Elevated Levels of a High Molecular Weight Antigen Detected by Antibody W1 in Sera from Breast Cancer Patients

Peter S. Linsley, Vincent Ochs, Sharon Laska, Diane Horn, David B. Ring, Arthur E. Frankel, and Joseph P. Brown

ABSTRACT

A novel screening assay was used to test 13 previously described anti-breast cancer antibodies for those which recognize antigens elevated in serum of breast cancer patients. Binding of three of these antibodies to breast or lung carcinoma cells was inhibited to a significantly greater extent by tumor patient serum than by normal serum, suggesting that the antigens might be useful serum markers. Two of these antibodies, W1 and W9, were shown to recognize nonoverlapping epitopes on a high molecular weight molecule(s) purified from serum from breast cancer patients. A sensitive double determinant immunoassay was developed to measure W1 antigen levels in sera from a total of 389 cancer patients and controls. Forty seven % (37 of 79) of individuals having breast cancer showed elevated serum levels of the W1 antigen, whereas only 4% (1 of 25) of normal controls showed elevated levels. These differences were statistically significant (P < 0.001). The percentage of breast cancer patients showing elevated serum levels was greater for individuals with metastatic disease. Statistically significant numbers of lung, ovarian, and prostate, but not colon, cancer patients also had elevated serum levels of the W1 antigen. These data suggest that measurement of the W1 antigen in serum might provide clinically useful information on the course of metastatic breast and other cancers.

INTRODUCTION

The progression of malignant diseases can in some cases be monitored by measuring serum levels of appropriate tumor markers. Several immunological assays for the detection of blood-borne tumor antigens have been developed. These can be categorized into two broad groups based on whether or not the antigen recognized has mucin-like properties. Non-mucin-like antigens are exemplified by α-fetoprotein, carcinoembryonic antigen, and prostate-specific antigen which can provide information on the clinical course of hepatic, colorectal, and prostate cancers, respectively (1, 2). Several promising non-mucin-like serum markers for breast cancer have also been described (3-5).

Mucin-like antigens are detected by many monoclonal antibodies having usefulness in serum diagnostic tests. Serum tests for gastrointestinal (6), pancreatic (6, 7), ovarian (8), and breast cancers (9-11) are all based on mucin-like antigens. A serum test for lung cancer based on a mucin associated determinant (12) has also been described (13).

Some of us have previously described a panel of monoclonal antibodies reactive with human breast cancer (14). The antibodies were originally developed with the goal of producing human breast cancer-specific immunotoxins. Because of the need for better procedures for monitoring and diagnosing breast cancer, we have evaluated these previously described antibodies for their usefulness in serum assays. In this paper, we show that two of these antibodies react with nonoverlapping epitopes on a high molecular weight component of sera from breast cancer patients. We present clinical data showing that elevated levels of this antigen are found in sera from significant numbers of patients having breast, lung, ovary, and prostate cancers.

MATERIALS AND METHODS

Cells

MCF7 cells were obtained from Dr. Marc Lippman, NIH, and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and insulin (0.6 µg/ml). Calu-1 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cell lines derived from Calu-1 were used for cell binding experiments. These lines bind elevated amounts of W1, W5, and W9 antibodies; their isolation and characterization will be reported elsewhere. Briefly, W1S4, W5S4, and W9S4 are enriched cell populations derived from Calu-1 by repeated selection on the fluorescence activated cell sorter for cells binding elevated amounts of W1, W5, and W9, respectively. W5-6 is a clonal line derived from the W5S4 population. These derivative cell lines bind more than 5-fold more 125I-labeled W1 and W5 antibodies than the parental Calu-1 cells and more than 50-fold more 125I-W9 antibody. All lines were periodically checked for the presence of mycoplasma. All experiments were performed with lines showing no detectable contamination.

Cell Binding Assays

Cells were harvested by trypsinization, seeded at 3-18 x 10⁴ cells/cm² in multiwell plastic dishes (48 or 96 well), and maintained at 37°C for 18 h prior to initiation of the experiment. Monolayers were washed and fixed in situ with 0.5% paraformaldehyde prior to antibody addition and blocking with binding buffer as previously described (15). Direct Binding Assay. Direct binding assays were conducted using a previously described procedure (15). 125I-labeled W1, W5, or W9 antibodies (specific activities were approximately 1 x 10⁶ cpm/ng) were added at the indicated concentrations in the presence or absence of competing solutions. The binding reaction was allowed to proceed for 1 h at 23°C, monolayers were washed with binding buffer, solubilized in 0.5 N NaOH, and counted in a gamma counter. Nonspecific antibody binding was measured in the presence of a 50-fold excess of unlabeled antibody and was generally less than 10% of total binding. Control experiments showed that all antibodies tested bound specifically to both untreated and formalin-fixed cells.

