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Sensitivity of Immunocytochemical Detection of Breast Cancer Cells in Human Bone Marrow[1]

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ABSTRACT

We have previously shown that occult micrometastases can be detected in the bone marrow of breast cancer patients, at the time of initial treatment, using a panel of epithelial specific monoclonal antibodies indirectly labeled with fluorescein. These monoclonal antibodies permit us to detect cancer cells at a concentration of two/million normal bone marrow cells. Immunofluorescence carries the disadvantage that detailed morphological examination of detected cells cannot be accomplished. A modification of the alkaline phosphatase anti-alkaline phosphatase method has been used to detect cancer cells and to observe their morphology in human bone marrow. The sensitivity of this method has been examined using an established human metastatic breast cancer cell line (MCF-7) mixed with normal bone marrow cells at various dilutions from 400 cancer cells/10^6 marrow cells to 10 cancer cells/10^6 marrow cells. The number of immunocytochemically stained MCF-7 cells counted at each concentration was related to the concentration by a simple nonlinear statistical model. At a concentration of 10 cancer cells/10^6 bone marrow cells, the model shows that this method has the sensitivity to detect between four and six MCF-7 cells 95% of the time. Extrapolation, using this model, predicts that at the very low concentration of one cancer cell/10^6 marrow cells, there is a 95% chance of detecting the cancer cell. This assay may be a very sensitive method for detecting cancer cells in the bone marrow of breast cancer patients.

INTRODUCTION

The long-term survival statistics for breast cancer patients are approximately 79% for Stage I ($T_1N_0M_0$)[3] (1), 83% for Stage IIA ($T_2N_0M_0$) (2), 73% for Stage IIB (T_1 and $T_3N_1M_0$) (2), and 20% for Stage III ($T_3N_1M_0$) (3). The commonest site for distant metastatic disease is the skeletal system, resulting from tumor cell dissemination to the bone marrow (4). Perioperative staging at the time of initial treatment, such as biochemical measurement of alkaline phosphatase, bone scanning, skeletal radiography, and routine cytological examination of bone marrow, fails to identify those patients who will relapse. Prediction of relapse currently rests on the determination of prognostic parameters in the primary tumor or regional lymph nodes (5, 6). Improved methods for detecting tumor cells in the bone marrow of patients with early stage disease at the time of diagnosis may allow a more accurate assessment of prognosis and aid in selecting candidates for adjuvant systemic therapy.

Prior studies using polyclonal antibodies to an epithelial cell membrane antigen (7-12) or monoclonal antibodies to epithelium (13-17) have shown that occult cancer cells are present in the bone marrow of patients with breast cancer at the time of diagnosis. In our previous studies we have used Mabs that recognize membrane (C26, T16) and cytoskeletal (AE1) antigens expressed by epithelial derived cells in an immunofluorescent assay (18) to detect cancer cells in the bone marrow aspirates of primary breast cancer patients (15). The sensitivity of this assay was shown statistically to be capable of detecting cancer cells at a concentration of $2/10^6$ normal bone marrow cells (18). However, the immunofluorescent method has the disadvantage that the cells observed cannot be studied morphologically to determine whether they are consistent with cancer cells. Morphological study may be important to exclude false positive cells and tumor cells that are not labeled by the antibody. In order to overcome this problem, we have used a nonfluorescent immunocytochemical method. This technique has been evaluated in a model system using a cell line derived from human metastatic breast cancer cells (MCF-7) mixed with normal human bone marrow.

MATERIALS AND METHODS

MCF-7 Breast Cancer Cells. The test cells used in this study were MCF-7 human breast cancer cells (Michigan Cancer Foundation, Detroit, MI). They were grown in Eagle's minimal essential medium at 37°C, supplemented with Hanks' buffered salts, nonessential amino acids, sodium pyruvate (100 μg/ml), L-glutamine (2 mM), gentamicin (50 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), Fungizone (2.5 μg/ml), bovine insulin (6.6 μg/ml), and 7% fetal calf serum. The cells were seeded into Petri dishes (60 x 15 mm) at a density of 0.5×10^6 cells/dish. The medium was changed every 3 days. Cells were obtained by scraping from the culture dishes; they were then suspended in RPMI 1640 and filtered through double 30-μm nylon mesh.

