Reactivity of Serum-armed Xenogeneic Macrophages to Breast Cancer Antigens

Lester F. Harris,¹ Lynda L. Miller, and David F. Hickok²

Cancer Research Laboratory, Abbott-Northwestern Hospital, Minneapolis, Minnesota 55407

ABSTRACT

Sera from breast cancer patients contained cytotoxicity to body which armed guinea pig peritoneal macrophages. These macrophages then exhibited specific adherence inhibition in the presence of tissue culture tumor antigens. These antigens were obtained from primary cultures of autologous or allogeneic breast cancer cells. Sera from control subjects for the most part did not induce macrophage adherence inhibition in the presence of these same antigens.

INTRODUCTION

Results of several studies have suggested that human breast cancers share common tumor-specific antigens (1–4, 7, 13, 16, 21). Studies in our laboratory have demonstrated the presence of blocking factors residing in the IgG serum fraction of patients tested with active breast cancer (14, 16). These observations were based on lymphocyte activation by breast cancer antigens and subsequent blocking of autologous tumor cytotoxicity with the patient’s serum. However, this assay is not practical as a screening test for breast cancer since it requires actively growing tumor cells and is difficult to interpret in allogeneic systems.

Halliday and Miller (11) developed a 2-hr in vitro test for antigen recognition by leukocytes, the LAI.³ Subsequently, Holan et al. (17), using a rat model, described an antigen-induced LAI assay performed in glass test tubes. Grosser and Thomson (9) adapted the test tube assay for studying cellular immunity in human breast cancer. Grosser et al. (8) demonstrated that antigen-induced LAI was mediated by circulating monocytes. Marti et al. (19, 20) reported that “arming” of human peripheral blood leukocytes with cancer patients’ sera was of value in specific tumor-antigen recognition in the LAI assay. In our laboratory, on reproducing Marti’s assay, we found that the differences in nonadherent cell counts were often too low to interpret readily and the false positivity rate was too high (20%). For this reason, we investigated the feasibility of using an enriched population of animal macrophages as receptor cells in arming studies with cancer patients’ sera. We devised a SA-MAI test which combines all of the above information. This test appears to be the most accurate serum test to date for diagnosis of early breast cancer.

MATERIALS AND METHODS

Subjects. Blood samples were taken with permission from hospital patients at the time of admission. This was a blind study. Assays were performed prior to surgery on patients with Stage I, II, III, and IV breast cancer. Stage I breast cancer is localized to the breast, Stage II has regional nodal involvement, Stage III has regional skin or muscle involvement, and Stage IV has nonregional metastatic involvement. Sera from control subjects consisted of patients with benign disease of the breast which includes fibroadenoma, benign cysts, and the variety of fibrocystic mastitis. Sera from patients with nonneoplastic disease and patients with cancers other than breast cancer were also studied. Each surgical specimen was examined histologically for final diagnosis.

Animals. Female Abyssinian guinea pigs weighing 250 g were purchased from Mogul-Ed, Oshkosh, Wis. Inbred Abyssinian guinea pigs were obtained from Abbott-Northwestern Hospital animal facility. Strain 2 and strain 13 guinea pigs were purchased from the National Cancer Institute. Outbred Hartley guinea pigs were purchased from Biolabs, Minneapolis, Minn.

Preparation of Guinea Pig Peritoneal Cells. Guinea pigs were given i.p. injections of 10 ml of light mineral oil. Five days postinjection, the peritoneal cavity was infused with 30 ml of Ringer’s lactate solution containing 5 units of preservative-free heparin per ml, and the macrophage exudate was collected by aspiration into a 50-ml syringe with a 16-gauge needle. The cells were washed twice in EMEM by centrifugation at 175 x g for 10 min. The residual cell pellet was briefly exposed to distilled water to lyse erythrocytes and was made isotonic with 2 x EMEM followed by centrifugation and resuspension of the cell pellet in EMEM. Viable cell count was determined in a hemacytometer chamber by exclusion of trypan blue. The cell concentration was adjusted to 1 x 10⁷ viable cells/ml.