Indirect Binding Assay. Formalin-fixed cell monolayers were incubated with unlabeled monoclonal antibodies at 0.5 µg/ml in binding buffer containing 25% by volume of serum pools from five normal individuals or breast cancer patients for a period of 1 h at 23°C. Monolayers were then washed and incubated with 125I-labeled goat anti-mouse immunoglobulin (Hyclone; specific activity, ~1 x 10⁷ cpm/ng) for an additional 1 h at 23°C. Finally, monolayers were washed twice with binding buffer, solubilized with 0.5 M NaOH, and cell-bound radioactivity was determined using a gamma counter. The difference in inhibitory activity of tumor patient sera as compared with normal sera was calculated as

\[
\text{cpm bound in normal sera - cpm bound in tumor sera}
\]

\[
\text{cpm bound in normal sera}
\]
W1 DDIA

Ninety-six-well plastic dishes (Immulon II) were coated with a solution of W1 antibody (0.5 \( \mu \)g antibody in a volume of 0.05 ml of 0.05 M Tris, pH 8.0) for 1 h at 23°C. Plates were then blocked with 1% gelatin in the same buffer for an additional 1 h at 23°C. A volume of 0.05 ml of serum (diluted as indicated in a solution of 5% (w/v) bovine serum albumin-0.1% (w/v) Na\(_2\)S\(_2\)O\(_3\) in 0.05 M Tris, pH 8.0) was added to each test well and the plate was incubated at 23°C for approximately 16 h. \(^{125}\)I-labeled W1 antibody (15 ng in a volume of 0.05 ml) was added to the wells, incubated for 30 min at 23°C, and the wells were washed three times with phosphate-buffered saline. Bound \(^{125}\)I-labeled W1 antibody was measured, and values were determined for individual sera using various dilutions of a reference serum from a breast cancer patient.

Production and Purification of Monoclonal Antibodies

The production, isotyping, purification, and antigen determination of the anti-breast tumor monoclonal antibodies used in this study have been previously described (14). All antibodies were used in purified form.

Analytical Procedures

Gel electrophoresis and immunoblotting were performed as described (15). Monoclonal antibodies bound to nitrocellulose-immobilized antigen were visualized by a colorimetric reaction following addition of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin serum (Tago) according to a published procedure (16). The procedure used for immunoprecipitation analysis was previously described (15).

Serum Collection

Blood was drawn from the indicated individuals and allowed to clot at 23°C for 10–90 min. Samples were then stored at 4–6°C for 0.5–5 h prior to centrifugation (16,000 \( \times \) g min) in a clinical centrifuge. Serum was separated from the clot, aliquoted, and frozen at −70°C.

Sera from a total of 395 individuals were used. The clinical stage of disease in these individuals at the time of serum collection was noted as disease free, only primary tumor present, regional lymph nodes involved, or metastases present.

Purification of the W1 Antigen from Serum

Antigenic activity was monitored at each step of the purification by assessing the ability of various fractions to inhibit the direct binding assay. One unit of activity was defined as the amount of material required to inhibit by 50% the binding of \(^{125}\)I-labeled W1 or W9 (0.4 \( \mu \)g/ml) to formalin-fixed monolayers of W5-6 cells.

Chloroform/Methanol Extraction. Serum (10 ml) was mixed sequentially with six volumes of CHCl₃ and three volumes of methanol. Phase separation was accomplished by a brief centrifugation, and the aqueous phase was carefully removed. The organic phase was twice reextracted with 10–15 ml of H₂O, the aqueous phases were pooled, assayed for activity, and lyophilized.

CsCl Density Gradients. The procedure used was as previously described (17). The lyophilized residue was dissolved in 6 M guanidine HCl, and solid CsCl was added to a density of 1.4. The mixture was subjected to centrifugation at 55,000 rpm in a Beckman Ti 70.1 rotor for a period of 42 h. Gradients were dripped into 10 equal fractions and the density of each was measured gravimetrically. All fractions were dialyzed versus distilled H₂O and assayed for activity. Fractions having a density of 1.33–1.47 (peak, 1.39) contained most of the antigenic activity, whereas the bulk of the protein was contained in fractions of density <1.30.

