Measurement of Ferritin-bearing Peripheral Mononuclear Blood Cells in Cancer Patients by Radioimmunoassay

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ABSTRACT

A radioimmunoassay has been developed to measure ferritin bound to the surface of isolated human peripheral blood mononuclear white blood cells (PBMs) in order to investigate the possible relationship of this phenomenon to breast and other forms of cancer. The assay measures the specific binding (%SP) of affinity-purified 125I-labeled rabbit anti-Hodgkin’s spleen ferritin antibody to isolated patient PBMs.

A preliminary prospective, preclinical trial on 300 patients was run which included: (a) normals, benign breast disease, and medical/surgical patients as non-cancer controls; (b) postoperative primary cancer and advanced cancer in clinical remission as post cancer controls; and (c) both early preoperative breast cancer patients and cancer patients with localized recurrences or active disseminated disease as test groups. The mean %SP for the non-cancer control groups was in the range of 4.3 to 5.1 (n = 187), which was identical to that for inactive cancer or postoperative cancer, which was no evidence of recurrence. Using a %SP normal cutoff level of 6.5, which resulted in a false-positive rate of approximately 10% for both non-cancer and post-cancer control groups, only 27% of early preoperative cancers (n = 22) gave elevated %SP values. These results suggest that measurement of ferritin-PBM is inappropriate for early disease diagnosis.

In contrast, 91% of patients with advanced active breast cancer and 73% of those patients with other types of advanced cancers, including tumors of ovarian, lung, colon or esophageal origin, showed elevated %SP values more than double those of post-cancer controls. The mean %SP value in active advanced cancer was 10.8 for breast (n = 12) and 10.6 for all other solid tumors investigated (n = 34). Paired patient comparisons of ferritin-PBM and plasma carcinoembryonic antigen in breast cancer showed elevations in 91% of the patients for ferritin-PBM and 67% for carcinoembryonic antigen. Overall, these results suggest that patients with advanced cancer display elevated ferritin-PBM levels of ferritin on the surface of their PBMs and that this measurement may be a useful adjunct in monitoring and evaluating the clinical status of cancer patients.

INTRODUCTION

For several years, it has been suggested that ferritin, a heat-stable, high-molecular-weight, intracellular iron storage protein, may be considered an oncofetal antigen. Isomers of ferritin with charge properties different than those of normal tissue have been found in ferritins derived from Hodgkin’s spleen (25), tumor cells in culture, hepatoma, and fetal tissue (5, 6). In clinical studies measuring ferritin in the serum of patients with a wide variety of tumors, a positive correlation has been seen in many cases between elevated levels and advanced cancer (12, 14). Elevated serum ferritin levels have also been described in breast cancer (2, 17). In breast cancer, the source of increased ferritin production has been ascribed directly to the tumor tissue (16).

Ferritin has also been linked to a variety of immunosuppressive effects involved in cellular immunity. Matzner et al. (18), have demonstrated inhibition of lymphocyte function including mitogen stimulation, concanavalin A capping, and mixed-lymphocyte reactions by splenic ferritin. Hancock et al. (11) have shown that ferritin from Hodgkin’s spleen interferes with the leucocyte migration inhibition assay performed on patients with malignant lymphoma. Further, in Hodgkin’s lymphoma, there is a substantial decrease in sheep erythrocyte rosette-forming lymphocytes (T-lymphocytes) (27), which has been attributed to a heat-stable, high-molecular-weight blocking factor found in the plasma of patients with active disease and could be absorbed out of the plasma with normal peripheral blood lymphocytes (7). When this substance was removed from isolated Hodgkin’s PBMs2 with the immunomodulatory drug levamisole (31), a restoration of erythrocyte rosette capability ensued. Cell surface labeling studies showed this substance to be ferritin (23). Extension of these studies into breast carcinoma, a disease in which T-cell levels appear reduced as measured by erythrocyte rosette techniques (33), has resulted in a similar finding of ferritin on the surface of PBMs when assessed by the technique of ADCC using RHF (21). In those studies, concurrent evaluation with anti-T-lymphocyte antisera showed that there was actually no reduction in the number of T-lymphocytes but rather a blockage of the sheep erythrocyte receptors on the T-lymphocytes by ferritin.

