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Detection of Rare MCF-7 Breast Carcinoma Cells From Mixtures of Human Peripheral Leukocytes by Magnetic Deposition Analysis

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Background: The presence of malignant breast cancer cells in bone marrow or peripheral blood is a prognostic factor. We tested the capacity of a novel magnetic cell analyzer to detect rare cancer cells in mixtures with human peripheral leukocytes.

Methods: Human peripheral leukocytes were spiked with cells of the MCF-7 line, and the cell mixture was labeled with anti-epithelial membrane antigen antibody and a magnetic colloid. The MCF-7 cells were selectively captured on a magnetic deposition substrate from the flowing leukocyte and MCF-7 cell mixture.

Results: The recovery of the MCF-7 cells from the original mixture ranged from 20% to 60%. The limit of detection of the MCF-7 cells was 10^{-6} ($n = 9$). The morphology of the captured cancer cells was well preserved and comparable to that observed in cytospin smears. All deposited cells were located in a small area of 1.4 mm × 6 mm and could be quickly identified with an optical microscope following Wright's staining.

Conclusions: This is a proof-of-principle study using a simplified model of rare cancer cells in a leukocyte mixture. The clinical relevance of the method will be tested in the future by extension to patient bone marrow samples and using antibody cocktails to increase specificity against the breast carcinoma cells. Cytometry 36:294–302, 1999. © 1999 Wiley-Liss, Inc.

Key terms: breast cancer; MCF-7 cell line; immunomagnetic separation; rare cell detection; cell separation

Breast cancer is the most common cancer in women. An estimated 181,600 new invasive cases in the United States were predicted in 1997, with an estimated mortality of 43,900 (1). Its incidence rate at approximately 27/100,000 women has been stable since 1930. In addition to surgery, high dose chemotherapy followed by autologous bone marrow (BM) transplantation (2,3) or peripheral blood stem cell (PBSC) grafting (4,5) appeared to be effective adjuvant treatments. However, the median 10-year survival rate is 65% (1).

Recent studies have shown that tumor cells may exist in the BM sample (6–8) or even in the peripheral blood (9,10) from operable breast cancer patients. These tumor cells are very likely to contribute to the disease recurrence, which had been proven by genetic analysis (6), cell surface phenotype analysis (11), and long-term follow-up in a large cohort of patients (8). Therefore, a method of detection of rare cancer cell in BM or blood is needed for identifying patients who are most likely to benefit from the adjuvant therapy. To be used in routine diagnostic tests, the cancer cell detection method should have the following characteristics: (a) low limit of detection (LOD), (b) short analysis time, (c) large cell volume capacity, and (d) low cost. The reported maximum, clinically significant frequency of the malignant cells in PBSC or BM is below one in 10^4 normal cells (12), and this determines the LOD of any new technique of breast cancer cell detection. The latest research reports have shown that reverse transcriptase–polymerase chain reaction (RT-PCR) (13,14), fluorescence-activated cell sorting (FACS) (12), automated image cytometry (15,16), and a combination of magnetic enrichment and FACS (17) have the ability to detect cancer cell frequency as low as 10^{-6} or even 10^{-7}, which certainly meets the criterion of clinical relevance. However, the clinical applications of these techniques have been hampered by the use of a large number of antibodies, long immunostaining procedures (up to 18 h) (12), slow quantitation process (15,16), difficulties in obtaining representative sample for analysis without contamination dur-

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ing surgery (13,14), high cost of the necessary state-of-the-art FACS apparatus (12), and the added complexity of magnetic enrichment (17).

In the present study, we report on the use of a novel magnetic separation apparatus, the Bio-Ferrograph, to isolate directly on the microscopic glass slide rare breast cancer cells (MCF-7 breast carcinoma cell line) from a model mixture with human peripheral leukocytes. The magnetic deposit was confined to a small area of the slide that was easily accessible to rapid scanning. In our hands, this method was relatively inexpensive to set up, quick, and relatively easy to perform. In the present configuration, five different deposits could be obtained on a single slide simultaneously. The method combines cell enrichment and immunocytology slide preparation in a single step. We determined the MCF-7 cell capture rate, which was the measure of the MCF-7 cell recovery from the original sample on the deposition substrate. We also measured the MCF-7 cell capture rate as a function of the decreasing frequency of the MCF-7 cell in the cell mixture, from 10^{-4} to 10^{-6}, which indicated that the method is able to detect one cancer cell in a model cell mixture of 1 million peripheral blood leukocytes.

MATERIALS AND METHODS

Breast Cancer Cell Line

The test cells used in this study were the MCF-7 breast carcinoma cell line, obtained from American Type Culture Collection (ATCC, Rockville, MA). The cells were maintained in continuous culture in RPMI 1640 medium (Gibco Co., Detroit, MI) supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY), 0.1% (v/v) insulin, 10 U/ml penicillin, and 10 μg/ml streptomycin. The cells were grown in a humidified incubator with 6.5% CO_2 at 37°C, and the culture medium was changed every 3 days.

The adherent growing cells were harvested by washing twice in Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS; Life Technologies) and exposure to 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) with 0.3% ethylene-diamine-tetraacetic acid for 10 min at room temperature. After two washes with reconstitution buffer containing Ca^{2+}/Mg^{2+}-free PBS with 0.1% bovine albumin solution (BSA; Ortho System, Inc., Raritan, NJ) and 0.045% sodium azide, the harvested cells were filtered through a 15-μm nylon mesh (Spectrum Co., Houston, TX) to obtain a single-cell suspension. The Trypan-blue exclusion test showed that the viability of the filtered MCF-7 cells was higher than 90%.

