21). These components have been shown to be effective targets for both in vivo antibody delivery and passive immunotherapy (2, 3). The present results further show that ductal carcinoma antigen is expressed within sera from patients with breast cancer, existing in a high-molecular-weight fraction.

All human sera examined have shown detectable levels of antigen activity. The source of antigen, as occurring in the circulation of normal individuals, is not known. Since a few normal ductal epithelial structures have been shown to express antigen (6, 21), their contribution to circulating immunoreactivity appears probable. However, the quantitative range of antigen levels in normal serum controls is narrow and may be discriminated from specimens of patients' sera.

**Discussion**

The highest incidence and levels of circulating DCA were associated with patients with clinical evidence of breast cancer. The incidence of elevated serum levels in this group (approximately 50%) may be based upon our previous observations, which indicate that only a subset of breast tumors expresses antigen (6). Since the use of McAbs is associated with the description of intertumoral antigenic heterogeneity (8, 13), this observed incidence is not surprising. There does appear to be, also, a strong correlation between the incidence of serum DCA levels and intratumoral expression of antigen. Tumor histotypes such as breast cancer, which exhibit a high incidence of tumor antigen (6), also show a related incidence of elevated circulating DCA values. These data predict that patients with mesenchymally derived tumors may present with low frequencies of circulating DCA (6, 21). Of clinical importance, these data also suggest that patients with ovarian ductal carcinomas may exhibit a high incidence of DCA serum elevations. As shown before, 100% of such tumors express antigen (6, 7), and serodetection for this group is currently underway. In any event, it may be possible to assist in the differential diagnosis of ductal carcinomas, as based upon serum values, in a manner analogous to our studies performed using solid tumor specimens (6, 7).

As presently described, using a limited number of mastectomy patients with primary localized disease, a significant decrease in serum DCA occurs postoperatively. These data indirectly indicate a relationship between serum DCA levels and tumor load, suggesting that such measurements may be of value for patient monitoring. Perhaps equally important, for more basic reasons, is the fact the DCA values in no instance were seen to increase postsurgically. This directly implies that the antigen under measurement does not represent a nonspecific "acute-phase reactant," the levels of which sharply increase following surgical therapy (12).

Some aspects of the enzyme immunoassay procedure are worth commenting upon. The format of the assay is dependent upon the occurrence of multiple antibody-combining sites on DCA, thereby permitting a sandwich-type of procedure. In our
experience, most McAbs react with singly expressed determinants and therefore are not amenable to detection using this format. Also, antigen recovery experiments were initially performed, wherein a constant amount of antigen was mixed with increasing concentrations of standard human serum. Results demonstrated a “masking” of antigen activity at high serum concentrations. For example, detection of input antigen (10 units) in 2-fold and 10-fold dilutions of serum was approximately 10 and 40%, respectively. Therefore, significant dilutions of test samples are required for both normal and pathological sera.

Physical studies indicate that, in the circulation, DCA represents glycoprotein material of high molecular weight. Thus, the antigen appears distinct from previously described serum antigens of breast or ovary cancer (1, 5, 16, 19). However, the high density and lectin-binding characteristics of serum antigen, along with its large size, allude to a proteoglycan or mucin-like structure (4, 11). Mucin-like material recently has been identified in the circulation of patients using anticolorectal carcinoma McAb 19-9 (18). Since the carbohydrate composition of mucin varies in different patients and also according to blood group systems (14), both antibodies may recognize separate polymorphic carbohydrate determinants carried by related mucin-like components.

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REFERENCES

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