Immunofinity Chromatography. The procedure for immunofinity chromatography has been previously described (15). Briefly, active fractions from the CsCl gradient were pooled and mixed with W1-conjugated Sepharose 4B (5 mg antibody/ml resin) at a volume ratio of 4:1 (serum:resin). The mixture was rotated overnight at 4°C, poured into a disposable syringe plugged with glass wool, and washed as described (15). Elution of bound antigen was accomplished by addition of 75 mM triethylamine.

RESULTS

Selection of Monoclonal Antibodies. A panel of antibodies which react with human breast cancer was tested to identify those which recognize antigens present at elevated levels in patient serum. Since this panel of antibodies appear to recognize a rather limited number of antigens (14), we used several criteria to select a subset of these antibodies for evaluation. Attempts were made to evaluate at least one antibody from each group of antibodies recognizing a single antigen. Antibodies recognizing antigens on normal liver and spleen were not evaluated, since normal serum levels of antigens present on these organs would be expected to be high; also, antibodies were selected which recognized antigens present on a high percentage of breast tumors. Finally, since many of the useful tumor serum markers discovered to date recognize a class of high molecular weight mucin-like molecules, we selected anti-breast tumor antibodies which recognize high molecular weight antigens (14).

Using these criteria, we selected a set of 13 monoclonal antibodies to evaluate for usefulness in serum assays. These antibodies, their isotypes, and designations, used previously and in the present study, are listed in Table 1.

Development of a Rapid Assay to Identify Serum Antibodies. In order to systematically and rapidly screen for antibodies present at elevated levels in tumor serum we developed the indirect competitive radiometric cell-binding assay described in “Materials and Methods.” This assay is based on the ability of antigen in serum to competitively inhibit the binding of antibodies to their antigens on formalin-fixed monolayers of cultured tumor cells. The binding of monoclonal antibodies in the presence or absence of serum is measured by the addition of an \(^{125}\)I-labeled secondary antibody. To minimize nonspecific effects of human serum on binding of the \(^{125}\)I-labeled antibody, the inhibitory activity of a pool of normal sera. Antigens present at elevated levels in sera from cancer patient will result in greater inhibition of antibody binding by tumor serum pools than by normal serum pools. It should be emphasized that factors other than antigen in serum (e.g., human antibodies to tumor antigens) conceivably could lead to apparent inhibition of the

<table>
<thead>
<tr>
<th>Designation</th>
<th>Previous</th>
<th>Isotype</th>
<th>Antigen (M₀)</th>
</tr>
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<tbody>
<tr>
<td>W1</td>
<td>3G3</td>
<td>KG1</td>
<td>High molecular wt.</td>
</tr>
<tr>
<td>W2</td>
<td>9C6</td>
<td>KM</td>
<td>70,000</td>
</tr>
<tr>
<td>W3</td>
<td>35E10</td>
<td>KM</td>
<td>80,000</td>
</tr>
<tr>
<td>W4</td>
<td>113F1</td>
<td>KG3</td>
<td>37,000; 60,000; 100,000</td>
</tr>
<tr>
<td>W5</td>
<td>120H7</td>
<td>KM</td>
<td>High molecular wt.</td>
</tr>
<tr>
<td>W6</td>
<td>219F3</td>
<td>KG1</td>
<td>Not determined</td>
</tr>
<tr>
<td>W9</td>
<td>24S7</td>
<td>KG1</td>
<td>High molecular wt.</td>
</tr>
<tr>
<td>W10</td>
<td>266B2</td>
<td>KG1</td>
<td>55,000</td>
</tr>
<tr>
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<td>43,000</td>
</tr>
<tr>
<td>W12</td>
<td>369F10</td>
<td>KM</td>
<td>High molecular wt.</td>
</tr>
<tr>
<td>W13</td>
<td>454C11</td>
<td>KG2a</td>
<td>110,000; 210,000</td>
</tr>
<tr>
<td>W18</td>
<td>87H7</td>
<td>KG1</td>
<td>230,000</td>
</tr>
<tr>
<td>W19</td>
<td>454A12</td>
<td>KG1</td>
<td>97,000</td>
</tr>
</tbody>
</table>

* Molecular weights refer to sizes of antigens identified in breast tumor cell lines.

Table 1 Characteristics of antibodies evaluated

Properties of the designated antibodies were previously reported (11) or have been more recently determined using published techniques.
binding assay. It should also be emphasized that this assay will
detect only antibodies recognizing antigens present in relatively
high levels in serum from tumor patients.