Tumor Antigens. Filtered tissue culture antigens from primary serum-free cell cultures of breast cancer and malignant melanomas were prepared as described previously (15). Protein concentrations of these preparations were determined by the method of Daughaday et al. (5) using human γ-globulin (Sigma Chemical Company, St. Louis, Mo.) as a standard. The optimal total protein concentration of the tumor antigens used in the assay was determined by titration. The threshold antigen concentration for reactivity ranged from 3 to 6 μg of protein. All antigens were stored undiluted at –70°C.

Isolation of IgG from Human Serum. Blood was obtained from patients preoperatively and was immediately stored at 4°C. After overnight retraction of the clot, the serum was separated and stored at –20°C. IgG was separated by the method of Webb (27). Briefly, 1 ml of serum was pipetted onto 5 ml of DEAE-Sephadex A-50 gel, and the stopped tube was rotated on a blood mixer at 30 rpm for 10 min. The tube was then centrifuged at 1000 rpm for 1 min. The supernatant IgG was removed, passed through a 0.45-μm filter, and used fresh. The IgG preparation was found to be free of IgM, IgD, and IgA by radial immunodiffusion on Behring Tri-Partigen plates.

Arming of Guinea Pig Macrophages by Serum from Breast Cancer Patients and Control Subjects. To test for arming, 0.5 ml of 0.45-μm freshly filtered serum or 0.25 ml of IgG was incubated at 37°C for 1 hr with 1.0 ml of guinea pig macrophage suspension (1 x 10⁷ cells) in EMEM, resuspended in 1 ml of EMEM, and dispensed at 1 x 10⁶ cells/tube (Falcon Plastics 3033) in triplicate with either 0.1 ml of breast cancer antigen or 0.1 ml of melanoma antigen or without antigen. The total volume per tube was brought to 0.5 ml by addition of EMEM. After either 1 or 2 hr incubation at 37°C in a horizontal
position, the tubes were positioned vertically, and the number of nonadherent cells in the medium was determined with hemacytometer and/or with a Coulter Counter. A NAI was calculated. Control 1

\[ \text{NAI} = \frac{- \text{No. of nonadherent cells with breast cancer antigen}}{- \text{No. of nonadherent cells with melanoma antigen}} \times 100 \]

Control 2

\[ \text{NAI} = \frac{- \text{No. of nonadherent cells with breast cancer antigen}}{- \text{No. of nonadherent cells with melanoma antigen}} \times 100 \]

A base line for breast cancer antigen reactivity was established; a positive reaction had to be above the base line of both controls. **Establishment of Base-line NAI.** Using sera from 10 known breast cancer patients and sera from 10 patients with benign disease of the breast, an index of positive reactivity was established comparing non-adherent cell counts in the presence of a standardized breast cancer antigen with non-adherent cell counts from cells treated with serum only. A minimum base-line NAI of 13 correctly differentiated the patients into benign and malignant categories. However, a minimum base-line NAI of 9 derived from Control 2 (above) was necessary to differentiate nonspecific false-positive reactivity.

A positive reaction for breast cancer required an NAI above both controls. When testing unknown sera, a known positive control serum was always used to validate each animal’s ability to react positively in the test. Statistical analyses were performed using Student’s t test (28).

**RESULTS**

Sera from preoperative patients with breast cancer armed guinea pig macrophages to react specifically with breast cancer antigen. Macrophages armed with preoperative serum from melanoma patients did not react with breast cancer antigen but were reactive with melanoma antigen. Macrophages armed with serum from nonneoplastic patients were not reactive with either antigen; representative results are shown in Table 1.

We began a blind study of 235 patients. We armed macrophages with an unknown preoperative serum and presented them with breast cancer antigen and melanoma antigen. The results of patients in 3 categories are shown in Chart 1. Fifty-one of 55 patients with Stage I or II breast cancer reacted positively with breast cancer antigen NAI ≥ 13. Twenty-five of 30 patients with cancers other than breast cancer and 18 nonmalignant control subjects were below the base-line NAI < 13.

