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Immunofluorescent Monoclonal Antibody Detection of Breast Cancer in Bone Marrow: Sensitivity in a Model System[1]

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ABSTRACT

We have previously shown that occult micrometastases can be detected in the bone marrow of breast cancer patients using a panel of epithelial specific monoclonal antibodies in an indirect immunofluorescent assay. The sensitivity of this assay has been examined using cells from an established human breast cancer cell line (MCF-7) mixed with normal bone marrow at various dilutions from 400 cancer cells/10^6 marrow cells ($400:10^6$) to $10:10^6$. MCF-7 cells were detected at the lowest concentration studied, namely $10:10^6$. The number of fluorescent labeled MCF-7 cells counted at each concentration was related to the concentration by a simple nonlinear statistical model. At the concentration of $10:10^6$, the model shows that this technique has the sensitivity to detect between two and four MCF-7 cells 95% of the time. Moreover, by extrapolation, the model predicts that even at the very low concentration of $2:10^6$, there is a 95% chance of detecting one cancer cell. Therefore, this assay may be a very sensitive method for detecting cancer cells in the bone marrow *in vivo*.

INTRODUCTION

The 10-year survival statistics for operable stage breast cancer patients treated by surgery and/or irradiation are approximately 83% for Stage I (T1N0M0)[3] (1), 73% for Stage II (T2N0M0) (2), 50% for Stage II (T1 and T2N1M0) (2), and 20% for Stage III (T3N1M0) (3). The commonest site of dissemination of breast cancer is to the bony skeleton via the bone marrow (4). Standard methods of 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, fail to identify those patients who are going to relapse. Improvement in the methods of detecting tumor cells in the bone marrow of patients with early stage disease at the time of diagnosis may aid in selecting candidates for adjuvant therapy.

Mabs which recognize membrane and cytoskeletal antigens expressed by epithelial derived cells (C26, T16, AE1) have been used in an immunohistochemical (immunofluorescent) method to find cancer cells in bone marrow aspirates of primary breast cancer patients (5). This method was evaluated in a model system using a human breast cancer cell line (MCF-7) and normal human bone marrow to assess clinically relevant sensitivities and to account for variations in the observations by statistical analysis.

MATERIALS AND METHODS

Human Breast Cancer Cell Line. MCF-7 human breast cancer cells (Michigan Cancer Foundation, Detroit, MI) were grown in Eagle's minimal essential medium at 37°C, supplemented with Hanks' buffered salts, nonessential amino acids, sodium pyruvate, α-glutamine (10 nM), gentamicin (50 μg/ml), penicillin (10 units/ml), streptomycin (1 mg/ml), Fungizone (2.5 μg/ml), bovine insulin (6.6 ng/ml), and 7% fetal calf serum. Cells were seeded into Petri dishes (60 x 15 mm) at a density of 0.5×10^6 cells/dish. Medium was changed every 3 days. Cells were obtained by scraping from the culture dishes and were then suspended in RPMI 1640 and filtered through double 30-μm nylon mesh.

Bone Marrow Aspirates. Bone marrow samples from normal donors were suspended in 2× volumes of RPMI 1640. The suspension was layered over a Ficoll-Hypaque solution and centrifuged at $400 \times g$ 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); concentrations (number of cancer cells/10^6 marrow cells) of MCF-7 cells at 10, 25, 50, 100, 200, 300, and $400:10^6$ marrow cells were made. These specimens were suspended in 50 μl PBS and thin smears were prepared and fixed in 100% ethanol and stored at −20°C. The experiments were repeated five to six times for each dilution of MCF-7 cells in normal nucleated bone marrow cells.

Monoclonal Antibodies. Three monoclonal antibodies, C26,[4] T16 (6), and AE1 (7–9) (Boehringer Mannheim, Indianapolis, IN) were studied in combination. Each monoclonal antibody reacts with distinct epithelial specific antigen; C26 and T16 react with dimeric cell surface glycoproteins with molecular weights of 40,000/28,000 and 48,000/42,000, respectively, while anti-cytokeratin (AE1) reacts with acidic cytokeratin cytoskeletal antigens. All are epithelial cell specific and react with the majority of human primary breast cancers tested, including mammary carcinoma cells from the established MCF-7 cell line.