We used this assay to screen the 13 anti-breast tumor anti-
bodies for those for which binding was inhibited to a greater
degree by breast cancer than by normal serum pools. Of these
antibodies, most detected antigens present on both the MCF-7
(breast carcinoma) and Calu-1 (lung carcinoma) cell lines. The
binding of the antibodies W1 and W9 to either cell line was
inhibited to a substantially greater extent (>50%) by sera from
tumor patients than by sera from normal individuals. The
binding of antibody W5 was sometimes substantially inhibited
by tumor sera depending on the particular serum pool used.
Binding of all other antibodies tested was not reproducibly
inhibited to a significantly greater extent (>25%) by sera from
tumor patients. We therefore chose antibodies W1, W5, and
W9 for further characterization.

Recognition of Different Epitopes by Antibodies W1, W5, and
W9. To determine whether the epitopes recognized by antibod-
ies W1, W5, and W9 were related, we compared the abilities of
each antibody to compete for binding of the other antibodies.
Each antibody was radiolabeled with \( ^{125}\text{I} \), and fixed concentra-
tions of the labeled antibodies were individually added to target
cells in the presence of increasing amounts of unlabeled anti-
bodies. As shown in Fig. 1, the binding of each radiolabeled
antibody was inhibited in a dose-dependent fashion by addition
of the corresponding unlabeled antibody, an indication that the
binding measured was specific. Unlabeled W9 antibody did not
inhibit binding of \( ^{125}\text{I} \)-labeled W1 antibody, even at a 50-fold excess of unlabeled to labeled antibody. At lower concentrations
of unlabeled W9, the binding of W1 was actually slightly, but
reproducibly, enhanced. Conversely, unlabeled W1 antibody did
not compete for binding of \( ^{125}\text{I} \)-labeled W9 antibody at high
concentrations, but actually stimulated binding at low concen-
trations. Since W1 and W9 do not compete with one another
for binding, we conclude that they recognize distinct epitopes.

The inhibition of \( ^{125}\text{I} \)-labeled W5 antibody binding is more
complex. W5 does not compete for binding of W1 or W9, but
in the reciprocal experiment, both W1 and W9 are able to
compete for binding of W5. This result is not easily explained
by the binding affinities of the respective antibodies (data not
shown). These results suggest that binding of the bulky W5
antibody (IgM isotype) may be inhibited by binding of other
antibodies but that it probably binds to a distinct epitope. Also
suggestive that W5 represents a distinct epitope is the finding
that W5 antibody binds to a significantly lower proportion of
breast tumors tested by immunohistology (14). In summary,
antibodies W1, W5, and W9 appear to recognize distinct epi-
topes.

Direct Demonstration of Antigens in Serum of Breast Tumor
Patients. To measure more precisely the antigen levels in sera,
we developed a direct competitive radiometric binding assay for
measuring each of the three antigens defined by antibodies W1,
W5, and W9. The methodology used was similar to that used
in the indirect binding assay described above. \( ^{125}\text{I} \)-labeled anti-
bodies were mixed with varying amounts of serum from normal
donors or patients with advanced breast cancer and added to
monolayers of target cells fixed in situ with formalin. As shown
in Fig. 2, sera from both normal volunteers and breast cancer
patients inhibited in a dose-dependent fashion in the binding of
\( ^{125}\text{I} \)-labeled antibodies W1, W5, and W9 to target cells. Sera
from breast cancer patients generally gave comparable levels of
inhibition at significantly higher dilutions than did normal sera.
These results confirmed and extended our observations made
using the indirect inhibition assay and indicated that all three
antibodies recognize antigen(s) present in elevated levels in
serum from breast cancer patients. Observations of this sort
indicated that the proportion of advanced cancer patients hav-
ing antigen levels elevated above normal was greater for W1
and W9 than for W5. Both the extent of antigen elevation and
the percentage of sera showing elevated antigen levels were
lower for W5 than for the other two antibodies indicating that
the former was less likely to be useful for serum tests.