In an expanded clinical study on 150 patients, using ADCC techniques, a 96% correlation was found between early preoperative breast cancer (TNM, Stage I and II) and elevated cell-bound ferritin (8). Interestingly, of those breast cancer patients without cell-bound ferritin, 20 of 22 were histopathologically diagnosed as having Stage III carcinoma. All benign breast disease patients and normal individuals tested gave negative results. However, that study did not address the potential of this tumor marker in Stage IV breast cancer or other types of malignancy, nor did it evaluate other nonmalignant disorders which may result in false-positive results. Further, methodology which suffers from being both labor intensive and potentially

2 The abbreviations used are: PBM, peripheral blood mononuclear white blood cell; RIA, radioimmunoassay; ADCC, antibody-dependent complement mediated cytotoxicity; RHF, rabbit anti-Hodgkin’s spleen ferritin antibody; %SP, percentage of specific binding of 125I-labeled rabbit anti-Hodgkin’s spleen ferritin antibody; CEA, carcinoembryonic antigen; TNM, tumor-nodes-metastases staging system; DPBS, Dulbecco’s modification of phosphate-buffered saline minus Ca++ and Mg++.
subjective in evaluation was used. This prompted us to investigate a RIA technique using radiiodinated affinity-purified RHF to measure ferritin bound to the surface of PBMs. Using this approach, we have conducted a preliminary study on over 300 patients with a variety of medical disorders including early and advanced breast cancer, other cancers, and nonmalignant diseases.

MATERIALS AND METHODS

Patient Samples. All patients in this study were drawn from clinics at Downstate Medical Center and Kings County Hospital, Brooklyn, NY, and sampled conformed to hospital procedures for human experimentation including informed patient consent. Twenty ml of fresh heparinized blood were collected by venipuncture, coded with a random number, and immediately sent to the laboratory for ferritin-PBM analysis. Clinical diagnoses were not known prior to testing, and patient diagnoses were not matched with test code numbers until the assay was complete. Since the initial intent of this study was to assess the accuracy of the marker with regard to breast cancer, patients comprised the following groupings: Group 1, non-cancer controls: clinically normal, benign breast disease, and medical-surgical patients including primarily endocrine abnormalities, but also such diseases as hypertension, schizophrenia, and arthritis; Group 2, preoperative primary breast cancer to assess the accuracy of the marker in early disease; Group 3, postoperative breast cancer with no evidence of recurrence; Group 4, advanced breast cancer with active clinical disease or clinical remission; and Group 5, other cancer, either active disease or clinical remission. Breast tumors were characterized pathologically according to the TNM staging method prescribed by the American Joint Committee on Cancer (1). Active clinical disease included localized recurrence and active disseminated disease. Remission implied a greater than 50% reduction in tumor burden. In some cases, the current clinical course of the metastatic cancer patient could not be assessed accurately, and these patients were categorized to reflect this status. Standard tests were used in the clinical work-up of the metastatic patient, including bone scans, chest X-rays, liver scans, and blood chemistries.

Preparation of 125I-labeled RHF. Ferritin was purified from the involved spleen of a patient with pathologically confirmed Hodgkin’s disease by the method of McKeen et al. (19). Analysis of Hodgkin’s ferritin by sensitive immunoradiometric assays to both normal spleen and purified acid ferritin isolated from placenta (P, 4.7 to 4.3), showed greater than 95% cross-reactivity with normal spleen ferritin and 8% cross-reactivity with placental acid ferritin. Normal spleen ferritin was not reactive in the acid ferritin assay. Rabbits were immunized with ferritin in complete Freund’s adjuvant by the method of Vaitukaitis et al. (30). Prior to iodination, RHF was affinity purified on Sepharose 4B CH (Pharmacia Fine Chemicals, Piscataway, NJ) to which both Hodgkin’s spleen and normal spleen ferritin had been covalently coupled according to the manufacturer’s procedure. Following successive batch washings with 0.1 M sodium carbonate (pH 8.0), 0.1 M sodium acetate (pH 5.0), and distilled water (pH 4.4), RHF was eluted with distilled water (pH 2.2) into tubes containing 0.5 M sodium phosphate, pH 7.7.