Human Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood samples (30–50 ml) were obtained by venipuncture from healthy volunteers at the Cleveland Clinic Foundation (CCF) who signed the informed consent statement approved by the CCF Institutional Review Board. The blood was anticoagulated by the addition of 10% heparin (Elkins-Sinn, Inc., Cherry Hill, NJ) at the time of blood withdrawal. The whole blood was mixed with an equal volume of Hank's Balanced Saline Solution (HBSS; Life Technologies) and centrifuged at 400*g* for 15 min. The plasma was replaced by an equal volume of HBSS and mixed with the blood cells. The cell mixture was layered over a Ficoll-Hypaque solution (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400*g* for 30 min at room temperature. The PBMC layer consisting mostly of lymphocytes (90% ≈ 95%), monocytes, and small amounts of granulocytes and red blood cells was collected and washed twice in 20 ml reconstitution buffer by centrifugation at 400*g* for 15 min.

Test Samples

Filtered MCF-7 cells were counted by using an improved Neubauer hemacytomer (six replicates); leukocytes were counted by using the Coulter Counter model Z-1 (Coulter Electronics, Hialeah, FL; five replicates). The MCF-7 cells were serially diluted and added to the PBMC preparation at ratios of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. The experimental design included two different sets of experiments. In one set of experiments, designed to test the theoretical limit of the MCF-7 cell recovery in the magnetic deposit, the MCF-7 cells were magnetically labeled before adding them to the leukocyte mixture. In another set of experiments, designed to test the practical limit of the MCF-7 cell recovery in a setting more relevant to clinical applications, the cell mixture was labeled after addition of the MCF-7 cells to the leukocyte mixture.

Monoclonal Antibody and Magnetic Colloid

The mouse anti-human monoclonal antibody (mAb) to epithelial membrane antigen (EMA; Biomeda Corp., Foster City, CA) was used as the MCF-7 cell targeting antibody, at a final concentration of 20 μl/ml in reconstitution buffer. The rat anti-mouse MACS microbeads (Miltenyi Biotec Inc., Auburn, CA) were used as the cell magnetizing reagent. The average microbead diameter was 50 nm, and it contained magnetite embedded in a dextran polymer matrix. The microbeads were covalently coupled to rat anti-mouse polyclonal antibodies. The final concentration of MACS microbeads used was 10 μl/ml in the reconstitution buffer.

Immunomagnetic Labeling

MCF-7 cells, alone or in a mixture with PBMC preparation, were centrifuged at 400*g* for 5 min. The pellets were incubated in 1 ml anti-EMA mAb solution with gentle rotation for 1 h at room temperature. After washing twice in the reconstitution buffer, the MCF-7 cells were magnetized in 1 ml MACS microbead solution with rotation for 45 min at 7°C. The excess antibodies were removed by two washing steps, and the pellets were suspended in 1 ml reconstitution buffer and stored on ice. Pure MCF-7 cell preparations were magnetized by the same staining procedure as that used for the cell mixture and were used as a positive control of the magnetic deposition.

Immunofluorescent Staining

An indirect immunofluorescence assay was used in the preliminary studies to maximize the specificity of anti-EMA mAb against MCF-7 cells (relative to human peripheral

mononuclear cells). One-and-a-half million MCF-7 cells or PBMCs were used for each test. The cells were incubated for 1 h at room temperature in 1 ml anti-EMA mAb solution at final concentrations of 20, 80, 140, and 200 µl/ml. After two washes in Ca^{2+}/Mg^{2+}-free PBS, cells were stained with 1 ml polyclonal antibody biotin-conjugate (Coulter Immunological Co., Hialeah, FL) at 1 µl/ml for 15 min at 7°C, washed, and then incubated in 1 ml avidin–fluorescein isothiocyanate (FITC; 20 µl/ml; Vector Inc., Burlingame, CA) for 5 min at 7°C. The final volume of the cell suspension was 1 ml. The fluorescence-labeled cells were fixed in 1 ml 1% (v/v) paraformaldehyde, centrifuged, and resuspended in 500 µl PBS with 4.4% BSA. The cells were kept at 4°C overnight prior to FACS analysis. The FACS analysis was performed with the FACScan Analyzer and CellQuest software (Becton Dickinson, San Jose, CA).

