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Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10^{-7}

(micrometastasis/rare-event detection/flow cytometry/genetic algorithm/ppm limit of detection)

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ABSTRACT **A flow cytometric assay was developed to detect rare cancer cells in blood and bone marrow. Multiple markers, each identified by a separate color of immunofluorescence (yellow and two shades of red), are used to reliably identify the cancer cells. Blood or bone marrow cells, which are not of interest but interfere in detecting the cancer cells, are identified by a panel of immunofluorescence markers, each of which has the same color (green). Thus, the rare cancer cells of interest are yellow and two different shades of red but not green. The requirement that the rare cancer cell be simultaneously positive for three separate colors (the specific markers) and negative for a fourth color (the exclusion color) allowed detection of as few as one cancer cell in 10^7 nucleated blood cells (a frequency of 10^{-7}). To test this rare-event assay prior to clinical studies, a model study was performed in which the clinical sample was simulated by mixing small numbers of cells from the breast carcinoma line BT-20 with peripheral blood mononuclear cells. We detected statistically significant numbers of BT-20 cells at mixing frequencies of 10^{-5}, 10^{-6}, and 10^{-7}. In control samples, no target events (BT-20) were observed when more than 10^8 cells were analyzed. For additional confirmation that the BT-20 cells in the model study were correctly identified and counted, the BT-20 cells (and only BT-20 cells) were covalently stained with a fifth fluorescent dye, 7-amino-4-chloromethylcoumarin (CMAC). CMAC fluorescence data were not used in the assay for detecting BT-20 cells. Only after the analysis using data from the specific and exclusion colors had been completed were the events identified as BT-20 cells checked for CMAC fluorescence. The putative BT-20 events were always found to be positive for CMAC fluorescence, which further increases confidence in the assay. Manual data analysis and an automated computer program were compared. Results were comparable with the manual and automated methods, but the automated "genetic algorithm" always found more BT-20 events. Cell sorting of BT-20 cells from samples that contained BT-20 at frequencies of 10^{-5}, 10^{-6}, and 10^{-7} provided further evidence that these rare cells could be reliably detected. The good performance of the assay with the model system will encourage further studies on clinical samples.**

Detection and quantitation of rare cancer cells in blood or bone marrow may be helpful in determining prognosis (1–5) and directing aggressiveness of therapy. High-dose chemotherapy (6, 7), which destroys the hematopoetic system, can be followed by autologous bone marrow transplantation to restore blood cell production. The patient's bone marrow or peripheral blood stem cells used for the autologous transplant must be obtained before high-dose chemotherapy and must therefore be purged of cancer cells before being infused back into the patient. Residual cancer cells in the bone marrow or peripheral blood stem cell preparations are a major concern (8, 9). It is thus vital to establish a sensitive method for detecting metastasized cells at presentation in bone marrow and in bone marrow or peripheral blood stem cell preparations prior to transplantation.

In model studies, bone marrow with 10% tumor cell contamination can be purged by a factor of 10^{-3} to 10^{-4} by state-of-the-art methods (10, 11). In a more realistic example with 0.1% tumor cell contamination, ≈3000 tumor cells could be returned into the patient. To detect those cells in purged bone marrow before infusion requires a detection level of one tumor cell per 10^6 to 10^7 cells, a level not achieved so far. Present day methodologies, including immunofluorescence and immunocytology, permit a level of detection by microscopy of one tumor cell per 10^5 cells at best. Detection with these cytological methods is limited by the relatively small number of analyzed cells (12), analysis time, and loss of cells during slide preparation. Common immunocytology indicator enzymes like horseradish peroxidase or alkaline phosphatase can give rise to false-positive cells because of endogenous enzymes (5).

In a previous study with hematopoietic cells (13), we addressed some of the difficulties in analyzing rare cells by flow cytometry. Nonspecific staining and autofluorescence are minimized with a well-chosen staining protocol, carryover is eliminated by an extensive cleaning procedure, and erroneous data acquired during bursts of events are removed by a computer algorithm. The present study used cytoplasmic staining (14) of cytokeratins in contrast to the surface staining used in our previous study (13). BT-20 breast cancer cells were characterized by a cocktail of three anti-cytokeratin antibodies, each tagged with a different color dye. Leukocytes, platelets, and traces of erythroid cells were characterized with a cocktail of monoclonal antibodies tagged with a fourth dye.

We also investigated a new approach to automated data analysis using a "genetic" algorithm for determining the best criteria to discriminate control and positive events. This is an optimization problem, where the function to be optimized has as independent variables the gate parameters and as an dependent variable the number of cells in the gate. The requirement that no event in the control data is allowed to fall in the gate is a boundary condition to the problem.

