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Circulating human mammary epithelial antigens in breast cancer

(human milk fat globule/radioimmunoassay/tumor marker/monoclonal antibody)

ROBERTO L. CERIANI*, MASAO SASAKI*, HOWARD SUSSMAN†, WILLIAM M. WARA‡, AND EDWARD W. BLANK*

*Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center, Oakland, California 94609; †Department of Pathology, Stanford University, Stanford, California 94305; and ‡Department of Radiation Oncology, University of California, San Francisco, California 94143

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ABSTRACT **Heterologous specific antisera against human mammary epithelial antigens (HME-Ags), which are present in the human milk fat globule membrane and breast epithelial cells, were used in a solid-phase radioimmunoassay to determine the presence of these antigens in the sera of patients with disseminated cancer of the breast and other organs. Breast cancer patients carry high levels of HME-Ags in their circulation, while patients with disseminated nonbreast cancer, as well as normal female controls, do not. A similar release of HME-Ags in the circulation was shown by us in a model system. To further corroborate these findings, a three-step procedure for the extraction and identification of HME-Ags from the sera was devised. In this analytical procedure, circulating HME-Ags are recovered on a solid phase carrying their corresponding antibody (anti-HME) and radioiodinated *in situ*. Later, the labeled HME-Ags are released from the solid phase and characterized by NaDodSO$_4$ gel electrophoresis. With this procedure, HME-Ags were isolated from sera of breast cancer patients but not from sera of nonbreast cancer patients or of normal female controls. The extracted HME-Ags had molecular masses of 150,000, 70,000, and 46,000 daltons. To further support these findings, a monoclonal antibody, BLMRL-HMFG-Mc3, directed to the 46,000-dalton HME-Ag was also used to extract its corresponding antigen from sera. Breast cancer patient sera contained such antigen while the sera of the other patients and controls did not. This highly sensitive methodology offers a specific approach to breast cancer diagnosis as well as further insight into the nature of circulating antigens with a view to increasing our understanding of breast cancer biology.**

Early detection and follow-up of breast cancer by noninvasive methodology has been the aim of many studies. Breast tissue markers such as casein (1) and α-lactalbumin (2) and purported cancer markers such as glycosyltransferases (3, 4), glycolipids (5), and phospholipids (6) have been measured in the circulation by a variety of methodologies, but to date none of them has gained widespread acceptance as a breast cancer marker. The markers with high specificity such as casein and α-lactalbumin (which rely for their synthesis on appropriate levels of hormonal stimulation) are expressed in few tumors and cancer markers such as glycosyltransferases and phospholipids lack specificity for breast (7, 8). In view of this, we propose, the use of human mammary epithelial antigens (HME-Ags) as high-prevalence specific markers for breast cancer. HME-Ags are detected by antibodies prepared against the human milk fat globule (HMFG) membrane (9). This membrane is derived from the apical membrane of breast epithelial cells during the process of milk secretion and envelops the fat of milk.

HME-Ags are considered cell surface differentiation antigens localized in breast epithelial plasma membrane (9), be these cells normal, neoplastic, fibroadenomatous, displastic, or obtained from male gynecomastias (10). The presence and the quantitative levels of HME-Ags on breast cells obtained from normal breast tissue, breast fibroadenomas and displasias, breast cancer, and breast epithelial cell lines (obtained from both solid and metastatic tumors) have been determined by us by indirect immunofluoresence (11), flow cytofluorimetry (12), and radioimmunoassay (RIA) (13).

In every case, neoplastic breast cells expressed HME-Ags, although at somewhat lower levels than the normal ones. Therefore, in view of the constant presence of HME-Ags in neoplastic breast epithelial cells and the fact that cell membrane components of mammary tumors are released in the circulation (11, 14, 15), we have investigated the presence of HME-Ags in the circulation of *nude* mice grafted with human breast tumors (16). HME-Ags were found at high levels in the circulation of these mice; these levels, however, disappeared after tumor ablation. *nude* mice without tumor grafts and those grafted with nonbreast human tumors did not have HME-Ags in their circulation (16).

In this paper, we demonstrate that HME-Ags are present and can be quantitated in the circulation of breast cancer patients and that they can be extracted from the sera and characterized with the help of polyclonal antisera and monoclonal antibodies.