Magnetic Cell Deposition in the Bio-Ferrograph

A prototype, high-gradient magnetic field separator (Bio-Ferrograph), developed in collaboration with the Institute Guilfoyle (Belmont, MA), was used in this study. An earlier version of this instrument was described in a previous publication (18). The magnetic field was generated by an interpolar gap of a permanent magnet assembly comprising ferrite magnets and a pair of 1016 low-carbon steel pole pieces (model C). The interpolar gap formed a magnetic barrier to the magnetically labeled cells and was sufficiently long to accommodate five flow channels (Fig. 1). The flow channels were formed by rectangular, round-edge cut-outs in a silicone rubber spacer sandwiched between a 150-µm-thick microscopic coverslip glass and an 2.4-mm-thick acrylic cover plate (Fig. 1; hereafter referred to as *substrate* and *platen*, respectively). The dimensions of the chambers were 0.5 × 6 × 16 mm. Cell sample volumes were 300 µl each. All five samples were held in 1-cc B-D sterile disposable syringes mounted on the syringe pump (Sage Instruments, Cambridge, MA). Each syringe was connected to its matching flow chamber through a 1,500-µl pipette tip, 10-mm-long Tygon tubing (inner diameter, or ID = 1⁄16″; outer diameter, or OD = 1⁄8″; Norton Performance Plastic Co., Akron, OH), and 60-mm-long Teflon tubing (ID = 1⁄32″, OD = 1⁄16″; Zeus Inc., Boise, ID). The same type Teflon tubing carried the eluate fractions to sample collection tubes. The syringe pump was modified by fitting it with a five-syringe receptacle and by extending the pusher plate to accommodate all five syringe plungers. The position of each plunger relative to the pusher plate was individually adjusted by thumb screws to allow the simultaneous start of all five samples. Before each experiment, the flow chambers and connecting tubings were primed with reconstitution buffer to ensure that the cells entered the magnetic deposition zone in a fully developed flow (Fig. 1). When the air–liquid interface of the test samples reached the Tygon tubings, the pump was stopped and the outlets were blocked. The upper syringes and pipette tips were replaced by new sets containing 100 µl 50% methanol partly filled with air to aid the evacuation of residual suspension, and then the pumping action was resumed. The flow of methanol

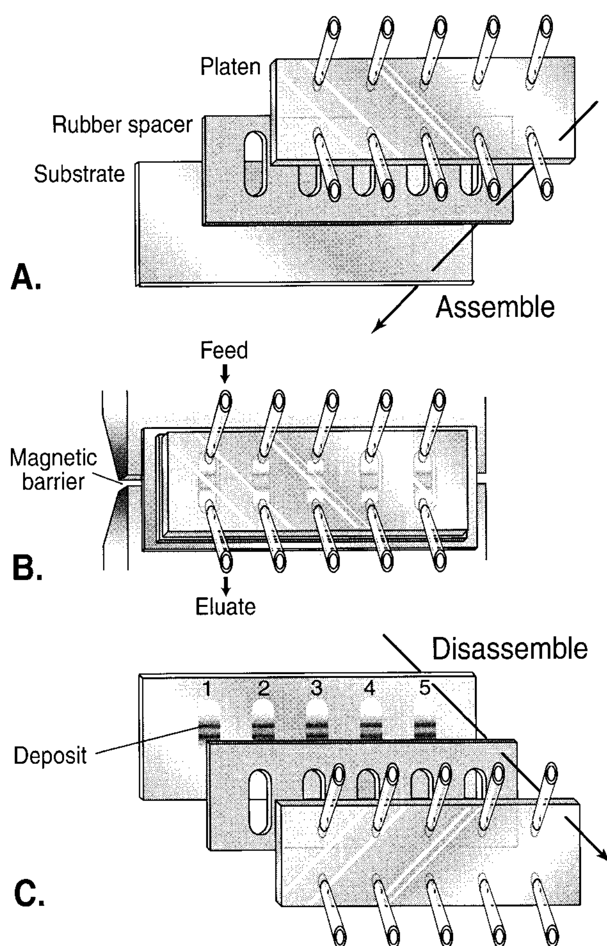

FIG. 1. Flow chamber. **A:** Assembly. **B:** Position of the flow chamber relative to th magnetic barrier. **C:** Disassembly. The magnetically labeled cells are captured on substrate (microscopic glass coverslip) in the area labeled magnetic barrier.

through the flow chambers fixed the magnetically deposited cells onto the substrate. After each experiment, the substrate with the deposited cells was carefully disassembled, stained with Wright's stain for nucleated cells, mounted on a microscopic substrate glass, and examined under an Olympus BX40 optical microscope (Olympus Co., Inc., Tokyo, Japan).

Data Analysis

Cytospin smears (Shandon Inc., Pittsburgh, PA) of 500 µl, 1 × 10^5 cells/ml, MCF-7 cell suspension were made by centrifugation at 500 rpm for 10 min before every experiment as a reference for morphology determination of MCF-7 cells and leukocytes on the Bio-Ferrograph slides. The presence of the MCF-7 cells on the Bio-Ferrograph substrates was confirmed only when the morphology of cells in the magnetic deposition bands and Cytospin smears was identical, as observed by two independent observers. Each magnetic deposition band was rapidly screened at low magnification (200×), and the preselected cells were inspected in detail at high magnification

(400×). This quantitation process of each sample was always completed in less than 5 min.

The isolation of rare MCF-7 cells with the Bio-Ferrograph was evaluated by using two different protocols, as mentioned above. The objective of the first protocol was to determine the theoretical capabilities of the Bio-Ferrograph to isolate the magnetized MCF-7 cells on the deposition slide. This objective required information concerning the exact number of the MCF-7 cells pumped into the deposition channel. The only method available to us to accomplish this task was to mix known amounts of the MCF-7 cells and PBMCs. We spiked suspensions of three different concentrations of PBMC (1×10^6 cells/ml, 1×10^7 cells/ml, and 1×10^8 cells/ml), with known number, 100, of the magnetically labeled MCF-7 cells. The measure of the power of the Bio-Ferrograph to isolate the MCF-7 cells from the mixture was the labeled MCF-7 cell capture rate, C_1, defined as follows:

$$C_1 = \frac{\text{MCF-7 cell count on substrate}}{\text{labeled MCF-7 cell count in solution}} \cdot 100\% \quad (1)$$