The goal of this study was to establish a very sensitive multiparameter flow cytometric assay for detecting micrometastases and to establish a sensitive cell-sorting technique that goes beyond the present day limit of 10^{-4} cells. To be able to control the experimental conditions accurately, we chose to use a model study. In the model study, we sorted cells from the breast carcinoma cell line BT-20 into peripheral blood mononuclear cells (PBMCs), a process referred to as "seeding." Seeding the rare cells into PBMCs by sorting allowed accurate

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Abbreviations: PBMC, peripheral blood mononuclear cells; CMAC, 7-amino-4-chloromethylcoumarin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; FITC, fluorescein isothiocyanate.

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control over their quantity. We then tried to detect the seeded cells. Our methods are based on detecting differences between control (containing no seeded cells) and test samples. The test samples contained rare cells at seeding or mixing frequencies of 10^{-5}, 10^{-6}, and 10^{-7} (i.e., 1 cancer cell per 10^5, 10^6, or 10^7 PBMCs).

MATERIALS AND METHODS

PBMCs. Buffy coats from four or five females having the same major blood group and rhesus factor subgroup were pooled. PBMCs were isolated by using Ficoll/Hypaque. After two washings with 0.45 μm-filtered Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), the cells were resuspended in 0.45 μm-filtered PBS with 20% (vol/vol) fetal calf serum (FCS) and rotated for 1 hr. An aliquot was taken to test viability with the propidium iodide (1 μg/ml) exclusion test. The viability was always greater than 97%. After the cells were counted with a hemocytometer, 4×10^8 PBMCs were placed in each of four 15-ml tubes. Each tube was supplemented with 0.45 μm-filtered fetal calf serum to a total of 14 ml.

BT-20 Cells. The breast carcinoma cell line BT-20 (15) was cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 50 units of penicillin G and 50 units of streptomycin per ml, 0.01 M Hepes buffer, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine (BioWhittaker) at 37°C in an atmosphere containing 7% CO_2. The adherent growing cells were harvested by mechanically detaching the cells from the tissue culture flask by a cell scraper. The cells were taken up by syringe to prepare a single-cell suspension. The cell suspension was washed once with fresh medium and was resuspended in 5 ml of fresh medium.

Prelabeling of BT-20 Cells. Before the seeding process, the BT-20 cells were covalently labeled with the UV-excitable dye 7-amino-4-chloromethylcoumarin (CMAC; Molecular Probes). Glutathione *S*-transferase mediates the conjugation of CMAC to intracellular thiol groups, thereby generating cell-impermeant fluorescent dye–thioether adducts (16). One-half milligram of the fluorochrome was dissolved in 100 μl of absolute ethanol; 50 μl of this solution was added to 5 ml of a prewarmed (37°C) BT-20 cell suspension and kept in an incubator (37°C, 7% CO_2/93% air) for 45 min. The cells were washed once and resuspended in fresh medium and incubated for another hour. The cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS. Prior to adding propidium iodide at 1 μg/ml, the cells were resuspended with a syringe. The viability was minimally 67%. Prior to seeding into PBMC, the propidium iodide was removed by two washes with Ca^{2+}/Mg^{2+}-free PBS.

Seeding the BT-20 into PBMC. A FACStar^{Plus} cell sorter (Becton Dickinson) was used in the "count" mode to deposit BT-20 cells, as characterized by a bright CMAC signal, into a suspension of PBMCs. Doublets were excluded by using width measurements in light scatter. The maximum number of BT-20 cells recovered in the analysis of the seeded sample is the number sorted into the tube. We seeded 0 cells as the control and 40, 400, and 4000 BT-20 tumor cells into 4×10^8 PBMCs. During analysis $\approx 1 \times 10^8$ PBMCs were measured.

Flow Cytometry Instrumentation. For cell sorting, a dual-laser FACStar^{Plus} was used. For cell analysis we used an experimental flow cytometer whose basic configuration has been described (17). The experimental flow cytometer was configured with three lasers (UV/325 nm/10 mW, red/633 nm/10 mW, and blue/488 nm/15 mW) focused at separate points along the stream of flowing cells. A cell event was acquired by the electronics only when the forward scatter signal and at least one predetermined fluorescence signal were simultaneously above threshold values.