Aliquots (150 µg/900 µl) of affinity-purified RHF were labeled with Na125I (Amersham, Chicago, IL) by the chloramine-T procedure of Greenwood et al. (8). Only fractions greater than 85% precipitable, as measured by cpm, in 10% trichloroacetic acid were pooled and utilized for analysis of ferritin bound to isolate patient PBMs. The final specific activity of labeled antibody was 1 to 2 µCi/µg.

Preparation of Ferritin-positive Control Cells. In order to develop a stable reference system which would best mimic binding to patient PBMs and control for interassay variability and reagent stability, normal spleen ferritins (JBL Chemical Company, San Luis Obispo, CA) were covalently coupled to human acute lymphoblastic leukemia cells (CCL-119; American Type Culture Collection, Rockville, MD) by the glutaraldehyde method of Rockoff et al. (28). After conjugation, approximately 50% of the starting cell population was recovered. Electron microscopy confirmed that these cells were uniform and retained lymphoblastoid morphology. These standards were stable for greater than 2 years without any loss in surface ferritin as assessed by %SP of 125I-labeled RHF. Prior to conjugation, no surface ferritin was demonstrable on these cells.

RIA for Ferritin-bearing PBM. PBMs were separated from fresh heparinized blood on Ficoll-Hypaque (Pharmacia). Following removal of the interfacial PBMs, cells were washed twice in DPBS at 100 x g for 10 min and resuspended in 1 x 10^8 cells/ml in balanced salt solution (20). To each of 2 duplicate sets of 12 x 75-mm polystyrene tubes were added 100 µl of cells, followed by addition of 100 µl of either 0.1% bovine serum albumin in DPBS to the first series of tubes or 3000 ng normal spleen ferritin in 0.1% bovine serum albumin-DPBS to the second series of tubes to assess the extent of nonspecific binding. This was followed by addition of 100 µl of 125I-labeled RHF.

Following incubation overnight with shaking at 4°C, cells were washed once in 3 ml DPBS and centrifuged at 1500 x g for 30 min. Supernatants were aspirated, and radioactivity bound to cell pellets was measured in a gamma spectrometer. %SP was determined by subtracting cpm bound in the presence of soluble ferritin (nonspecific binding) from cpm bound in the absence of soluble ferritin (total binding) divided by total cpm added to the assay system minus nonspecific cpm x 100. Precise details of the binding characteristics of this RIA will be described elsewhere.

Assay Sensitivity and Specificity. Determinations of sensitivity and specificity of the ferritin-PBM assay as a cancer marker were measured using cumulative distribution analysis where percentage cumulative distribution equaled

\[ \frac{\sum n \leq x}{\text{Total samples} + 1} \]

where n is the number of patients with a defined %SP less than or equal to x in a given disease or control category

and total samples were the total number of patients in a given classification. Cumulative distributions at each level of %SP were plotted for a variety of test populations (early and advanced breast cancer, other metastatic cancers) and compared to their appropriate non-cancer control groups (normals, benign breast disease, non-cancer medical-surgical patients, and postoperative cancer with no evidence of recurrence). A cumulative distribution plot of the control group denotes the specificity of the assay at any selected %SP level, while a cumulative distribution of the test group denotes the sensitivity of the assay. By plotting cumulative distribution of both a test and a control population on the same graph and by inverting the test curve only, an intersection of the 2 curves results which describes the maximum sensitivity and specificity that can be expected from the assay. This statistical approach allows one to easily determine sensitivity and specificity characteristics at any %SP chosen. Percentage of sensitivity is defined as the percentage of positive results in patients with disease, while specificity is considered to be the percentage of negative results in patients without disease. The rate of false-positives in a given control group can therefore be defined as 100% − % specificity.

Tests of statistical difference between various patient groups were assessed using Student’s t-test.