MATERIALS AND METHODS

Human Serum Samples. Human sera from breast and nonbreast cancer (brain tumor, colon and lung carcinomas, and melanoma) patients were collected and kept frozen at −80°C until used. Sera from healthy adult volunteers were also collected and were used as controls. The sera of cancer patients with disseminated disease (both of breast and nonbreast origin) for both the RIA and the "three-step" radioimmunodetection were obtained from patients during relapse and prior to any therapy for it. The sera from patients with benign breast disease and primary breast cancer were obtained before surgical intervention. The sera of normal men and women were drawn from apparently healthy active subjects.

Polyclonal Antiserum and Monoclonal Antibody Production. Preparation and screening for polyclonal anti-human mammary epithelial (anti-HME) antisera have been described (9, 10). A monoclonal antibody (BLMRL-HMFG-Mc3 to be called Mc3) was also used to detect breast epithelial cell components in circulation. This monoclonal antibody recognizes a cell surface component of HME cells (M_r, 46,000) and was prepared and used as follows. Briefly, as described (17), the defatted HMFG membrane (9) was used as a source of immunogen. Hybridomas were prepared by fusion of a mouse myeloma line (X63A) with

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Abbreviations: HME-Ag, human mammary epithelial antigen; HMFG, human milk fat globule; RIA, radioimmunoassay.

spleen cells from BALB/c mice immunized with HMFG in the presence of 50% polyethylene glycol. A solid-phase screening procedure was used to obtain monoclonal antibodies with breast epithelial specificity. This procedure was based on specific binding of the antibody to HMFG membranes and not to membranes from human mammary fibroblasts, and HeLa, HT-29 (a colon carcinoma cell line) (18), and Bristol-8 (a human lymphoid cell line) (19) cells. These membranes were bound to microtiter plates and recognition of monoclonal antibody binding to the membrane-coated plates was monitored with radioactively labeled anti-mouse total immunoglobulin antibody (Antibodies, Davis, CA), which had been affinity purified. The anti-mouse total immunoglobulin antibody was radioiodinated by the chloramine-T method (20). For the affinity purification, total mouse immunoglobulin was used as the solid phase (21).

Immunobeads. Cyanogen bromide-activated Sepharose 4B (Pharmacia) was used to prepare polyclonal anti-HME-bound, monoclonal anti-HME-bound, and anti-human serum albumin-bound Immunobeads according to the manufacturer's suggested procedure.

HME-Ags Radioimmunoassay. A solid-phase "sandwich" RIA was developed and used to quantitate the HME-Ags present in sera from breast cancer and nonbreast cancer patients. All dilutions were made in RIA buffer (0.5% bovine serum albumin/0.3% Triton X-100/0.05% NaN_3/phosphate-buffered saline, pH 7.4). Polystyrene tubes (12 × 55 mm, Sarstedt, Federal Republic of Germany) were used for the assay. The HMFG standard antigen stock solution (100 μg of protein/ml of RIA buffer) was sonicated, before each use, with a Biosonic sonicator (Bronwill Scientific, Rochester, NY). To construct a standard curve, tubes containing 100 μl of packed anti-HME beads and 200 μl of RIA buffer received, in triplicate, 0.3, 1, 3, 10, 30, 100, or 300 ng of sonicated HMFG solution in a volume of 50 μl. To eliminate any contribution of serum components to the assay, all tubes for standard dosage curves were also supplemented with 100 μl of pooled adult male sera and protease inhibitors. Tubes containing 100-μl samples of unknown serum from patients in triplicate were similarly prepared, supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM ε-aminocaproic acid, and aprotinin at 20,000 kallikrein inhibitor units/ml), and rotated end-over-end at 20°C for 4 hr or at 4°C overnight. Beads were then transferred into new polystyrene tubes, washed five times with RIA buffer, and incubated with biotin-conjugated anti-HME antibody for 3 hr at 20°C. The latter was custom prepared by E. Y. Laboratories (San Mateo, CA), using biotinyl-*N*-hydroxysuccinimide ester as an intermediate reagent (22). The binding of avidin, the egg white protein, for biotin is remarkably rapid, stable, and quite strong, having a K_a of 10^{15} M^{-1} (23), which is far larger than the one for antigen–antibody binding.

After incubation, the beads were washed five times with RIA buffer, incubated at 20°C for 3 hr with 30 μl of ^{125}I-labeled avidin ($10–15 \times 10^4$ cpm), and washed five times with RIA buffer, and their radioactivity was assayed with a gamma scintillation counter. Iodination of avidin (Sigma) was performed by the chloramine-T method (20).