The results shown in Chart 2 illustrate the overall arming reactivity of breast cancer patients’ sera in early and advanced (metastatic) disease. Only 2 of 11 patients with Stage III to IV metastatic breast cancer gave a positive reaction with breast cancer antigen, while 51 of 55 patients with Stage I or II breast cancer gave a positive reaction.

A summary of results with preoperative sera from patients with breast cancer and from patients with benign disease of the breast is shown in Table 2. Four patients of the 55 patients with Stage I and II breast cancer were apparently deficient in breast cancer antigen-specific cytotoxic antibody. Thirty-five patients undergoing mastectomy were followed postmastectomy for macrophage-arming reactivity. Serum samples taken immediately postmastectomy were consistently negative. This activity was variable with time; usually by 2 to 3 weeks postmastectomy, a patient’s serum would once again arm macrophages to react with breast cancer antigen. After 2 to 6 months postsurgery, the serum-arming reactivity usually turned negative. However, 13 patients showed negative serum arming 2 to 6 months postsurgery which then became positive once again. To date, 5 of these patients have developed early clinical recurrence of cancer. The other 8 remain clinically free of cancer, and their sera have turned negative again. There were 85 patients with benign disease of the breast, and the sera of 72 of these patients were negative in the assay. Thirteen patients gave positive reactions to breast cancer antigen. However, with subsequent follow-up serum sampling, 4 of these patients tested negatively.

Histological examination revealed that of 55 Stage I and II
Macrophage Reactivity to Breast Cancer Antigens

STAGE I & II BREAST CANCER PATIENTS
STAGE III & IV BREAST CANCER PATIENTS

Chart 2. Macrophages (1 x 10⁷) were incubated with patient's serum at 37° for 15 min. Serum-armed macrophages (1 x 10⁶) were then incubated for 2 hr at 37° in triplicate with breast cancer antigen, with melanoma antigen, and without antigen. The mean number of nonadherent cells is based on triplicate determinations with each antigen. NAI was calculated as described in "Materials and Methods." NAI = 13 compared to serum-treated macrophages without antigen; points on or above broken line are positive for cytophilic antibody to breast cancer antigen.

Table 2
Summary of results of specific arming of guinea pig macrophages with serum from breast cancer patients and from patients with benign disease of the breast

<table>
<thead>
<tr>
<th>Donor serum</th>
<th>Significant NAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I and II breast cancer</td>
<td>55 4</td>
</tr>
<tr>
<td>Postmastectomy</td>
<td>35 22</td>
</tr>
<tr>
<td>Stage IV</td>
<td>11 9</td>
</tr>
<tr>
<td>Benign disease of the breast</td>
<td>85 72</td>
</tr>
</tbody>
</table>

*a* Serum samples were taken 2 to 6 months postsurgery.
*b* Five of these patients showed early clinical recurrence of cancer.
*c* Benign disease of the breast includes only those patients with biopsy-diagnosed fibroadenoma and fibrocystic mastitis.
*d* NAI of 4 of these patients were negative with follow-up serum samples.

breast cancers, all were of ductal origin except for one infiltrating lobular carcinoma with a marked scirrhous reaction.

A summary of reactivity to breast cancer antigen among subjects free of breast disease (normal) and patients with tumors other than breast cancer is shown in Table 3. Significant cross-reactivity was seen in all patients with thyroid cancer. Four of 5 patients' sera gave clearly positive reactions and the other patient's serum gave a borderline reaction. The remaining tumor patients' sera gave negative results except for that of one melanoma patient, who experienced active benign disease of the breast (mastitis) at the time of testing. None of the 18 normal patients' sera was positive.