In practical applications, the number of cancer cells in the nucleated cells specimen is, of course, unknown. This more realistic situation was simulated by the second protocol, in which we labeled the cell mixture after it was spiked with the known number of the MCF-7 cells. In this protocol, due to a series of cell washing steps involved in the antibody staining, during which the ratio of MCF-7 cells to leukocytes may have had changed, and the lack of an absolute method of the MCF-7 cell concentration measurement, the exact number of the MCF-7 cells in the mixture, at the time of pumping it onto the deposition substrate, was unknown. The information about the number of the MCF-7 cells on the substrate as compared with the number of the MCF-7 cells in solution before labeling provided the measure of the MCF-7 cell recovery on the substrate, including confounding effects unrelated to the deposition process in itself such as cell loss during cell washing following antibody incubation. We used the term unlabeled MCF-7 cell capture rate, C_2, to describe the percentage of the deposited MCF-7 cells out of the unlabeled MCF-7 cell sample:

$$C_2 = \frac{\text{MCF-7 cell count on substrate}}{\text{unlabeled MCF-7 cell count in solution}} \cdot 100\% \quad (2)$$

Error analysis included determination of error of the MCF-7 cell concentration in suspension, error of the lymphocyte concentration, and error of the MCF-7 cell count in the magnetic deposition. The MCF-7 cells were counted in quadruplicate by using the hemacytometer, and the concentration was adjusted to 10^5 cell/ml and diluted serially 1:10 in three steps to 100 cells/ml. The coefficient of variation (CV) of hemacytometer counts was 10%. The serial dilutions were prepared by using micropipets at CV = 3% for each dilution; thus, CV = 9% at the lowest concentration. By adding the errors of the MCF-7 cell concentration determination and the error of dilutions, one obtains a CV = 19% for the concentration of MC-7 cell suspension used for spiking of the lymphocyte suspensions. For lymphocyte suspensions, CV = 5% at the cell concentration of 10^4 cell/ml as measured by the Z-1 Coulter Counter. The error of serial dilutions or pelleting required to obtain the desired final lymphocyte concentration was estimated to be no greater than 5%; thus, the total error of the lymphocyte concentration was 10%. The maximum error of the MCF-7 cell frequency in the mixture (ratio of MCF-7 cells to PBMC concentrations) was calculated as a compound error of function and was equal to $\sqrt{(0.10)^2 + (0.19)^2} \cdot 100\% = 21\%$. The number of MCF-7 cells deposited on the slide was reported as an arithmetic mean for replicate experiments at the same MCF-7 cell-to-lymphocyte ratio; the error was equal to the standard deviation of the mean.

The LOD was defined as the lowest MCF-7 cell frequency in the original sample for which we were able to detect at least 10 MCF-7 cells on the deposition substrate in at least three experiments. In the actual diagnostic applications of this method, more stringent LOD criteria may be required.

RESULTS

Anti-EMA mAb Specificity

Anti-EMA antibody specificity against MCF-7 cells was maximized by antibody titration as measured by flow cytometry. Representative examples of FACS histograms of PBMC and MCF-7 cells stained at different concentrations of anti-EMA mAb are shown in Figure 2. The leukocyte staining served as an indicator of the specificity of the primary antibody, the secondary antibody biotin, and avidin-FITC sandwich. No anti-EMA mAb staining of leukocytes was detected up to 200 μl/ml. Results for low antibody concentration of 20 μl/ml and 4× low concentration (80 μl/ml) are shown in Figures 2B and 2E, respectively. The anti-EMA mAb staining was sensitive to MCF-7 cells for both antibody concentrations (Fig. 2C–D,F–G, respectively). Subsequently, the anti-EMA mAb concentration of 20 μl/ml and incubation time of 1 h at room temperature were used. The FACS histograms shown in Figure 2 were obtained by acquiring and analyzing samples of 10,000 cells each.

Capture Rates of the Bio-Ferrograph

To determine the labeled MCF-7 cell capture rate (C_1; equation 1) in the Bio-Ferrograph, we mixed 100 magnetized MCF-7 cells with 1×10^6, 1×10^7, and 1×10^8 unlabeled PBMCs in 1 ml reconstitution buffer and used 300-μl aliquots for the magnetic captured experiments with the Bio-Ferrograph. The flow rate was set at 50 μl/min during the magnetic deposition stage; the flow rate was changed to 10 μl/min during the methanol fixation stage. The results are shown in Table 1 and Figure 3A. The labeled MCF-7 cell capture rate, C_1, was 31–32% for the three different MCF-7 cell frequencies (10^{-4}, 10^{-5}, and 10^{-6}). The lack of dependence of MCF-7 cell capture rate on leukocyte concentration indicated that the presence of