CEA and Ferritin Levels. Plasma CEA and ferritin levels were determined on selected patients to study the possible relationship of ferritin-PBM values to CEA and also to examine the relationship of cell-bound and soluble ferritin. Plasma aliquots were taken prior to PBM isolation

and frozen at −20° for subsequent measurement of CEA by RIA (Abbott Diagnostics, Chicago, IL). Soluble ferritin levels were measured by an immunoradiometric assay (Coming Medical and Scientific, Medfield, MA). Comparison of ferritin-PBM and soluble ferritin or CEA values were determined by regression analysis of the natural logarithmic values of patient test results.

RESULTS

Ferritin-bearing PMBs in Non-Cancer Populations. The normal range for ferritin-bearing PMBs was established by examining 3 separate control populations: normals; benign breast diseases, which are the most appropriate control group for comparison to breast cancer; and medical disorders other than cancer. Individual results and appropriate group statistics are shown in Table 1 and Chart 1. Of 187 total non-cancer controls examined in the 3 groups, there was very little difference in the overall mean %SP (4.3% normals, 4.4% benign breast disease, and 5.1% medical-surgical disorders). A normal population cutoff of 6.5 %SP was chosen for the purposes of appropriate comparison to malignant diseases. At this level, the normal and benign breast groups gave a false-positive rate of approximately 10%. The control group composed of medical-surgical patients had an overall false-positive rate of 23% at this cutoff level. A wide spectrum of nonmalignant diseases were examined, including endocrinopathies, autoimmune disorders, obesity, and nephritis. There was no definite correlation between a particular nonmalignant disease state and elevated ferritin-PBM. Also, there was no correlation between ferritin-PBM and age, race, or sex in the entire control population (data not shown). In this study, greater than 90% of the patients studied were women.

Ferritin-PBM in Early Breast Cancer. In order to adequately assess the entire spectrum of breast disease, 5 separate categories for patient distribution were established. These were: Category 1, benign breast disease; Category 2, preoperative or pretreatment primary breast cancer, TNM Stages 0 to III; Category 3, postoperative primary breast cancer with no evidence of recurrence; Category 4, clinically active advanced breast cancer; and Category 5, advanced breast cancer in clinical remission. There was no statistical difference between %SP levels in either normals or benign breast disease and preoperative breast cancer, Stages 0 to III (Table 1; p = 0.35). Only 27% of breast cancer patients assessed preoperatively showed elevated levels of ferritin-PBM using a cutoff of 6.5 %SP.

Cumulative distribution analysis (Chart 2) showed that, for

Table 1

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of subjects</th>
<th>%SP</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td>21</td>
<td>4.3 ± 3.0 (1.1–13.0)</td>
<td>10</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td>73</td>
<td>4.4 ± 3.3 (1.5–19.0)</td>
<td>11</td>
</tr>
<tr>
<td>Medical disorders</td>
<td>93</td>
<td>5.1 ± 3.2 (1.0–16.0)</td>
<td>23</td>
</tr>
<tr>
<td>Breast cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative primary breast cancer</td>
<td>22</td>
<td>5.5 ± 3.6 (2.1–14.3)</td>
<td>27</td>
</tr>
<tr>
<td>Postoperative primary breast cancer, no evidence of recurrence</td>
<td>26</td>
<td>4.3 ± 2.6 (1.1–11.0)</td>
<td>15</td>
</tr>
<tr>
<td>Active advanced breast cancer</td>
<td>12</td>
<td>10.8 ± 4.1 (3.5–18.0)</td>
<td>92</td>
</tr>
<tr>
<td>Advanced breast cancer with clinical remission</td>
<td>3</td>
<td>5.1 ± 0.8 (4.2–5.8)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Based on a normal cutoff level of 6.5 %SP.

[Table 1 continued...]

Chart 1. Scattergram of individual patient test results. Ferritin-PBM %SP values were determined on 1 x 10⁶ patient PMBs. Following decoding of clinical (CLIN.) data, patients were classified into the following groups: non-cancer controls (A); postoperative (POST-OP) cancer with no clinical evidence of recurrence or advanced cancer with definite evidence of clinical remission (REMIS.) (B); early preoperative (PRE-OP) cancer (C); or advanced cancer in a clinically active disease state (D). ——, cutoff value for the normal population of 6.5 %SP. ——, 95% confidence intervals for the mean (%SP).
early breast cancer, regardless of TNM staging, the maximum sensitivity that could be expected when compared to benign breast controls was 65% for TNM Stage 0 to II (n = 16) and 60% for TNM Stage III (n = 6). However, this level of sensitivity could be reached only at a false-positive rate of 35%, making ferritin-PBM inappropriate as a marker for early breast cancer diagnosis.