Copurification of W1 and W9 Antigens from Serum. In order

![Fig. 1. Recognition by antibodies (ab) W1, W5, and W9 of distinct epitopes on Calu-1 cells. Cells (1 x 10⁶) were grown in 48-well plates and fixed in situ as described in "Materials and Methods." \( ^{125}\text{I} \)-labeled antibodies (0.3 μg/ml) were mixed with the indicated concentrations of unlabeled antibodies, added to cells, and binding of \( ^{125}\text{I} \)-labeled antibody was measured. Top, W1S4 cells were used; middle, W5S4 cells; bottom, W9S4 cells. In the absence of competitor, 12,180 cpm of \( ^{125}\text{I} \)-labeled W1 was bound, 2,560 cpm of \( ^{125}\text{I} \)-labeled W5, and 4,828 cpm of \( ^{125}\text{I} \)-labeled W9.

![Fig. 2. Dose-dependent inhibition of W1, W5, and W9 antibody binding by breast cancer patient and normal sera. Direct binding of \( ^{125}\text{I} \)-labeled W1, W5, and W9 antibodies was measured in the presence of the indicated amounts tumor patient serum (○) or normal serum (○).](https://cancerres.aacrjournals.org/doi/10.1158/0008-5472.CAN-86-1143)
found on the same molecule (Fig. 3). We chose therefore to would be predicted from the fact that W1 and W9 epitopes are epitopes are on the same molecule. Interestingly, the W5 anti body, whose epitope on cultured cells is spatially related to W1 tion of the alternative antigen, thereby indicating that both levels of W1 and W9 antigens in a number of sera from cancer patients and normal controls. There was a general correlation between W1 and W9 values determined on the same sera, as as would be predicted from the fact that W1 and W9 epitopes are found on the same molecule (Fig. 3). We chose therefore to characterize the antigens recognized by W1 and W9 antibod to further investigate this possibility, we subjected the 125I- and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3), revealed three main components: a diffuse high molecular weight component (estimated M, 330,000; see Fig. 4 legend), and two others of M, 68,000 and 50,000. Western blotting (not shown) and immune precipitation experiments (Fig. 3) indicate that the high molecular weight component was immunologically active. The M, 50,000 component was precipitated in the absence of monoclonal antibody indicating that it was nonspecifically precipitated. We conclude that the W1 antigen found in serum is a high molecular weight protein which because of its increased buoyant density is most likely heavily glycosylated.

The fractionation procedure used for the W1 antigen resulted in a substantial copurification of W9 antigenic activity (Table 2). The ratio of W1: W9 activity was increased by a factor of three during the purification procedure, indicating that either (a) W9 activity is more labile during purification or (b) some W9 activity is contained on molecule(s) other than those carrying W1 activity; nonetheless, these data suggested W1 and W9 epitopes most likely were carried on the same molecule.

To further investigate this possibility, we subjected the 125I-labeled immunoaffinity-purified preparation to sequential immuno precipitation analysis. Purified W1 antigen (50 inhibitory units of activity) was radiolabeled with 125I as described previously (5). Aliquots of 5% of the total 125I-labeled preparation were then subjected to immune precipitation (IP) analysis as indicated (first immune precipitation). The supernatant from the first immune precipitation was subsequently treated with the indicated antibodies during the second immune precipitation. The monoclonal antibody was omitted from the reaction. No IP, not subjected to immunoprecipitation; this sample contained 38% of the 125I-labeled antigen analyzed in other lanes. Indicated molecular weights were estimated by comparison with mobility of standards: ovalbumin, 43,000; serum albumin, 68,000; phosphorylase b, 92,400; $\beta$-galactosidase, 117,000; myosin, 200,000; spectrin, 215,000 and 240,000. The molecular weight of the W1 antigen (330,000) represents an extrapolated value which must be considered as an estimate due to the expected heavily glycosylated nature of the antigen and the unavailability of standard proteins in that size range.

develop more precise immunoassays for only one of these antibodies, W1.

To more accurately determine the usefulness of W1 epitope as a serum diagnostic, we developed a DDIA. Titration curves for several normal and breast cancer patient sera were determined using this assay, as shown in Fig. 4. All sera tested gave relatively linear results over a wide concentration range. At higher serum concentrations, "hook" effects were seen with some sera, particularly those from breast cancer.

Serum levels of W1 antigen from a larger group of individuals were then determined using DDIA. Bound radioactivity determined for individual sera was converted to arbitrary units using a standard curve derived from a frozen reference serum, which was arbitrarily assigned an antigen concentration of 1000 units/ml. The data obtained for various patient groups are presented in Fig. 5, and are statistically analyzed in Table 3.