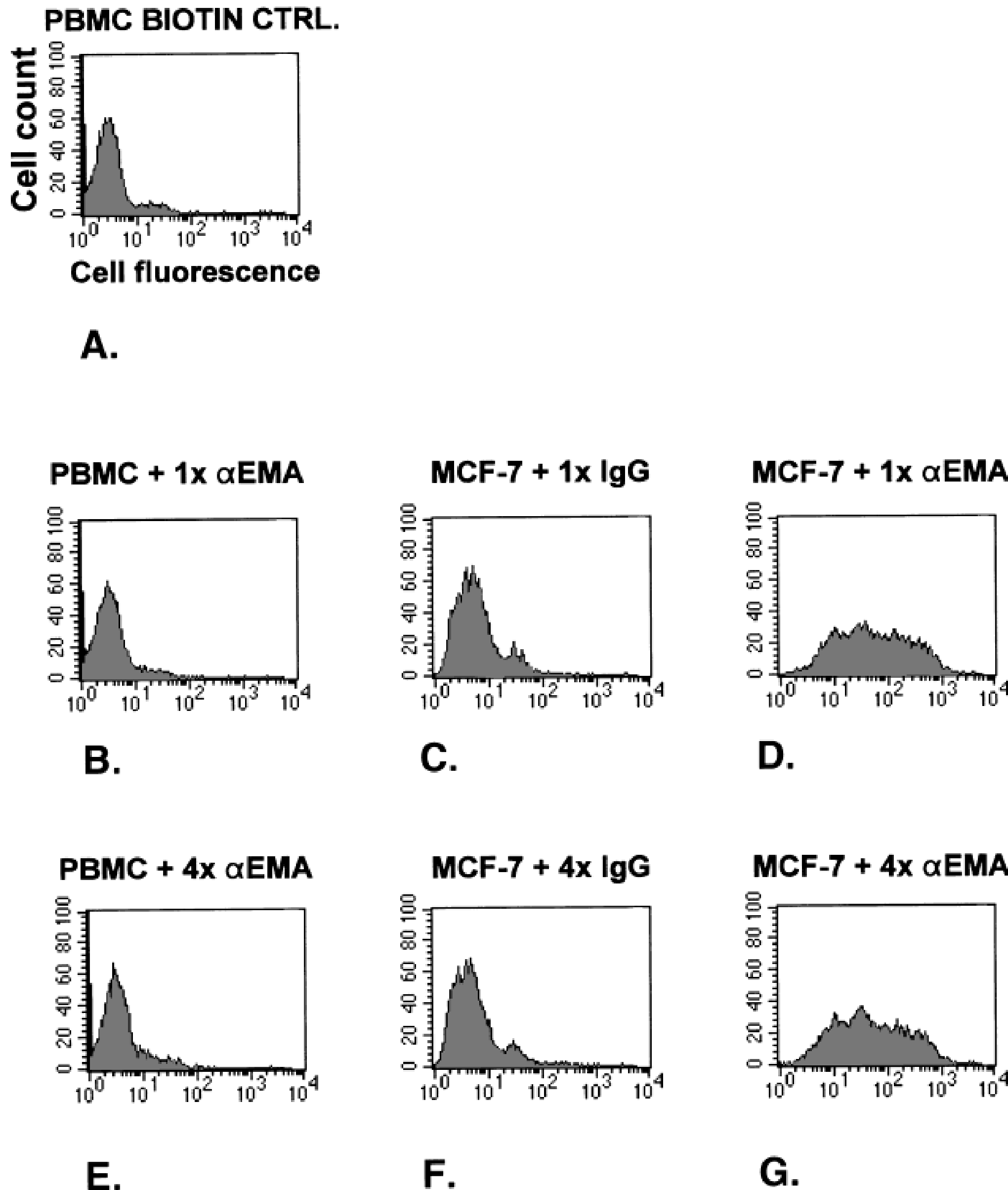

FIG. 2. Fluorescence-activated cell sorting histograms representative of the specificity and sensitivity of the anti-epithelial membrane antigen monoclonal antibody (anti-EMA mAb) to MCF-7 cells in a mixture with human peripheral blood mononuclear cells (PBMCs). **A:** PBMC negative control (no immunoglobulin, only avidin–fluorescein isothiocyanate added). **B:** PBMC anti-EMA mAb control (low immunoglobulin concentration, 20 μl/ml). **C:** MCF-7 control with the anti-EMA mAb isotype (IgG1). **D:** MCF-7 and anti-EMA mAb (low immunoglobulin concentration, 20 μl/ml). **E–G:** Same as B–D, respectively, at high immunoglobulin concentration (80 μl/ml). There is a lack of anti-EMA mAb binding the PBMCs in B and E versus that of the biotin control in A. There is specific binding of the antibody to the MCF-7 cells in D and G versus that to the IgG1 controls in C and F. There is a partial overlap between PBMC and MCF-7 cell fluorescence distributions (B and D or E and G, respectively).

leukocytes in the samples did not affect the magnetic deposition of MCF-7 cells (for up to 0.3×10^8 cells in 300-μl sample). The relatively small number of captured leukocytes ($\approx$50 for the total of 0.3×10^8 cells fed into the deposition area) confirmed the specificity of the immunomagnetic binding to MCF-7 cells.

To determine the performance of the Bio-Ferrograph in a more realistic experimental setting, mixtures of MCF-7 cells and PBMCs at different MCF-7 cell frequencies were prepared before rather than after the magnetic label was applied. One hundred MCF-7 cells were mixed with 1×10^6, 1×10^7, and 1×10^8 PBMCs and labeled sequentially by anti-EMA mAb and immunomagnetic colloid, after which the cell mixtures were separated in the Bio-Ferrograph. The unlabeled MCF-7 cell capture rate (C_2; equation 2), was 32–50% and did not show significant dependence on the MCF-7 cell concentration (Table 2, Fig. 3B).