Immunofluorescent Staining Procedure. The immunofluorescent staining procedure has been described previously (10). The smears were thawed and washed with PBS for 10 min and incubated with suppressor serum (5% rabbit serum in PBS-0.1% BSA) for 30 min as a blocking step. The suppressor serum was drained off and the mouse monoclonal antibody mixture was applied (200 μl/slide) as described previously (5). The slides were incubated for 1 h at room temperature. The slides were washed 3 times with PBS (containing 0.1% BSA and 0.5% Tween 20) and then incubated for 1 h at room temperature in the dark with fluorescein-conjugated rabbit anti-mouse antiserum (200 μl/slide) (Dako Corporation, Santa Barbara, CA) at a concentration of 1:20. The smears were washed 3 times with PBS (containing 0.1% BSA and 0.5% Tween 20) and wet mounted in 90% glycerol in PBS. The immunofluorescent staining procedures were performed at room temperature in wet chambers. The slides were either examined immediately or stored in the dark at 4°C for no more than 12 h prior to examination with a Nikon Optifot fluorescence microscope. Slides were scanned at ×40 and the number of cells observed was counted. Negative controls of PBS-0.1% BSA in place of the antibody cocktail and positive controls consisting of smears of MCF-7 cells were included in each assay.

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. Plots of *NUM* on power transformation of *CONC*, namely $CONC^P$, $0 < P < 1$, suggest that for concentration less than $400:10^6$,

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[3] The abbreviations used are: TNM, tumor-nodes-metastasis classification; Mab, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NLS, nonlinear least squares; WNLS, weighted nonlinear least squares.

[4] Manuscript in preparation.

an appropriate model for the data is given by

$$\text{NUM} = aCONC^b + e$$

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 $aCONC^b$. The parameter a gives the mean number of MCF-7 cells detected at concentration of $1{:}10^6$. The parameter b represents the change in mean *NUM* on a logarithmic scale per unit change in *CONC*, also expressed on a logarithmic scale.

A NLS procedure was used to obtain the NLS estimates of a and b. Residual analysis revealed that the variance of e increased linearly with concentration. In order to obtain optimal NLS solutions, 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 standard errors and construct confidence intervals.

RESULTS

Fig. 1 shows a MCF-7 bone marrow mixture after incubation with T16, C26, and AE1 anti-epithelial mouse Mabs and then fluorescein-conjugated rabbit anti-mouse antiserum. Under UV microscopy the MCF-7 cells can be identified; both cell surface and cytoplasmic components show positive fluorescence (Fig. 1*A*). The MCF-7 cells showed strong and uniform staining when the antibody cocktail was used with over 90% of cells showing reactivity. The same field is seen under phase-contrast microscopy in Fig. 1*B*. Note that normal bone marrow elements are not identified by the immunofluorescence technique.

Table 1 shows that the number of detectable positive cells increases with increasing concentration of MCF-7 cells in normal nucleated bone marrow cells and that the number of positive smears increases with increasing number of MCF-7 cells. In this experiment, at the lowest concentration measured, MCF-7 cells constitute only 0.001% of the population of two cell types or 1 cancer cell (MCF-7) in 100,000 ($1{:}10^5$) normal bone marrow cells. Fig. 2 shows the plot of *NUM* on *CONC* for all the measurements from the six experiments. It is evident that there was a considerable amount of variation of *NUM* at each concentration and that the variation increases as concentration increases. The mean values shown on the plot (Fig. 2) suggest that a linear model cannot satisfactorily fit the data, at least not at the level of sensitivity required by this kind of experiment. The reason why the plot is nonlinear is unknown. Moreover, the plot also indicates that the relationship between *NUM* and *CONC* for $CONC < 400{:}10^6$ can be quite different from that for $CONC \geq 400{:}10^6$.