Forty seven % (37 of 79) of the breast cancer patients tested had circulating levels of W1 antigen greater than 60 units/ml.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total (units)*</th>
<th>Specific (units/mg)$b$</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>21,000 (100)</td>
<td>35.3</td>
<td>1</td>
</tr>
<tr>
<td>CHCl$_3$ extract</td>
<td>16,700 (80)</td>
<td>161</td>
<td>4.5</td>
</tr>
<tr>
<td>CsCl pool</td>
<td>9,650 (46)</td>
<td>386</td>
<td>10.9</td>
</tr>
<tr>
<td>W1 affinity</td>
<td>2,080 (9.9)</td>
<td>67,100</td>
<td>1,900</td>
</tr>
</tbody>
</table>

* One unit is defined as the amount of material required to inhibit by 50% binding of $^{125}$I-labeled antibodies added at 0.4 $\mu$g/ml to 2 x 10$^6$ W5C16 cells.

$^{a}$ Numbers in parentheses, percentage initial recovery.

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Table 2 Copurification of W1 and W9 antigens from serum

<table>
<thead>
<tr>
<th>Step</th>
<th>Total (units)</th>
<th>Specific (units/mg)</th>
<th>Purification factor</th>
<th>Ratio, W1: W9</th>
</tr>
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<tbody>
<tr>
<td>Serum</td>
<td>10,600 (100)</td>
<td>17.8</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CHCl$_3$ extract</td>
<td>8,640 (81)</td>
<td>83.0</td>
<td>4.7</td>
<td>1.9</td>
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<tr>
<td>CsCl pool</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>6.34</td>
</tr>
<tr>
<td>W1 affinity</td>
<td>350 (3.3)</td>
<td>11,300</td>
<td>Not determined</td>
<td></td>
</tr>
</tbody>
</table>

* Protein was determined according to Markwell et al. (25).

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To characterize the antigens recognized by W1 and W9 antibodies, we purified the W1 antigen as described in "Materials and Methods" and summarized in Table 2. Antigenic activity was monitored using the direct competitive binding assay shown in Fig. 2.

Precursor labeling and carbohydrate staining of the W1 antigen purified from cultured cells had indicated that the antigen was a large, heavily glycosylated molecule (data not shown). These findings suggested that the W1 antigen in serum might be separable from the bulk of serum proteins by CsCl density gradient centrifugation in guanidine HCl (17). Serum was first delipidated to improve separation during subsequent fractionations and then subjected to density gradient fractionation. Fractions enriched for W1 antigenic activity were then subjected to final purification by immunoaffinity chromatography using W1-Sepharose (Table 2).

The purified preparation, which had been labeled with $^{125}$I and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3), revealed three main components: a diffuse high molecular weight component (estimated M, 330,000; see Fig. 4 legend), and two others of M, 68,000 and 50,000. Western blotting (not shown) and immune precipitation experiments (Fig. 3) indicate that the high molecular weight component was immunologically active. The M, 50,000 component was precipitated in the absence of monoclonal antibody indicating that it was nonspecifically precipitated. We conclude that the W1 antigen found in serum is a high molecular weight protein which because of its increased buoyant density is most likely heavily glycosylated.

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To further investigate this possibility, we subjected the $^{125}$I-labeled immunoaffinity-purified preparation to sequential immune precipitation analysis (Fig. 3). Depletion of the preparation for W1 or W9 activity resulted in the concomitant depletion of the alternative antigen, thereby indicating that both epitopes are on the same molecule. Interestingly, the W5 antibody, whose epitope on cultured cells is spatially related to W1 and W9 does not immunoprecipitate the high molecular weight W1 antigen from serum (data not shown).

Circulating Levels of W1 Antigen in Cancer Patients and Controls. The direct inhibition assay was used to measure serum levels of W1 and W9 antigens in a number of sera from cancer patients and normal controls. There was a general correlation between W1 and W9 values determined on the same sera, as would be predicted from the fact that W1 and W9 epitopes are found on the same molecule (Fig. 3). We chose therefore to develop more precise immunoassays for only one of these antibodies, W1.

To more accurately determine the usefulness of W1 epitope as a serum diagnostic, we developed a DDIA. Titration curves for several normal and breast cancer patient sera were determined using this assay, as shown in Fig. 4. All sera tested gave relatively linear results over a wide concentration range. At higher serum concentrations, "hook" effects were seen with some sera, particularly those from breast cancer.