Our LOD criterion required detection of at least 10 MCF-7 cells on the deposition substrate. The LOD of the method, established by the data shown in Table 2 and in Figure 3B, was one MCF-7 cell in 10^6 leukocytes. It is

Table 1
Labeled MCF-7 Cell Capture Rate (C_1) in the Bio-Ferrograph

n[a]	PBMC count in feed[b] (±10%)[c]	Labeled MCF-7 count in feed (±19%)	Labeled MCF-7 cell frequency in feed (±21%)	MCF-7 cell count on substrate			Mean %C_1 (CV)
				Range	Mean	S.D.	
6	3×10^5	30	10^{-4}	7 ~ 12	9.2	1.8	31 (20)
12	3×10^6	30	10^{-5}	7 ~ 13	9.6	2.0	32 (21)
6	3×10^7	30	10^{-6}	5 ~ 18	9.7	5.3	32 (55)

[a]Number of cell deposits evaluated.
[b]Feed sample volume was 300 μl. PBMC, human peripheral blood mononuclear cell.
[c]Coefficient of variation (CV).

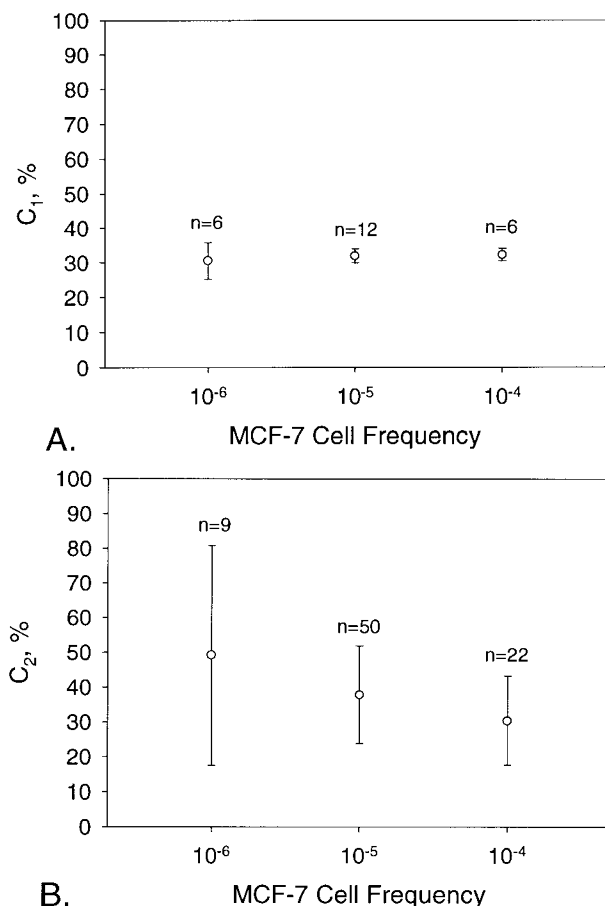

FIG. 3. Magnetic MCF-7 cell capture in the Bio-Ferrograph. **A:** Labeled MCF-7 cell capture rate (C_1) as defined by equation 1. **B:** Unlabeled MCF-7 cell capture (C_2) as defined by equation 2.

interesting to note that the LOD was the same for the labeled MCF-7 cells as that for the unlabeled MCF-7 cells (compare cell capture rates in Tables 1 and 2, respectively), although in the latter case the magnetic deposition was performed after numerous cell incubations and washings. A plausible explanation of this effect is that the MCF-7 cells were recovered in pellet in equal (or possibly higher) proportion to leukocytes as compared with that in suspension. The larger error bars associated with mean C_2 values (Fig. 3B) versus those associated with the C_1 values (Fig. 3A) are related to the greater uncertainty of the true MCF-7 cell concentration in feed solutions used for C_2 determination versus that in solutions used for C_1 determination.

In the present study, all deposition slides obtained at different MCF-7 cell frequencies (10^{-4}, 10^{-5}, and 10^{-6}) contained MCF-7 cells (there were no false negative results). The morphology of magnetically deposited MCF-7 cells was well preserved and comparable to the morphology of the cells in the cytospin smears (Fig. 4C,D, respectively). The rare MCF-7 cells (at a frequency of 10^{-6}) could be easily identified on the magnetic deposition slide at low magnification (Fig. 4A). The average number of leukocytes retained in the magnetic deposition was 50, and this small number did not interfere with the search for the MCF-7 cells in the magnetic deposit. In comparison, the lowest frequency at which MCF-7 cells could be consistently identified on the cytospin slides was 10^{-4} because of the leukocyte overcrowding at lower MCF-7 cell frequencies (Fig. 4B).

The LOD of the method at lower ratios of MCF-7 cells to leukocytes, i.e., 10^{-7} and lower, was not tested because of the high volume of the leukocytes and of the whole blood (in excess of 50 ml) required for such testing.

DISCUSSION

The current commercial magnetic separation systems that have been used for diagnostic cell isolation include MACS (Miltenyi Biotec GmbH, Bergisch-Gladbach, FRG), Dynal (Dynal Co., Trondheim, Norway), and Immunicon (Huntingdon Valley, PA). Their effectiveness was tested in the isolation of rare cancer cells from leukocytes (19), from human mammary gland (20), and breast carcinoma and prostate cancer cells from whole blood (17). The sensitivities achieved in those systems were as high as 10^{-6} (19). The differences between the current commercial magnetic separation systems and the Bio-Ferrograph are the following: (a) current systems typically can separate only one sample at a time, (b) separation often has to be staged to obtain the highest sensitivity, and (c) all current systems are negative selection (depletion) systems that do not provide a means for visual inspection and preservation of the cellular material at the site of the magnetic capture. In comparison, the Bio-Ferrograph is a true positive cell selection system, capable of depositing labeled cells directly on a substrate suitable for immediate microscopic examination. Moreover, in the Bio-Ferrograph, one processes multiple samples in parallel, e.g., five samples at a time in the version used in the present study.