Bone Marrow Aspirates. Bone marrow samples from normal volunteer donors were suspended in 2× volumes of RPMI 1640. The suspension was layered over a Ficoll-Hypaque solution and centrifuged at 400 × *g* for 20 min. The resultant interface contained nucleated bone marrow cells and the pellet contained RBC and damaged cells. The interface was collected and washed in RPMI 1640.

Test Specimens. MCF-7 cells were serially diluted and added to normal nucleated bone marrow cells. The marrow cells were maintained at a constant concentration (1×10^6); suspensions of MCF-7 cells at 10, 25, 50, 100, 200, 300, and 400 cancer cells/10^6 marrow cells were made. These specimens were suspended in 50 μl PBS; thin smears were prepared, fixed in 100% ethanol, and stored at −20°C. The experiments were repeated 6 times for each dilution of MCF-7 cells in normal nucleated bone marrow cells. In addition, six cytospin preparations were made for each of two low concentrations (10 and 25 cancer cells/10^6 marrow cells) to test immunocytochemistry and immunofluorescence on the same batches of cells in order to make a direct comparison.

Monoclonal Antibodies. Three monoclonal antibodies, C26,[4] T16 (19), and AE1 (20-23) (Boehringer Mannheim, Indianapolis, IN), were studied in combination. Each monoclonal antibody reacts with distinct epithelial specific antigens; C26 and T16 react with dimeric cell surface glycoproteins with molecular weights of 40,000/28,000 and 48,000/42,000, respectively, while anticytokeratin (AE1) reacts with acidic cytokeratin cytoskeletal antigens. All are epithelial cell specific and

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[3] The abbreviations used are: TNM, tumor, nodes, metastases classification; Mab, monoclonal antibody; APAAP, alkaline phosphatase anti-alkaline phosphatase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; NLS, nonlinear least squares; WNLS, weighted nonlinear least squares; EMA, epithelial membrane antigen; CI, confidence interval.

[4] Manuscript in preparation.

individually react with the majority of human primary breast cancers tested (15), including mammary carcinoma cells from the established MCF-7 cell line (18).

Immunocytochemical Staining Procedure. Stored, prefixed smears were brought to room temperature and washed for 10 min in TBS (pH 7.6). The monoclonal antibody mixture (200 μl/slide) was applied for 30 min (T16 and C26, 25 μg/ml; and AE-1, 3:100 dilution). After two 10-min washes in TBS, smears were incubated with rabbit anti-mouse immunoglobulin (linking antibody) (200 μl/slide). The same washing pattern was repeated, and then APAAP complexes were applied for 30 min (linking antibody and APAAP complexes were used from Dako APAAP Kit System 40; Dako, Santa Barbara, CA). The slides were then washed in TBS (pH 7.6) for 15 min. Alkaline phosphatase substrate was prepared by dissolving 2 mg of naphthol As-Mx in 0.2 ml of dimethyl formamide in a glass tube, and 1 mg of Fast Red Tr salt was dissolved in 9.8 ml of TBS (pH 8.2). The two solutions were mixed and filtered through Whatman No. 1 filter paper. The filtered solution (200 μl) was applied to the slides for 20 min. The slides were then washed for 1 min in distilled water and counterstained with Mayer's hematoxylin. All immunocytochemical staining procedures were performed at room temperature in humid chambers. Smears were then wet mounted in crystal mount and left in an oven at 60°C for 15 min to dry. Dry smears were mounted in Permount for permanent record. Slides were examined with a light microscope at ×40, and the number of red staining cells was counted. Negative controls of PBS-1% BSA in place of the antibody mixture and positive controls consisting of smears of MCF-7 were included in each assay. The method for indirect immunofluorescence has been previously described in detail (18).