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Forty seven % (37 of 79) of the breast cancer patients tested had circulating levels of W1 antigen greater than 60 units/ml.
HIGH MOLECULAR WEIGHT ANTIGEN DETECTED BY ANTIBODY W1

Fig. 4. Double-determinant immunoassay for W1 antigen. The W1 double-determinant immunoassay was performed on indicated dilutions of three different tumor patient (•, △, ■) and normal sera (○, ▽, ▼). Serum arbitrarily assigned a concentration of 1000 units/ml and used as a reference standard. Units on upper x-axis, arbitrary units assigned to the reference serum.

Fig. 5. Levels of W1 antigen in sera from cancer patients and controls. Sera from a number of individuals from the indicated patient groups were analyzed for W1 antigen using DDIA. Bound radioactivity was converted to arbitrary units by comparison to a standard curve obtained with a reference serum which was assigned a concentration of 1000 units/ml (see Fig. 4). Antigen levels for individual sera were determined at a dilution corresponding to the linear portion of the standard curve; thus, values for sera having antigen concentrations in the normal range were determined at a 200-fold dilution, whereas those for sera having higher concentrations were determined at a greater dilution. ——, cutoff value of 60 units/ml.

In contrast, only 4% (1 of 28) of normal individuals and 2% (1 of 47) of individuals hospitalized with nonmalignant diseases had elevated levels; likewise, only 14% (3 of 22) of patients with nonmalignant breast diseases had elevated serum levels, indicating that the elevations were associated with malignant disease.

The percentage of breast cancer patients having elevated serum levels was related to the extent of disease (Table 3). W1 levels were lower in patients with nonmetastatic disease. In contrast, two thirds (33 of 49) of patients with metastatic disease showed elevated levels. The site of metastasis was an important determinant of serum antigen levels, since greater numbers of patients having metastases to internal organs (lungs, liver, etc.) had elevated levels. No obvious correlation was noted between W1 levels and estrogen or progesterone receptor levels, age, sex, or therapeutic regimen.

Significant numbers of patients having elevated antigen levels were seen with cancers other than breast. Statistically significant numbers of prostate cancer patients showed elevated levels (13 of 30 positive; 

DISCUSSION

We have described a rapid assay to select anti-tumor monoclonal antibodies recognizing blood-borne tumor antigens. We used this assay to screen a panel of 13 previously described antibodies reactive with breast cancer (14) for those detecting antigens elevated in serum of breast cancer patients. Of the antibodies tested, only those which detected high molecular weight antigens appeared to have potential usefulness in serum assays. Two antibodies, W1 and W9, were shown to recognize the same molecule in serum.

The screening assay we have described represents a tool for identifying useful antibodies. Preliminary results have sug-
gested that this assay will work with hybridoma supernatants as the source of antibody. The assay is thus applicable to routine screening of hybridomas, thereby minimizing the work involved in selecting antibodies useful for serum assays. We hope in this way to select new antibodies detecting elevated serum levels of tumor-associated antigens; hopefully, by examining a number of antibodies selected only for their ability to detect antigens released into the serum, we will discover antibodies having greater specificity and sensitivity for those previously described. It may also be possible, by using serum pools from individuals who do not have elevated levels of a given epitope, to select antibodies which will complement the specificity of existing antibodies.

The antibody, W1, was used as the basis for a sensitive DDIA which has apparent potential as a serum diagnostic test. Since serum antigen levels were higher in patients with metastatic disease, it would appear that the most likely use for the test would be to monitor the clinical course of breast cancer, as has been done with other monoclonal antibody based serum tests (8–11). Serum antigen levels may have prognostic usefulness for patients where stage of disease cannot accurately be assessed. Antigen levels might also be of use for monitoring the progression of disease in patients in clinical remission. Finally, serum antigen levels could be an indication of response to tumor therapy. The data presented in this paper will allow design of appropriate experiments to test these possibilities.

Many groups have described monoclonal antibodies which recognize high molecular weight mucin-like antigens from different tumor types (6–8, 10–11, 18–22). A number of these antigens have been used as serum markers for different types of cancer, including pancreatic and gastrointestinal (6), ovarian (8), pancreatic (6, 7), and breast (9–11). High molecular weight antigens thus appear to be highly immunogenic components of a number of different tumor types. It is not yet clear whether these antibodies all detect different epitopes or if the epitopes detected are found on the same or different molecules.