Table 2
Unlabeled MCF-7 Cell Capture Rate (C_2) in the Bio-Ferrograph

n[a]	PBMC count in sample (±10%)[b]	MCF-7 cell count in unlabeled sample (±19%)	MCF-7 cell frequency in unlabeled sample (±21%)	MCF-7 cell count on substrate			Mean $\%C_2$ (CV)
				Range	Mean	SD	
22	3×10^5	30	10^{-4}	5 ~ 18	9.5	3.8	32 (40)
50	3×10^6	30	10^{-5}	6 ~ 28	11	4.2	37 (38)
9	3×10^7	30	10^{-6}	6 ~ 29	15	9.1	50 (61)

[a]Number of cell deposits evaluated.
[b]Coefficient of variation (CV). PBMC, human peripheral blood mononuclear cell.

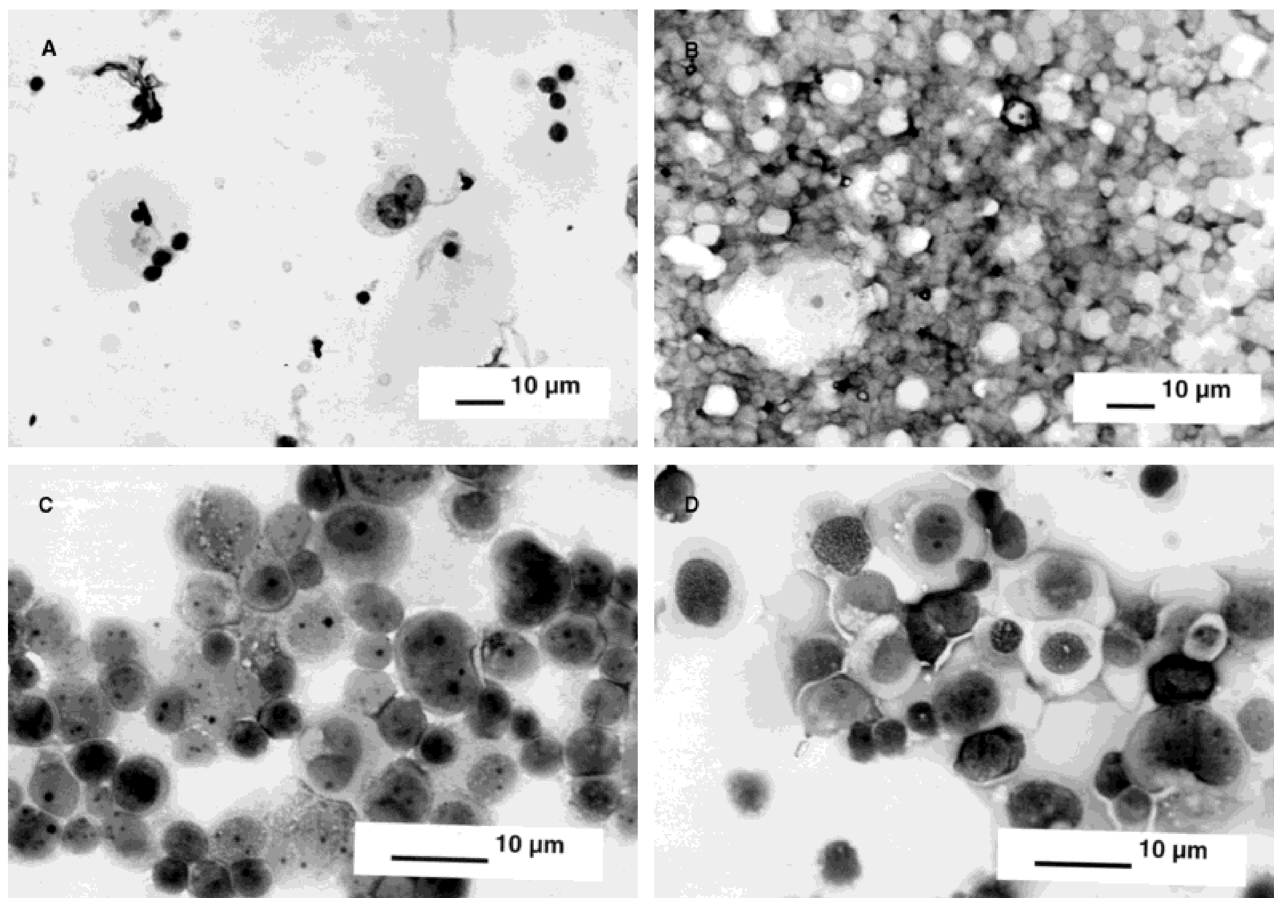

FIG. 4. Photomicrographs of MCF-7 cells in human peripheral blood mononuclear cell mixtures. **A:** Magnetic deposition on the Bio-Ferrograph slide at an MCF-7 cell frequency of 10^{-6}. Two MCF-7 cells (large nucleus, nuclear inclusions) can be readily recognized among the few captured leukocytes (small dense nuclei). **B:** Cytospin smear at an MCF-7 cell frequency of 10^{-4}. The MCF-7 cell could not be detected readily among the large number of leukocytes. Lower MCF-7 cell frequencies were not practical because of either too high MCF-7 cell dilution or cell overcrowding. **C:** Morphology of the MCF-7 cell on the Bio-Ferrograph slide. **D:** Reference morphology of the MCF-7 cell on the cytospin smear (magnification 1,000×). Comparison of C and D indicates that the magnetic labeling and deposition technique does not significantly alter the morphology of the MCF-7 cell.