Three-Step Radioimmunodetection of HME-Ags. An analytical procedure using immunoaffinity beads that is based on extraction, *in situ* radioiodination, release, and electrophoretic separation of antigens has been developed to extract and identify HME-Ags from the sera of patients. For this purpose, 200 μl of the selected sera was rotated end-over-end at 20°C for 5 hr or at 4°C overnight with 200 μl of packed Immunobeads and 200 μl of buffer A (RIA buffer lacking bovine serum albumin). The Immunobeads carried covalently bound anti-HME, Mc3, or anti-HSA; the serum came from breast cancer patients with positive values determined by RIA for HME-Ags (Table 1), from patients with disseminated nonbreast cancer, or from normal men and women. Beads were then transferred to new tubes and washed five times with buffer A. The Immunobeads, now carrying the HME-Ags recovered from the sera, were labeled with ^{125}I by the chloramine-T method (20). Then, they were washed five times with buffer A to remove unreacted radioactive iodine. The bound and labeled HME-Ags were released from the antibodies attached to the beads by incubating the beads for 20 min at 20°C with 300 μl of 1.0 M acetic acid or 2.0 M NaSCN and immediately dialyzed against phosphate-buffered saline overnight. When experiments were carried out with the aim of eliminating labeled human serum albumin from the eluate, the dialysate was further incubated with 100 μl of packed anti-human serum albumin beads at 20°C for 4 hr. Then, the beads were decanted and the dialysate was lyophilized for $NaDodSO_4$ gel electrophoresis.

Table 1. HME-Ag levels of sera used for radioimmunodetection determined by RIA

Patient	Condition	HME-Ags, ng/ml of serum
1	Breast tumor with metastasis	280
2	Breast tumor with metastasis	250
3	Breast tumor with metastasis	140
4	Breast tumor with metastasis	110
5	Lung carcinoma	<30
6	Colon carcinoma	45
7	Melanoma	<30
8	Normal control, female	<30
9	Normal control, female	<30
10	Normal control, female	<30
11	Normal control, male	<30

$NaDodSO_4$ Gel Electrophoresis. Lyophilized ^{125}I-labeled proteins released as described above from the Immunobeads were dissolved, placed in boiling water for 5 min, and separated by electrophoresis on 8% polyacrylamide/0.1% $NaDodSO_4$ slab gels as reported (24). After electrophoresis, the lanes containing the labeled proteins were cut into 1.8-mm sections and their radioactivities were determined. Molecular weight standards and a sample of HMFG were coelectrophoresed in each run.

RESULTS

The RIA described here permits measurement down to 3 ng equivalents of the HME-Ags present in the HMFG (Fig. 1). Because the antigen used to construct the standard curve is total delipidated HMFG and the anti-HME has been absorbed to remove crossreactivity with other tissues, only some components of the HMFG are recognized by the antiserum. From previous determinations, HME-Ags comprise approximately 15% of all the proteins in the HMFG (unpublished results); therefore, this RIA can be considered capable of detecting HME-Ags down to the picogram level. Despite the several antigens involved, a smooth and reproducible curve could be obtained in every assay (Fig. 1). The only limitation to the assay was the shelf life of the radioiodinated anti-HME (less than 10 days). In addition, to account for the effect of nonspecific serum proteins on the assay, male serum was added to each assay mixture of the standard dosage curve. High levels of HME-Ags were found in the circulation of patients with disseminated breast cancer. These levels were statistically significantly higher than the background levels found in the circulation of normal women and men and in female patients with benign breast disease, primary breast cancers, disseminated cancer of the lung, nervous tissue, and colon, and melanomas (Fig. 2). The maxi-

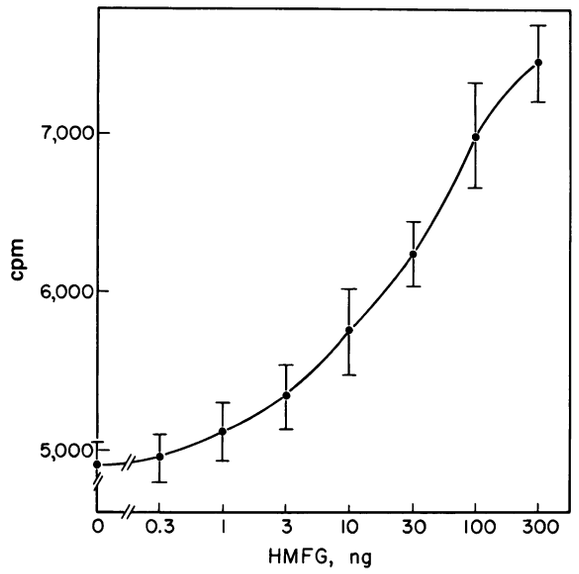

FIG. 1. Typical RIA standard dosage curve for HME-Ags. Assay mixtures contained anti-HME Immunobeads and various amounts of HMFG proteins. Mixtures were incubated and treated with biotinylated anti-HME, and then the amount of HME-Ags on the Immunobeads was determined by ^{125}I-labeled avidin. Results are mean ± SEM.