Results with IgG-armed macrophages are shown in Chart 3. IgG from sera of patients with Stage I and II breast cancer induced specific macrophage nonadherence in the presence of breast cancer antigens (p < 0.05). IgG from patients with benign disease of the breast did not induce macrophage reactivity at a significant level (p > 0.05) in the presence of breast cancer antigens. We included a melanoma patient's IgG to demonstrate specificity and to ensure that the melanoma antigen was capable of reacting. Although not shown, this patient's IgG induced macrophages to significant reaction with melanoma antigen (p ≤ 0.05) but did not with breast cancer antigen (p > 0.05). This substitution of IgG for serum in the test gave results similar to the above results using whole serum (Charts 1 and 2).

The effect of varying the concentration of IgG reacting with a constant concentration of antigens is shown in Chart 4, A and B. Chart 4A shows that IgG from a Stage I breast cancer patient's serum induced macrophages to display nonadherence in an optimal proportion curve to breast cancer antigens.

Table 3
Summary of SA-MAI reactivity to breast cancer antigen with guinea pig macrophages armed with serum from normal subjects and from patients with cancers other than breast cancer

<table>
<thead>
<tr>
<th>Donor serum</th>
<th>No. of sera tested</th>
<th>Significant NAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>0 18</td>
</tr>
<tr>
<td>Thyroid cancer</td>
<td>5 4</td>
<td>1²</td>
</tr>
<tr>
<td>Hypernephroma</td>
<td>4 0</td>
<td>0 4</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>6 0</td>
<td>0 6</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>1 0</td>
<td>0 1</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>1 0</td>
<td>0 1</td>
</tr>
<tr>
<td>Melanoma</td>
<td>10 1   ⁵</td>
<td>9 1</td>
</tr>
<tr>
<td>Osteogenic cancer</td>
<td>2 0</td>
<td>0 2</td>
</tr>
<tr>
<td>Bronchogenic cancer</td>
<td>1 0</td>
<td>0 1</td>
</tr>
</tbody>
</table>

* ⁵ This patient gave NAI = 11 versus cells alone.
  ∗ This patient had active mastitis at time of testing.
The maximal reaction to breast cancer antigens was obtained with 75 μg of IgG ($p \leq 0.01$), while nonadherence of IgG-treated macrophages to melanoma antigens paralleled the reactivity of macrophages with IgG and no antigen. Reactivity of macrophages treated with IgG from a patient with benign disease of the breast is shown in Chart 4B. The nonadherence reactivity was essentially identical in the presence of both melanoma and breast cancer antigens.

**DISCUSSION**

Our results indicate that patients with Stage I and II early breast cancer have cytophilic antibody in their sera which can arm guinea pig macrophages to react specifically with human breast cancer antigens. This antibody becomes undetectable for approximately 2 weeks following anesthesia and surgery, after which the antibody returns for an indeterminably brief period and subsequently disappears, only to reappear just prior to the onset of clinical recurrence in some patients. The antibody is also undetectable in patients undergoing chemotherapy and radiation therapy.

Based on SA-MAI-positive results and subsequent histological findings, 55 patients were placed in the Stage I and II breast cancer category. Four of these patients were initially negative by clinical examination and xeromammography. Our SA-MAI-positive findings led to a repeat clinical examination and a breast biopsy. These patients showed early Stage I breast cancer confirmed by histological examination of the surgical specimen.

Patients with metastatic breast cancer showed diminished serum arming reactivity. Only 2 of 11 Stage III and IV patients (18%) were reactive. Similar findings have been reported by others using the LAI assay (10, 26). The failure of sera from advanced cancer patients to arm guinea pig macrophages may be due to a lack of free antibody to bind with breast cancer antigen in vitro resulting from specific immune complexes preformed in antigen excess in vivo. Onyewotu et al. (23) demonstrated that the binding of human immunoglobulin to guinea pig macrophages can be blocked with immune complexes. On the other hand, Marti and Thomson (20) reported that sera from patients with advanced melanoma would not block binding on human leukocytes of antibodies from patients.
with early breast cancer. Hence, they concluded that the blocking phenomenon seen in advanced cancer patients was tumor specific and was not due to immune complexes per se but was a function of excess antigen.