The WNLS procedure described in "Materials and Methods" was applied to the data for $CONC < 400{:}10^6$. The estimated equation was

$$NUM = 0.4003\ CONC^{0.869}$$

The standard errors of the WNLS estimates of a and b were 0.10 and 0.055, respectively. About 92% of the total variation of *NUM* about zero was explained by its nonlinear regression on *CONC*. The diagnostic plot in Fig. 3 indicates the appropriateness of the model.

Table 2 summarizes the WNLS fit to the data at six observed concentrations. Extrapolation of the model to four low concentrations is also given. The entries of Columns 2 and 3 show that a very good fit to the data at the range of concentrations investigated was obtained with the simple nonlinear model. The high level of sensitivity of the assay is demonstrated as the 95% confidence interval at concentration equal to $2{:}10^6$ contained 1. It should be pointed out that this model also appears to closely fit recently described data but the extent of variability associated with the sensitivities described are unknown since no statistical analysis was presented (11).

DISCUSSION

A significant proportion of patients presenting with apparently local primary breast cancer have micrometastases undetectable by conventional staging procedures. The most reliable prognostic indices, axillary lymph node status and primary tumor diameter, cannot predict which individual patient has systemic micrometastases that will ultimately progress to relapse and death.

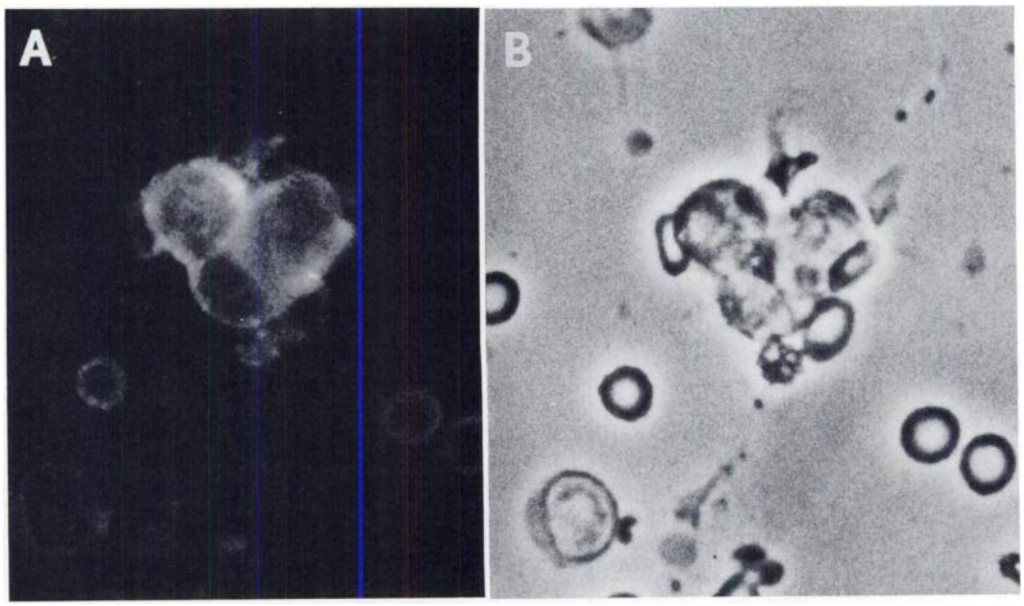

Fig. 1. Breast cancer cell line MCF-7 mixed with normal bone marrow mononuclear cells. (*A*) cluster of MCF-7 cells stained by the indirect immunofluorescent technique; (*B*) the same cluster as seen under phase contrast. Note that the bone marrow elements are not visualized by the immunofluorescent method.