mum level of HME-Ags obtained from the sera of disseminated breast cancer patients was 280 ng/ml. In this group of patients, 75% had positive values that were statistically different from the control group ($P < 0.05$). Among the primary breast carcinoma patients, 25% had levels statistically different from the control ($P < 0.05$).

Because the type of RIA used was not competitive, an effort was made to ascertain whether HME-Ags could be isolated from the circulation of those patients showing high levels of HME-Ags by RIA. Our three-step immunodetection method was first tried on delipidated HMFG. The result of exposing polyclonal anti-HME Immunobeads to HMFG is shown in Fig. 3. The material released corresponds in electrophoretic migration pattern and molecular masses to the HME-Ags previously separated by us by other affinity procedures (9, 13). Peaks corresponding to molecular masses of 150,000, 70,000, and 46,000 daltons are found. Breast cancer patients whose RIA values are shown in Table 1 were selected to isolate HME-Ags from their sera. Assorted normal controls, both male and female, and several disseminated cancer patients with nonbreast cancers were studied simultaneously (Table 1). As shown in Fig. 3, peaks corresponding to the three components previously assigned to HME-Ags (when detected by the polyclonal antisera in previous work) (9, 13) were extracted from the sera of breast cancer patients by polyclonal anti-HME on the solid phase by the affinity procedure. It is noteworthy that the molar ratio of the HME-Ags extracted from patient sera is similar to that of the antigens recovered from the HMFG membrane by polyclonal anti-HME (9, 13). When sera of patients having nonbreast tumors and normal sera were subjected to similar affinity procedures, none of the HME-Ags were recovered (Fig. 4). Control beads with no protein attached on them or carrying human serum albumin failed to recover HME-Ags from the sera (data not shown). However, from these sera, a smaller mound centered around 70,000 daltons is recovered in every sample while none of the 150,000- and 46,000-dalton components are detected in the nonbreast cancer serum controls (Fig. 4). To test the nature of this 70,000-dalton mound, sera from normal women were incubated with Sepharose 4B beads previously treated with 0.2 M glycine to inactivate the cyanogen bromide-functional residues. After incubation, the beads were washed, radioiodinated, and treated with 2 M NaSCN, and the released materials were subjected to gel electrophoresis. In every instance, a radioactive peak corresponding to the 70,000-dalton mound was observed (data not shown), indicating that the Sepharose 4B beads retain component(s) from every sera that can be labeled with iodine and subsequently released by high molarity solution. To further identify these compound(s), materials released from the beads were absorbed with anti-human serum albumin-coated-Sepharose 4B beads before gel electrophoresis. The 70,000-dalton mound was appreciably reduced (data not shown), indicating

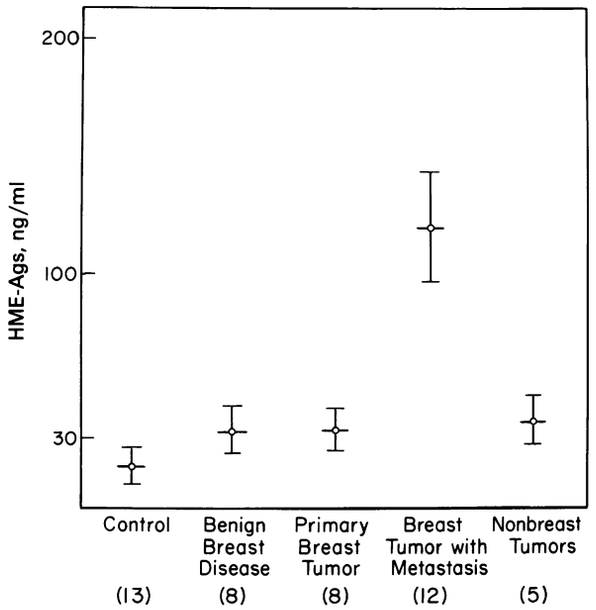

FIG. 2. HME-Ags in the circulation of patients with breast and nonbreast diseases. A solid-phase sandwich RIA was used to quantitate HME-Ag levels in sera from various patients and healthy normal women (controls). Results are mean ± SEM; numbers in parentheses are numbers of patients and controls.