In the case of breast cancer mucins, it is unclear how many breast cancer specific epitopes exist, largely because structural data is lacking on the epitopes recognized by existing antibodies; however, since differences in antibody specificity have been reported, current data would suggest that there may be many different epitopes with different specificity patterns. It is therefore of interest to compare the specificity of the W1 DDIA with those of previously described serum tests. Although we recognize that the various assays were performed on different serum samples, comparisons of specificity may suggest possible relationships which can then be tested in side-by-side fashion at a later date.

Both Papsidero et al. (10) and Hayes et al. (11) have described serum tests for breast cancer based on high molecular weight antigens. Both of these tests detected elevated antigen levels in sera from advanced breast cancer patients. Papsidero et al. (10) described elevated levels of antigen F36/22 in serum of 56% of late stage breast cancer patients, while Hayes et al. (11) reported elevated levels of the DF3 antigen in 72–76% of similarly advanced patients. Using the W1 DDIA, we detected elevated antigen levels in 67% of patients having metastatic breast cancer, a figure which compares favorably with previous reports. Hayes et al. (11) also show a higher percentage of positives in patients with visceral as compared with nonvisceral metastases, as we have demonstrated for the W1 antigen (Table 3). Our test differs from that of Papsidero et al. (10) in its ability to detect elevated antigen levels in sera from prostate cancer. F36/22 antigen levels were not found elevated in a statistically significant number of patients with prostate cancer. In contrast, the W1 DDIA detected elevated antigen levels in 13 of 30 prostate cancer patients, a value statistically significant at the P < 0.001 level. Our test may differ from that of Hayes et al. (11) in its ability to detect elevated antigen levels in lung cancer patients. Hayes et al. (11) reported elevated antigen levels in only 1 of 11 lung cancer patients. We examined a larger series of lung cancer patients (N = 70) and found elevated serum levels in 13 of 70, a value significant at the P < 0.05 level.

Thus, although there are similarities between the W1 DDIA and previously reported tests, there are apparent differences as well. These differences may be of clinical usefulness. It may be possible, for instance, to use the W1 DDIA in conjunction with previously described assays for breast (10, 11), prostate (2), and lung (13) to detect increased numbers of patients. Further studies will be required to determine which tests are superior and if useful combinations exist.

Many of the antibodies with specificity for breast tumor mucins recognize a high molecular weight component(s) on the milk fat globule membrane (18–23). We have also detected W1, W5, and W9 epitopes on high molecular weight components of the milk fat globule membrane (data not shown); however, since this component is quite heterogeneous in nature (23) it could conceivably contain several different antigenic species. In an experiment identical to the one shown in Fig. 3, W9 antibody was also shown to react with W1 affinity-purified mucin from human milk (data not shown); thus, there are at least two distinct epitopes on the W1 antigen found in breast cancer patient serum and in human milk, indicating that these molecules, while physically heterogeneous, may contain common antigenic determinants. It is of interest that the epitope recognized by the W5 antibody is also carried on the W1 affinity-purified milk mucin* in contrast to the case with the serum mucin, where we have been unable to detect W5 antigenic activity on W1 affinity-purified mucin from several different individuals.

These data are similar to the findings of Burchell et al. (24) who showed that the relative levels of epitopes recognized by antibodies HMFG-1 and HMFG-2 varied with the source of antigen. Taken together, these data suggest that epitopes may differ in their densities on mucin-like molecules from different sources. This possibility raises the question of whether antibodies can be identified which recognize epitopes which vary independently in their serum levels from those previously described. By using combinations of antibodies having this property, it may be possible to develop better serum tests for a number of human cancer types.

ACKNOWLEDGMENTS

We wish to thank Dr. Michael Bean and Rissa Sanchez of Virginia Mason Research Center for providing serum samples; Jeff Kallestad and Diane Decker-Ihle for technical help; Anne Little and Bonnie Kirk for computer data entry and for typing the manuscript; Dr. Karl-Erik Hellström for critically reviewing the manuscript; and Dr. George Todaro for his help in coordinating the initiation of these experiments.

NOTE ADDED IN PROOF

Since the submission of this paper, another report has been published describing the use of antibodies to a high-molecular-weight mucin antigen for serum assays. (Hilkens, J., Kroezen, V., Bonfrer, J. M. G., De Jong-Bakker, M., and Bruning, P. F. MAM-6 antigen, a new serum marker for breast cancer monitoring, Cancer Research, 46: 2582–2587, 1986.)

* Unpublished observations.
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Elevated Levels of a High Molecular Weight Antigen Detected by Antibody W1 in Sera from Breast Cancer Patients

Peter S. Linsley, Vincent Ochs, Sharon Laska, et al.


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