Table 1 *Sensitivity of immunofluorescent assay*

MCF-7 cells:10^6 BMC[a]	% of MCF-7 cells	No. of slides positive	% of positive slides	Sample mean MCF-7 cells
10	0.001	6/25	24	3.83
25	0.0025	11/25	44	5.17
50	0.005	15/25	60	11.83
100	0.01	18/25	72	24.00
200	0.02	19/25	76	38.40
300	0.03	13/16	81	56.80
400	0.04	25/25	100	109.60

[a] BMC, bone marrow cells.

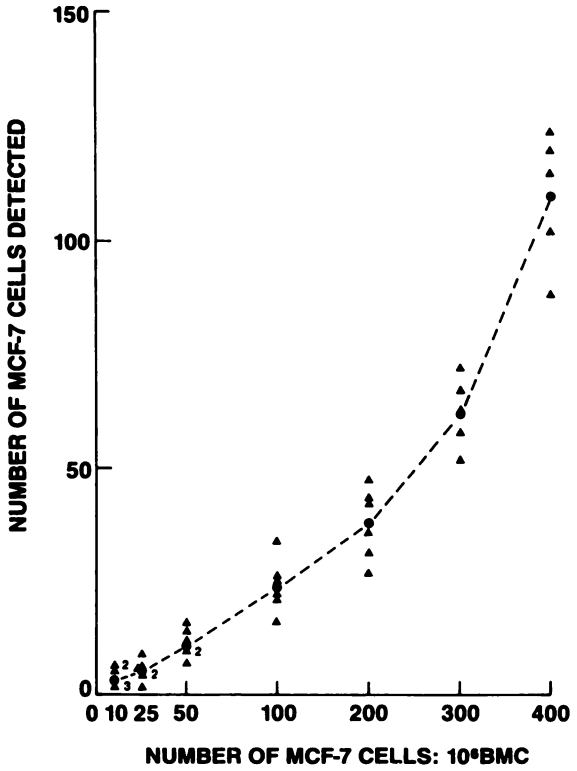

Fig. 2. MCF-7 cells detected against concentration in replicate assays. ▲, individual values; ●, sample mean.

The skeleton is a common site of distant metastases from breast cancer and is frequently the first organ in which metastases develop. Conventional cytology of bone marrow fails to detect micrometastases in early stage patients (12). An immunocytochemical technique designed to detect low numbers of breast cancer cells in the bone marrow of patients with operable disease has been studied by the Ludwig Institute for Cancer Research group (13–20). We have recently reported on a similar immunocytochemical method which detected the presence of breast cancer cells in the bone marrow of patients with early stage disease (1). Unlike the Ludwig group, which used rabbit antiserum to epithelial membrane antigen (21, 22), we have used a mixture of mouse Mabs directed against antigens. The specificity of these antibodies has been well characterized, and in addition to reacting with most breast cancers, they do not react with normal bone marrow elements (5). As reported previously, the antibodies used here did not react with bone marrow cells from 44 subjects undergoing diagnostic and staging work-ups for nonepithelial malignancy such as leukemia and lymphoma (5). The antibodies react with most breast cancers and, when used in combination, react with over 90% of breast cancer cells in frozen sections of primary breast cancers. Although there is some variation in the intensity of staining from case to case, all breast cancers tested showed good reactivity. In addition, rather than use light-microscope methods, we have used an indirect immunofluorescent method to identify extrinsic epithelial cells, with the belief that it might be easier to detect fluorescent cells against a dark background (Fig. 1) and thus improve the likelihood of detecting extrinsic cells. The purpose of the current investigation is to demonstrate and statistically validate the sensitivity of the immunofluorescent method. We have shown in our prior clinical studies a comparable ability to detect extrinsic cells using only two to three sites for bone marrow aspirations by this method (5, 23) as opposed to the eight sites used by the Ludwig group (17). We were able to detect breast cancer cells in the bone marrow of breast cancer patients without evidence of metastases to lymph nodes in 23% of patients with T1N0M0 and 33% of patients with T2N0M0 disease (5).