Statistical Methods. Data from six sets of experiments were used to establish a mathematical relationship between number (NUM) of MCF-7 cells detected and concentration (CONC) of MCF-7 cells expressed as number of the cells diluted in 10^6 normal nucleated bone marrow cells as previously described (18). Plots of NUM on power transformation of CONC, namely $CONC^{p<p<1}$, suggest that for concentrations less than 400 cancer cells/10^6 marrow cells, an appropriate model for the data is given by

$$NUM = a CONC^b + e$$

where a and b are unknown parameters, and e is a random error term with zero mean representing measurement errors and biological variations of observed values of NUM about the population mean value $a CONC^b$. The parameter a gives the mean number of MCF-7 cells detected at a concentration of 1 cancer cell/10^6 marrow cells. The parameter b represents the change in mean NUM on a logarithmic scale per unit of change in CONC, also expressed on a logarithmic scale.

A NLS procedure was used to estimate the parameters a and b. Residual analysis revealed that the variance of the error term e increased linearly with concentration. To obtain more precise estimates of the parameters, it was necessary to weight the NLS procedure by an appropriate set of weights. A linear regression of the standard deviation of NUM on CONC was used to determine the weights. Standard asymptotic statistical arguments were applied to the WNLS results in order to obtain approximate standard errors and construct confidence intervals.

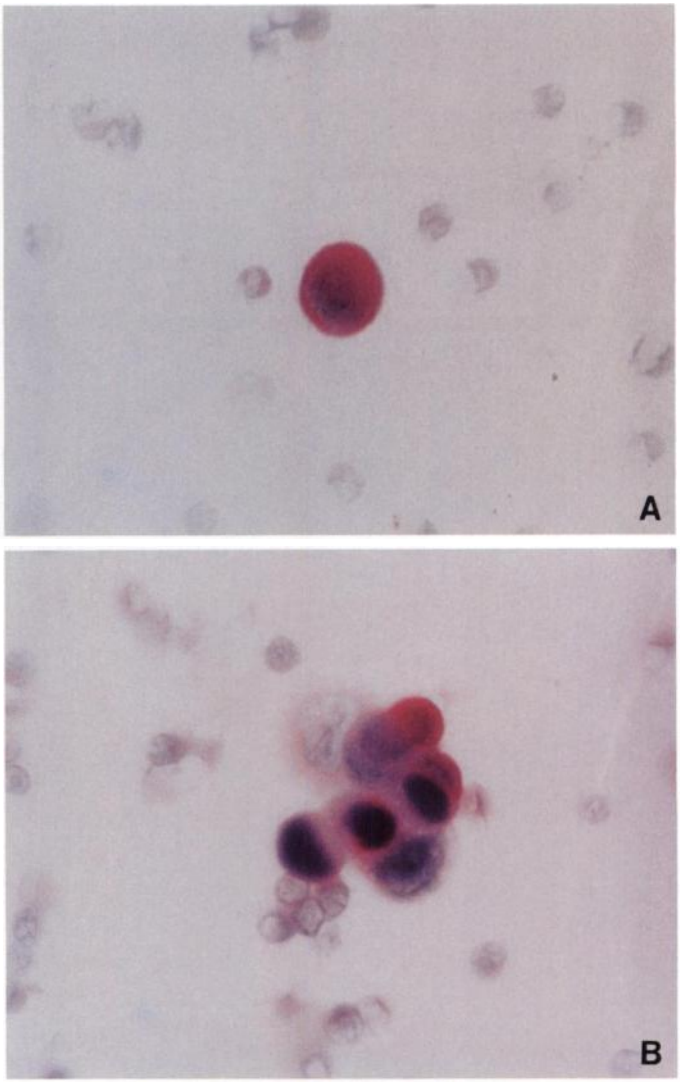

Fig. 1. Photomicrograph showing a single MCF-7 cell stained red (*A*) and a cluster of MCF-7 cells stained red (*B*). Background shows unstained bone marrow cells. × 800.

Table 1 *Sensitivity of immunocytochemical method*

No. of MCF-7 cells/10^6 BMC[a]	% MCF-7 cells	Sample mean no. of MCF-7 cells
10	0.001	6.33
25	0.0025	10.60
50	0.005	19.66
100	0.01	37.80
200	0.02	60.16
300	0.03	86.16
400	0.04	156.33

[a] BMC, bone marrow cells.