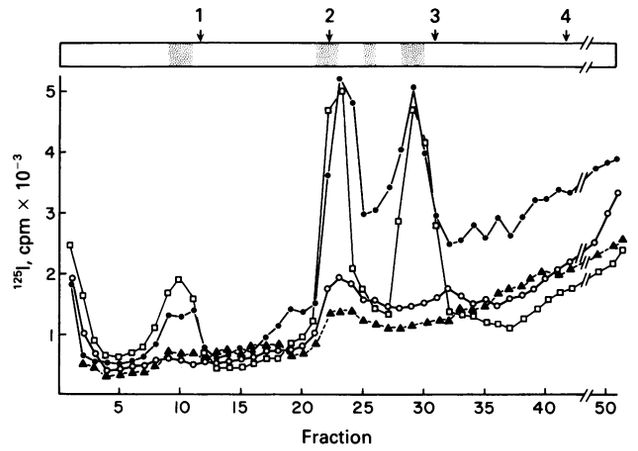

FIG. 3. Radioimmunodetection of HME-Ags in sera by using heterologous anti-HME Immunobeads. (*Lower*) Profile of circulating HME-Ags in serum from a metastatic breast cancer patient (●) is compared with profiles of HME-Ags extracted from native HMFG (□) and healthy controls (○, ♀; ▲, ♂). (*Upper*) Electrophoretic pattern of HMFG stained with Coomassie brilliant blue R. ↓, M_r marker proteins: 1, bovine serum albumin dimer, 136,000; 2, bovine serum albumin, 68,000; 3, ovalbumin, 45,000; 4, ribonuclease, 13,700.

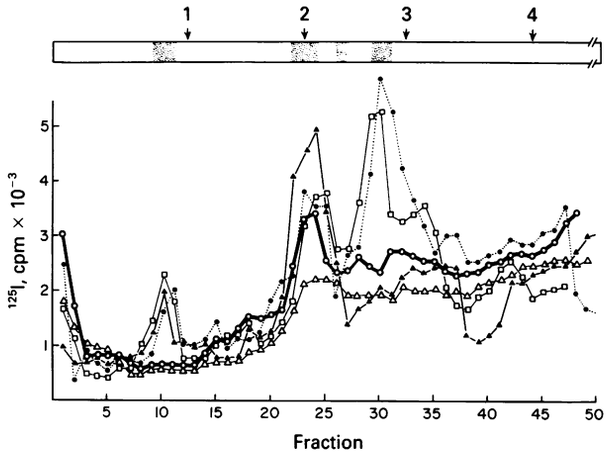

FIG. 4. Radioimmunodetection of HME-Ags in sera by using heterologous anti-HME Immunobeads. (*Lower*) Profiles of circulating HME-Ags in sera from three metastatic breast cancer patients (●, □, and ▲) are compared with profiles of HME-Ags from sera of a lung tumor patient (○) and a colon carcinoma patient (△). (*Upper*) As in Fig. 3.

that it was composed mainly of nonspecifically bound human serum albumin.

The breast epithelial membrane component recognized by monoclonal Mc3 was also found in the circulation of breast cancer patients. For this purpose, Mc3 was conjugated to the Sepharose 4B beads without loss of its binding activity for its antigen. By using our three-step radioimmunodetection procedure, a 46,000-dalton component was isolated from total delipidated HMFG solution (Fig. 5). This component was also extracted from the sera of those patients from whom HME-Ags could be extracted by using polyclonal anti-HME Immunobeads (Fig. 5). Sera from nonbreast cancer patients and from normal women did not have the component recognized by the monoclonal antibodies (Fig. 5).

DISCUSSION

It has been suspected for some time that cell membrane components are released by breast tumors into the general circulation (25). High levels of glycosyltransferases (3, 4) have been found in the sera of these patients. However, it was not possible to assign with certainty the tissue of origin of the tumor, because these enzymes are not organ specific. In the present case, tissue-specific antigens have been used to prove that breast tumors release components of their cell membrane into the circulation. The certainty of the detection of HME-Ags in the sera of breast cancer patients is due to both positive RIA values and affinity extraction of cell membrane components and their subsequent characterization by molecular mass.