The formal statistical approach taken here is important. First, conclusions about the sensitivity of our assay are valid if inherent laboratory as well as biological variations are accounted for in the analysis. In this study, the extent of variation across six sets of experiments was substantial and increased with higher concentrations; this may be due to biological factors such as

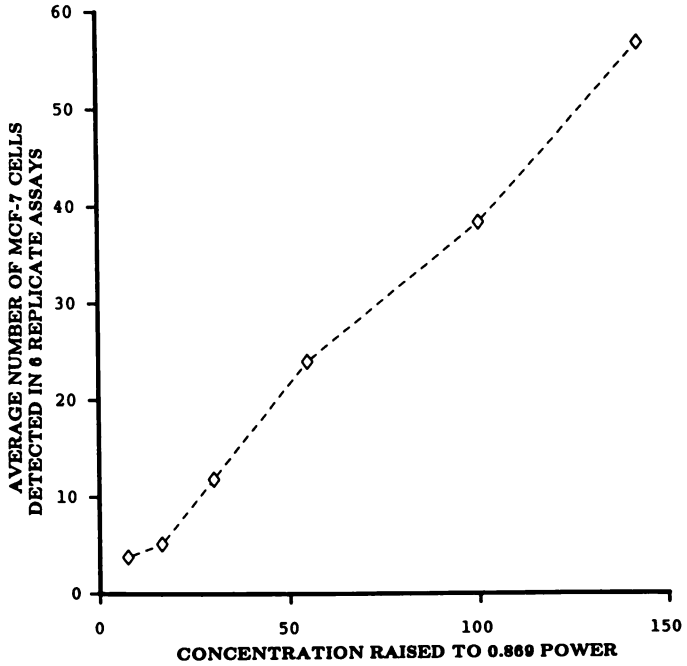

Fig. 3. Diagnostic plot for nonlinear model.

Table 2 *Summary of nonlinear fits*

MCF-7 cells:10^6 BMC[a]	Sample mean[a]	Estimated mean[a]	Asymptotic SE of estimated mean	95% confidence interval for the mean
2	—[b]	0.73	0.16	0.40, 1.06
4	—	1.34	0.24	0.85, 1.83
6	—	1.90	0.31	1.27, 2.53
8	—	2.44	0.36	1.71, 3.17
10	3.83	2.96	0.40	2.15, 3.78
25	5.17	6.56	0.60	5.35, 7.78
50	11.83	11.99	0.76	10.43, 13.54
100	24.00	21.89	1.13	19.59, 24.19
200	38.40	39.99	2.57	34.75, 45.22
300	56.80	56.87	4.52	47.66, 66.08
400	109.60	[c]	[c]	[c]

[a] BMC, bone marrow cells; Sample mean, sample mean number of MCF-7 cells detected in 5–6 independent experiments; Estimated mean, estimated mean number of MCF-7 cells detected.
[b] —, assay not performed at this concentration.
[c] Model not applicable at this concentration.

settling or clumping of MCF-7 cells or other unknown causes. Second, the statistical analysis led to a simple mathematical model which succinctly delineates the relationship between number of cancer cells detectable and concentration. More importantly, the fitted model has enabled us to make statistical confidence statements about sensitivity of the assay at any concentration, particularly at the low concentrations of clinical relevance.

Further studies are necessary to determine whether this method of determining the prevalence of cancer cells in the bone marrow independently predicts for site of relapse and prognosis. The development of more specific monoclonal antibodies used in optimum combinations may be required (24). Utilization of strict criteria to distinguish extrinsic (epithelial) cells from normal bone marrow constituents will enhance sensitivity. Improvement of complementary immunocytochemical staining techniques may be accomplished by using alkaline phosphatase (14, 25) or glucose oxidase following *in vitro* culture (26). Peroxidase staining methods are less suitable due to high levels of endogenous peroxidase in bone marrow elements. Should our highly sensitive method be proved to be a predictor of prognosis, then automated methods such as computerized fluorodensitometry may allow more efficient processing of larger numbers of specimens.

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