RESULTS

Fig. 1 shows MCF-7 cells mixed with bone marrow after incubation with C26, T16, and AE1 anti-epithelial mouse Mabs. The specimen had been stained with Fast Red Tr using the modified APAAP method. The MCF-7 cells could be identified by light microscopy since their cytoplasm and cell membranes were strongly stained by Fast Red Tr. More than 90% of the cells were reactive.

Table 1 shows that the number of immunoreactive cells increased with an increasing concentration of MCF-7 cells in normal nucleated bone marrow cells. The lowest concentration measured in this experiment was 10 cancer cells/10^6 bone marrow cells. Fig. 2 shows the plot of NUM related to CONC for all measurements in six experiments. The plot shows that there was a considerable amount of variation in NUM at each concentration and that the variation increased as concentration increased. The mean values shown on the plot (Fig. 2) suggest that a linear model cannot satisfactorily fit the data. The graph also shows that the relationship between NUM and CONC for CONC <400 cancer cells/10^6 marrow cells can be quite different from that for CONC ≥400 cancer cells/10^6 marrow cells. The WNLS procedure described in "Materials and Methods" was applied to the data for CONC <400 cancer cells/10^6

marrow cells. The estimated equation was

$$NUM = 0.8749\ CONC^{0.8040}.$$

The standard errors of the WNLS estimates of a and b were 0.12 and 0.028, respectively.

Table 2 summarizes the WNLS fit to the data at six observed concentrations. Extrapolation of the model to low concentrations is also given. The entries of Columns 2 and 3 indicate that a very good fit to the data within the range of concentrations investigated was obtained with the nonlinear model. The high level of sensitivity of the assay is demonstrated as the 95% CI at a concentration of 1 cancer cell/10^6 marrow cells includes 1.

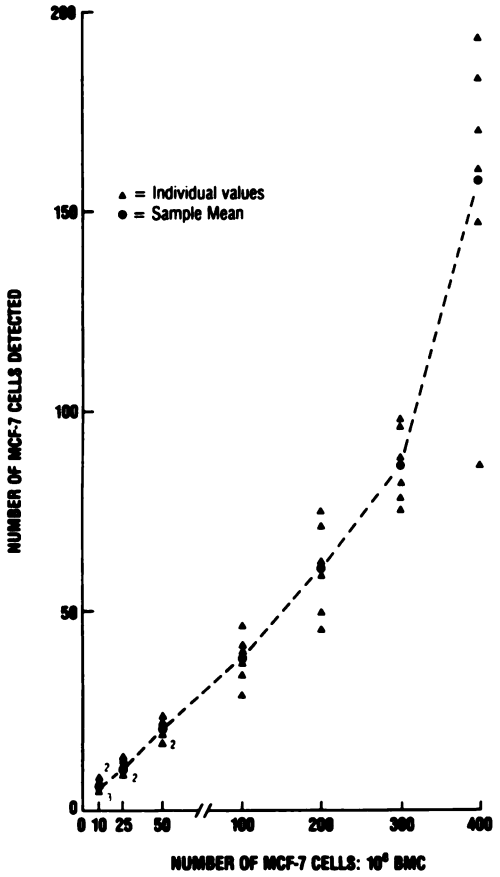

Fig. 2. MCF-7 cells detected against concentration in replicate assays. *BMC*, bone marrow cells.

Table 2 *Summary of nonlinear fits*

No. of MCF-7 cells/10^6 BMC[a]	Sample mean[b]	Estimated mean[c]	Asymptotic SE of estimated mean	95% confidence interval for the mean
1	—[d]	0.88	0.12	0.64–1.11
2	—	1.53	0.18	1.18–1.88
4	—	2.67	0.26	2.15–3.18
6	—	3.70	0.32	3.06–4.33
8	—	4.66	0.37	3.93–5.38
10	6.33	5.57	0.41	4.77–6.38
25	10.60	11.64	0.59	10.48–12.79
50	19.66	20.32	0.72	18.91–21.73
100	37.80	35.48	0.94	33.63–37.32
200	60.16	61.94	1.86	58.29–65.59
300	86.16	85.82	3.18	79.59–92.04
400	156.33	[e]	[e]	[e]

[a] BMC, bone marrow cells.
[b] Mean number of MCF-7 cells detected in 6 independent experiments.
[c] Estimated mean number of MCF-7 cells detected.
[d] —, assay not performed at this concentration.
[e] Model not applicable at this concentration.