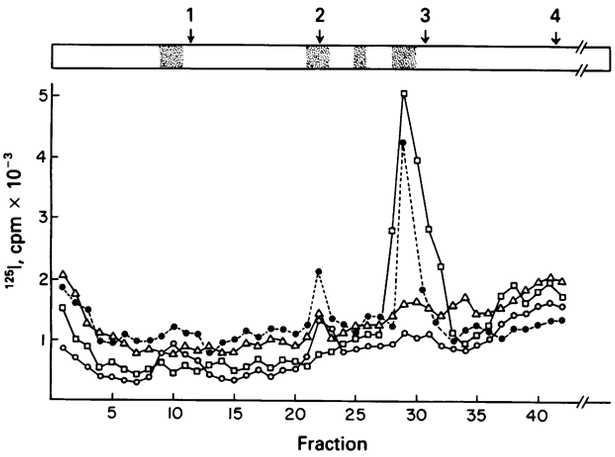

FIG. 5. Radioimmunodetection of HME-Ags in sera by using monoclonal anti-HME Immunobeads. A monoclonal antibody that recognizes a 46,000-dalton component of HME-Ags was used to label the beads and characterize the HME-Ags. (*Lower*) Profiles of HME-Ags in sera of patients with breast tumor metastasis (●) and melanoma (△) are compared with profiles of HME-Ags from HMFG (□) and a healthy female control (○). (*Upper*) As in Fig. 3.

Further, these breast-specific normal components are detected by both a heterologous polyclonal antiserum raised against the complete delipidated breast epithelial cell membrane and a monoclonal antibody raised to a 46,000-dalton component of the same membrane. These same reagents were able to fractionate the HME-Ags from a mixture represented by a delipidated preparation of HMFG membrane cleanly, thus attesting their effectiveness. Another important finding to support the hypothesis that the HME-Ags recovered in the serum originated in the breast tumor cell membranes is that the molar ratio of HME-Ags recovered by the heterologous polyclonal anti-HME was similar to the ratio of these antigens in the native HMFG membrane (Figs. 3 and 4 and refs. 9 and 13).

The certain identification of low concentrations of HME-Ags in sera requires highly sensitive affinity binding and *in situ* radiolabeling as the one we used, which theoretically should detect any HME-Ags present. The high sensitivity provided by the three-step immunodetection method mainly rests on the level of specific activity of radioiodine that can be obtained when labeling the bound antigens with chloramine-T *in situ*. However, only by appropriately characterizing the radiolabeled antigens released will we be assured that we are dealing with HME-Ags. The separation step by polyacrylamide gel electrophoresis provides a stringent test of identification by molecular mass of the released antigens. The latter are compared in the gels to the known molecular masses of HME-Ags from native HMFG membrane preparations. In addition, this high sensitivity is not obtained at the sacrifice of specificity.

There are several implications of these findings. A RIA, such as the present one measuring levels of HME-Ags, can be used with definite specificity in the follow-up of breast cancer patients and, if higher sensitivity were obtained and studies supported it, screening the entire female population would be possible. The present RIA, which can be performed rapidly and reasonably, makes it a very attractive proposition.

Similar considerations apply to the immunodetection method, which might be a more specific and more sensitive method for the follow-up of breast cancer than the RIA. Although this separation technique is currently not quantitative, it represents a potentially useful diagnostic tool for identification of the type of tumor carried by a given patient (breast or nonbreast) by a noninvasive immunodiagnostic procedure. The high sensitivity and specificity of both the polyclonal and monoclonal immunodetection procedures make them powerful assays in cancer diagnosis. At present, the polyclonal antiserum procedure may be more reliable; all the breast tumors and breast cell lines examined to date have some expression of HME-Ags, although usually lower than their normal counterparts (12, 13). In contrast, several breast tumors and breast cell lines examined for the presence of the antigens identified by monoclonal components have not expressed them (ref. 17; unpublished information). This problem might be solved by the use of a mixture of monoclonal antibodies directed to all the HME-Ags.

The makeup of the breast epithelial cell surface may be relevant to the future prognosis of breast cancer (26). Thus, the proposed methodology, requiring only a serum sample, might

be useful in detecting and possibly quantitating noninvasively the level of expression of antigens present on the cell surface of breast tumors. A molecular classification based on the cell surface components of breast tumors could guide in the staging and choice of therapy for the patient.

Finally, this methodology, which could be extended to other organs, can be used for the detection of circulating tumor-associated antigens of membrane origin that could aid in assessment of the immunological status of breast cancer patients and for further study of the biology of breast cancer.

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