In principle, the number of channels could be increased by lengthening the interpolar gap of the permanent magnet. In addition, mounting of the substrate with the cell deposition on a microscopic substrate glass slide provides a permanent record of the patient's sample.

All cells (including small numbers of leukocytes) were deposited in the areas of 1.4 × 6 mm. No cell overlap was observed within these areas. Small examination areas and obvious morphological differences between MCF-7 cells and leukocytes enabled us to take only a few minutes to identify all positive cells in each sample. Such an examination could be made even more effective if stains more specific than the modified Wright's stain were used for the MCF-7 cell detection. The entire period of analysis from sample procurement to final results was shorter than 4 h for five samples; therefore, the effective time for MCF-7 cell detection was less than 50 min per sample.

The cell deposition event in the Bio-Ferrograph is the result of an interplay between the viscous drag forces of the flowing medium and the magnetic forces acting on the

cell. Such interactions strongly favor the deposition of the magnetically labeled cells because of the very low intrinsic magnetic susceptibility of the unlabeled cells. In fact, in the process of the magnetic deposition, we observed losses of the leukocytes on the slide by a factor of 6×10^5 (from 30×10^6 leukocytes in the sample to 50 leukocytes on the slide). The accompanying loss of the MCF-7 cells was much lower, by a factor of less than 2 to up to 6 (from 30 MCF-7 cells in the sample to $18 \approx 5$ MCF-7 cells on the slide, respectively). The resulting MCF-7 cell enrichment rate on the slide relative to sample, calculated as the ratio of leukocyte losses to the MCF-7 cell losses, was equal to 1×10^5 or higher. In our hands, this translated into high sensitivity of the rare MCF-7 cell detection, comparable to that reported for the RT-PCR (13) or automated image cytometry (15,16) but at shorter time and lower costs. Our studies indicate that the immunomagnetic cell analysis is potentially as sensitive, or perhaps more sensitive, than cell detection systems based on flow cytometry, an observation made previously by others (21).

The reason for the incomplete MCF-7 cell recovery in the magnetic deposit and a partial contamination of the deposit by the leukocytes could be elucidated by a discussion of the anti-EMA antibody specificity with the use of Figure 2. A comparison of Figures 2A (no primary antibody) with 2B (with primary antibody) indicates that there is no detectable binding of the anti-EMA antibody to the leukocytes, as expected. The presence of a comparable fluorescence signal between 10^1 and 10^2 fluorescence units in both panels implicates the biotin and avidin-FITC binding rather than the anti-EMA antibody binding as a cause of the non-zero fluorescence signal in that region. The existence of the nonspecific anti-EMA binding to the leukocytes could not be excluded entirely on the basis of those two histograms, however. It is plausible that a few rare leukocytes may bind an amount of the anti-EMA antibody equivalent up to 10^2 fluorescence units, which would make them as likely candidates for the magnetic colloid binding as approximately half of the MCF-7 cells (Fig. 2D). Therefore, the results summarized in Figure 2 do not preclude leukocyte magnetization and capture on the magnetic substrate, and contamination of the MCF-7 deposit by the rare leukocytes is possible, as seen in Figure 4A. Moreover, the broad MCF-7 cell fluorescence distribution indicates that there is a substantial fraction of the MCF-7 cells that bind low amounts of the primary antibody, equivalent to less than 10^1 cell fluorescence units (Fig. 2D) or equivalent to the leukocyte autofluorescence (Fig. 2B). Such MCF-7 cells are likely to escape the magnetic capture with the majority of the leukocytes, resulting in the decrease of MCF-7 cell recovery.

What is the required cell volume for the optimum diagnostic method? The BM is the most common site of distant metastases of breast cancer, and it is frequently the first organ in which metastases are detected (22). The reported ratio of malignant cells to normal cells determining the risk of relapse following BM autologous transplantation is lower than one in 10^4 normal cells, depending on clinical symptoms. In diagnostic tests, multiple BM aspirates taken from different sites are strongly recommended to eliminate sampling errors; the volume of each aspirate is usually $2 \sim 5$ ml (23), yielding $1 \approx 5 \times 10^7$ leukocytes following Ficoll-Hypaque separation. Thus, the final number of cells in the pooled sample could be from 0.5×10^8 to 2×10^8 cells. The modified Bio-Ferrograph has a maximum loading capacity per channel of 1×10^8 cells (maximum sample volume is 1 ml), which is sufficiently high to determine cancer cell contamination at the level of 10^{-6} sensitivity or better.

This is a preliminary study to test the feasibility of the magnetic cell capture directly on the microscope slide for the potential, future application in rare cancer cell detection. The present study has indicated that such cell capture is feasible and has identified areas of further investigations when the method is extended to BM samples. We plan to extend these studies to patient BM samples and a cocktail of antibodies against breast cancer cells.

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