Our preliminary experiments show that IgG isolated from the sera of patients with early breast cancer will arm guinea pig macrophages to react specifically with a dose-response curve to breast cancer antigens. IgG isolated from sera of patients with benign disease of the breast did not significantly induce macrophages to nonadherent reactivity with the same breast cancer antigens. Inchley et al. (18) demonstrated that human myeloma proteins of Subclass IgG bind on guinea pig macrophage Fc receptor sites at the same location as guinea pig myeloma proteins of Subclass IgG. This reactivity could be due to autoimmunity.

To date, we have shown that Stage I and II breast cancer can be detected with 93% accuracy in a blind study. There was an initially positive rate of 15% in patients with benign disease of the breast. Of the false positives with negative xeromammography and negative clinical examination, with follow-up serum samples, 9 patients remain positive in the test giving a definitive false-positive rate of 10%. At the time of preparation of this manuscript, one patient of the 9, who was initially considered a true false positive, underwent surgery. One breast contained lobular carcinoma in situ. The other breast showed a 1-cm infiltrating lobular carcinoma. Other investigators have reported false-positive reactivity of 10 to 24% in the tube LAI assay with leukocytes from patients with benign disease of the breast reacting with breast cancer antigens (9, 26). This reactivity could be due to autoimmunity in benign disease of the breast with the patient reacting to normal breast antigens contained in the tumor antigen preparations. Alternatively, in some patients benign disease of the breast is thought to be a precursor of neoplastic disease (6, 22). There was no positive reactivity among the 18 normal patients in our study. However, significant positive reactivity was seen with sera from patients with thyroid cancer four-fifths (80%) of whom were positive. The reason for this cross-reactivity is unknown; perhaps thyroid carcinoma and breast cancer share common tumor-associated antigens.

The ability of macrophages to respond positively in the SA-MAI test is predominantly a characteristic of female guinea pigs. This suggests a female sex hormone-dependent mode of action.

In the present study, 31 of 65 female guinea pigs did not yield peritoneal macrophages that were reactive in arming with known positive sera from patients with Stage I or II breast cancer. For this reason, a known positive breast cancer serum must be included as a control in each test to ensure the ability of each animal to respond positively.

It is possible that the presence of Fc receptors on guinea pig macrophages available to bind certain subclasses of human IgG is a genetic recessive trait. In this regard, we have selectively inbred Abyssinian guinea pigs for reactivity. We have increased positive reactivity from 25% in outbred Abyssinian guinea pigs to approximately 75% in our inbred Abyssinian colony. Strain 2 and strain 13 guinea pigs were consistently negative reactors, and only 25% of Hartley guinea pigs were positive reactors. We also found that macrophages from rabbits, mice, rats, hamsters, and goats were negative reactors in the SA-MAI test.

The results shown in this study are based for the most part on one standardized breast cancer antigen preparation; however, sera from breast cancer patients were also reactive in the SA-MAI test against autologous and allogeneic breast cancer antigens. Other investigators have also reported similar cross-reactivity with allogeneic breast cancer antigens, which indicates that common tumor-associated antigens are shared in human breast cancers.

During preparation of this manuscript, a paper dealing with activation of guinea pig peritoneal macrophages with supernatant from breast cancer patients' lymphocytes pretreated with breast cancer antigens appeared in the literature (25) and was in agreement with our observations reported here and in part elsewhere (12). Thus, it appears that soluble lymphokines as well as cytophilic antibodies of human origin can specifically program guinea pig macrophages to respond in a macrophage adherence inhibition test to breast cancer antigens.

ACKNOWLEDGMENTS

We wish to thank Leslie Knoph and Sandra Nylen for technical assistance. We also thank Julie Mihm for secretarial help with the manuscript.

REFERENCES


Reactivity of Serum-armed Xenogeneic Macrophages to Breast Cancer Antigens

Lester F. Harris, Lynda L. Miller and David F. Hickok


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/42/12/4985

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.