Ferritin-PBM in Advanced Breast Cancer. In contrast to early disease, evaluation of active advanced breast cancer, either localized or metastatic disease, when compared to either benign breast disease, postoperative disease with no evidence of recurrence, or advanced cancer in clinical remission showed significance (p ≤ 0.001), regardless of to which control group it was compared. The mean %SP of active advanced breast cancer was greater than double that of any of the control groups to which it was compared (10.6 %SP versus 4.4 %SP benign breast, 4.3 %SP postoperative with no recurrence, or 5.1 %SP advanced breast cancer with clinical remission) (Table 1). As shown in Chart 2, an assay sensitivity in the range of 90% could be expected with concurrent specificity of 90% when compared to appropriate controls.

Ferritin-PBM in Other Cancers. A variety of patients with other solid tumors were also sampled to assess the potential utility of ferritin-PBM by RIA as a diagnostic measure of advanced disease. A total of 50 other advanced cancer patients classified as clinically active or inactive were measured for ferritin-PBM. Overall results were similar to those for breast cancer, with a mean %SP of 10.6 for other cancers in an active disease state, while those in clinical remission had a mean %SP similar to that of non-cancer control groups (Table 2; Chart 1). The mean %SP in those patients whose clinical status could not be adequately assessed was also quite high, with a value of 11.8. Cumulative distribution analysis suggested findings similar to those in breast cancer, with a maximum sensitivity and specificity of 83% when compared to post-cancer control groups.

Chart 3 presents a breakdown of results based on tumor type. Although in most cases the number of patients in each group was too small for statistical analysis, the overall trend suggests possible utility of this test for management of a variety of carcinomas and also sarcoma.

Plasma CEA, Ferritin, and Ferritin-PBM Levels in Cancer. Individual patients with various breast diseases were assessed for plasma CEA and both soluble and PBM-bound ferritin to study their relationship to each other. There was no correlation of the quantitative amount of plasma ferritin and the %SP of 125I-labeled RHF (r = 0). Only 4 of 10 plasma ferritin values were elevated for advanced breast cancer patients, whereas 11 of 12

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**Table 2**

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of subjects</th>
<th>%SP</th>
<th>% positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced cancer, clinical remission</td>
<td>16</td>
<td>5.0 ± 2.4</td>
<td>(1.2–12.6)</td>
</tr>
<tr>
<td>Active advanced cancer</td>
<td>22</td>
<td>10.6 ± 7.2</td>
<td>(1.2–30.1)</td>
</tr>
<tr>
<td>Active advanced cancer; clinical status unknown</td>
<td>12</td>
<td>11.8 ± 8.3</td>
<td>(1.2–26.8)</td>
</tr>
</tbody>
</table>

* Based on a normal cutoff level of 6.5 %SP.
** Mean ± S.D.
Numbers in parentheses, range.

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**Chart 2.** Cumulative distribution analysis comparing sensitivity and specificity of ferritin-PBM by RIA in early preoperative and advanced breast cancer. The cumulative distribution of the benign breast disease control group (C) is determined from the left ordinate. Preoperative primary breast cancer, TNM Stages 0 to II (○), preoperative primary breast cancer, TNM Stage III (△), or active advanced breast cancer (□) are determined from the inverted right ordinate. Extrapolation of any %SP value on the X abscissa to the control group cumulative distribution line determines the assay specificity as read from the left Y axis, while extrapolation of any test population line determines sensitivity of the assay at any given %SP value. The intercept of control and test cumulative distribution lines determines the maximal assay sensitivity and specificity.