The mean number of cells counted in six cytospin preparations at 25 cancer cells/10^6 marrow cells was 10.6 MCF-7 cells using immunocytochemistry and 8.6 using immunofluorescence. At a concentration of 10 cancer cells/10^6 marrow cells, a mean of 5.8 MCF-7 cells were counted using immunocytochemistry and 4.0 using immunofluorescence.

DISCUSSION

Micrometastases due to tumor cell dissemination prior to diagnosis are undetectable by current staging procedures at the time of initial treatment. The subsequent progression of micrometastases results in distant relapse after treatment of an apparently localized primary tumor. Tumor diameter, lymph node status, and some biological factors are currently used to predict prognosis and determine the need for systemic drug treatment. New methods are needed to improve the accuracy of estimates of prognosis.

Skeletal metastases from breast cancer are frequently the first and commonest site of relapse. Skeletal metastases occur owing to dissemination of tumor cells which lodge in the bone marrow and then clonally expand to invade adjacent bone (4). Efforts to detect tumor cells in the bone marrow of early stage patients, using conventional cytological examination of bone marrow aspirates, have not been successful (24). An immunocytochemical technique for staining bone marrow aspirates for extrinsic cells was developed by the Ludwig Institute for Cancer Research group using a rabbit polyclonal antiserum to the milk fat globule membrane called epithelial membrane antigen, EMA (7, 8). Although the presence of EMA-staining cells in the bone marrow has been shown to be a predictor of prognosis and site of relapse (9–12), concerns have been expressed about the epithelial specificity of EMA (16, 25, 26).

We have used a mixture of mouse Mabs directed against antigens on the epithelial cell membrane and cytoskeleton. The specificities of these antibodies have been well characterized. They each react against the majority of tumor cells in most primary breast cancers but not with normal bone marrow elements and in combination react with almost all breast cancers (15).

Our initial hypothesis was that immunofluorescence would permit easy identification of tumor cells; the current experiment shows that tumor cells can be detected with comparable, if not slightly greater, accuracy using an immunocytochemical method. We have shown that, at each concentration, the 95% CI of the immunocytochemical method is higher than that of our previously published immunofluorescent method (18). For example, at CONC = 10, the 95% CI = 4.77–6.38, which is higher (greater probability of detecting cells) than for immunofluorescence, where the 95% CI = 2.15–3.78. This is true also for CONC = 25, 50, 100, 200, and 300. Direct comparison of the two methods at the low concentrations of 10 cancer cells/10^6 marrow cells and 25 cancer cells/10^6 marrow cells, using the same batch of samples, also showed a consistently higher detection rate using immunocytochemistry compared with immunofluorescence. The ability to examine the morphology of immunoreactive cells as well as to maintain a permanent record using immunocytochemistry is a significant advantage. The statistical model applied to these experiments also appears to closely fit recently described data, but the extent of variability associated with the sensitivities described is unknown as no

formal statistical analysis was reported (14).

The purpose of the present investigation was to determine the sensitivity of an immunocytochemical method in a model system *in vitro* to detect occult breast cancer cells prior to applying it to a prospective clinical trial in patients to determine whether the presence of these cells is a predictor of prognosis. However, it is not known whether metastatic cells *in vivo* will have the same uniform staining characteristics as the test system using MCF-7 cells. It is possible that metastatic cells *in vivo* are more heterogeneous than cultured MCF-7 cells *in vitro* and that this assay may not be as sensitive in clinical practice. We anticipate that it will be possible to detect very low levels of metastatic involvement of bone marrow using the immunocytochemical method.

A pilot clinical study of the immunofluorescent method revealed that a third of patients with operable breast cancer without clinical evidence of distant metastatic disease had fluorescent cells in their bone marrow (27). These patients had an increased relapse rate when compared with patients who did not have these cells present in their bone marrow (28).

We therefore conclude, using the statistical approach applied in these experiments, that immunocytochemistry is a highly sensitive and suitable method for detecting breast cancer cells in human bone marrow. Evaluation of this method in a prospective clinical research study to determine its prognostic value is in progress.

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