**Chart 3.** %SP of 125I-labeled RHF to PBM's from patients with various solid tumors. □, disease in a clinically active state; ○, those breast cancer patients with postoperative primary tumor with no clinical evidence of recurrence, or advanced breast cancer in clinical remission to compare the difference in ferritin-PBM values in active versus inactive disease. X, those advanced cancer patients whose clinical status could not be precisely determined.
ferritin-PBM values were elevated in the same patient population. These results indicate that the concentration of soluble ferritin was not proportional to the amount of ferritin bound to the PBM surface and suggest that physiological processes other than simple adsorption phenomena may be responsible for the elevation of ferritin on the surface of PBM.

Evaluation of elevated CEA alone as a tumor marker in active advanced breast cancer resulted in 67% accuracy in comparison to the clinical status of the patient. However, 2 of 3 patients in clinical remission also continued to have markedly elevated CEA values. In contrast, 91% of those with active advanced breast cancer had elevated ferritin-PBMs, and all 3 of those in remission showed normal ferritin-PBM levels, suggesting that ferritin-PBM alone may be a better indicator of clinical status than is CEA in breast cancer patients.

In preoperative breast cancer, of those patients sampled for both ferritin-PBM and CEA, neither test was accurate for early detection of disease, with only 36% positive findings for ferritin-PBM and 6.7% positive for CEA. In general, both tests were negative in benign breast disease. The overall correlation of quantitative values of ferritin-PBM and plasma CEA was 0.49.

DISCUSSION

In the preceding study, we have evaluated a RIA designed to measure ferritin bound to the surface of isolated PBMs. Using this technique, we were able to discriminate with a high degree of specificity and sensitivity between individuals without cancer, postoperative primary cancer patients, and advanced cancer patients in clinical remission from those patients found to have clinically active disease. Although the major focus of these studies was on breast carcinoma, initial results on other advanced cancers such as those of ovarian and esophageal origin suggest that this measurement may be useful in evaluating other solid tumors as well.

We were not able to discriminate with a high degree of sensitivity between benign disease and early breast cancer, indicating that this test will not be useful in screening or early diagnosis. These findings are in contrast to the early work of Giler et al. (8) who, using ADCC, claimed 96% sensitivity and 100% specificity for early preoperative Stage I and II breast cancer as compared to benign breast disease and normal controls.

In a recently published report by the same group (22) utilizing a refined ADCC technique including monoclonal antibody to placental ferritin and an expanded patient population (n = 447), positive ferritin-PBMs were found in up to 29% of the normal and benign breast population. These results are consistent with the findings presented here and suggest that the ADCC test will not be useful in a screening capacity. Initial evaluation of results in over 1000 patients reconfirms the findings as a potential new tumor marker. These levels of sensitivity and specificity have recently been corroborated using indirect immunofluorescence techniques to detect ferritin-PBMs (26). In that study on 65 patients, 16% of normal and non-cancer controls showed positive values, while greater than 80% of cancer patients showed elevations.

At present, there is no single reliable test for monitoring breast cancer patients. Perhaps the most widely used test is CEA. In single-point determinations in advanced breast cancer, the percentage of those patients with elevated CEA varies from 54% to 74% (3, 29) comparable to CEA results described in this study. Use of elevated CEA values as a predictor of breast cancer recurrence following primary surgical intervention has conflicting results (4, 10). It has been recommended that the most efficacious use of tumor markers is in serial determinations to monitor response to therapy (24). A recent report also suggests that simultaneous measurement of multiple tumor markers is superior to any single marker in the monitoring of the breast cancer patient (15). It is therefore noteworthy that, in this preliminary study, 91% of locally advanced and disseminated breast cancer patients had elevated ferritin-PBMs, whereas only 67% showed increases in CEA.

On the basis of preliminary findings reported here, we have intensified our study on the clinical relationship of ferritin-PBMs in cancer as measured by RIA. Efforts are presently focused on the behavior of the marker in: (a) long-term serial studies on individual patients; (b) analysis of tumor burden and quantitative binding of 125I-labeled RHF to PBMs; (c) comparisons of values in benign and malignant diseases by organ site; (d) marker elevation in other types of solid and hematological tumors; and (e) enhanced correlation with disease status when used in conjunction with other parameters of disease progression. Initial evaluation of results in over 1000 patients reconfirms the findings presented here and will be described in future reports.

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