Staining. After seeding the BT-20 cells into PBMCs, the cells were rotated for 1 hr to ensure dispersion of seeded cells. Cells were centrifuged, the supernatant was taken off to about 75 μl, and 125 μl of normal mouse serum (X 910; Dako) was added. The cells were incubated for 1 hr; 150 μl of the exclusion staining cocktail containing 20% normal mouse serum and the following fluorescein isothiocyanate (FITC)-conjugated antibodies was added: 14.9 μg of anti-(CD45) HLe1-FITC per ml, 54 μg of anti-platelet glycoprotein gpIX (CD42a)-FITC per ml, 56.6 μg of anti-pan-platelet (CD61)-FITC per ml, 2.2 μg of anti-(CD34) HPCA-2-FITC per ml, and 0.7 μg of anti-glycophorin-FITC per ml. The final concentrations are listed. The cells were resuspended and rotated for 1 hr at 4°C, washed twice with 14 ml of Ca^{2+}/Mg^{2+}-free PBS, and placed in 250 ml of PBS containing 0.5% paraformaldehyde. After 20 min, 1 ml of PBS containing 0.4% Triton X-100 was added. The fixation process continued for 14 hr. The cells were harvested and washed once with Ca^{2+}/Mg^{2+}-free PBS. The supernatant was depleted to a volume of about 50 μl, and 350 μl of PBS containing 30% (vol/vol) normal mouse serum was layered over the cell pellet. After 1 hr we added a staining cocktail containing 25% normal mouse serum and the following anti-cytokeratin antibodies conjugated to peridinin chlorophyll protein (PerCP) or phycoerythrin (PE) or allophycocyanin (APC): 2.6 μg of NCL-5D3 anti-cytokeratin components 8 and 18 conjugated to PerCP per ml; 1.8 μg of NCL-LP34 anti-cytokeratin components 5, 6, and 18 conjugated to PerCP per ml; 0.27 μg of CAM 5.2 anti-cytokeratin components 8 and 18 conjugated to PE per ml; and 1.43 μg of AE1 anti-cytokeratin components 10, 14, 15, 16, and 19 conjugated to APC per ml. The final antibody concentration is listed. The cell suspension was stained for 1 hr at 4°C and washed once with Ca^{2+}/Mg^{2+}-free PBS. The cell suspension was resuspended in 45 ml of Ca^{2+}/Mg^{2+}-free PBS containing 0.1% fetal calf serum and 10 mM NaN_3 and rotated until the analysis.

Data Analysis. We used both manual and automated data analysis. In either case the goal of the analysis was to find upper and lower limits (regions) of fluorescence intensities that best discriminated BT-20 cells of interest from all other cells. Combining the analysis regions for each fluorescence color so that an event is counted only if it were within a defined region for each color produces a "gate" that selects events of interest. The optimal gate contains no cells in the control data (from samples not seeded with BT-20 cells) and maximizes the number of BT-20 cells it contains. Control samples in a clinical study will consist of pooled normal bone marrows or peripheral blood stem cell preparations. We therefore pooled the control data of all four experiments into one large control data set.

Manual data analysis required fluorescence analysis regions to be set by the user. Data from all of the cells in a set of experiments were then analyzed with this set of regions. The analysis regions could be varied to try to optimize the gate. The automated analysis method used a "genetic" algorithm that attempts to obtain an optimal solution to the gate by sequentially "evolving" a set of selection parameters (in this case the fluorescence analysis regions) through a procedure similar to the biological process of mutation and selection of genetic information in chromosomes.

Genetic Algorithm. Genetic algorithms solve optimization problems by randomly generating solutions and evaluating how good the solutions are. More solutions are then generated close to the ones that are best (18). A genetic algorithm encodes solutions to a problem on a data structure, a "chromosome." In our case the data structure simply specifies the four gate parameters. At first a pool of 200 random "chromosomes" is generated. These are allowed to change until 20,000 chromosomes have been generated. Chromosomes are changed by operators. We randomly used three kinds of operators: mutation, creep, and crossover. Mutation randomly changes one of the gate parameters. Creep randomly incre-

ments or decrements one of the parameters. Crossover combines the parameters of two chromosomes randomly to generate a new chromosome. After an operator generated a new chromosome, its fitness is compared to the fitness of the old chromosome(s). The new chromosome replaces the old chromosome if the new chromosome's fitness is better than the fitness of the old chromosome. The final, most fit chromosome is the set of four gate parameters that gives the greatest discrimination between control and test data sets.

To speed up the processing, the data were prefiltered with a gate that broadly selected events that were negative for FITC but positive for PE, PerCP, and APC. This gate directs the genetic algorithm and prevents it from optimizing a gate in a region that is biologically not of interest. In the control files a few hundred events survived the prefiltering.

When the algorithm is allowed to run more than once on the same data, different optimal solutions will be found. The results are very similar though (coefficients of variation from 0 to 25% at the 10^{-7} seeding frequency and 0–2% at the 10^{-6} and 10^{-5} seeding frequencies). All data presented in the results section are averages of five runs through the genetic algorithm.

RESULTS

We used both a manual and an automatic method to analyze the data. In both instances the goal was to find a gate defined by the fluorescent intensities of FITC, PE, PerCP, and APC so that no events fell in the gate in the control samples and so that the number of events in the gate in the test samples was maximal.

Fig. 1 shows the manually and automatically selected gates applied to an experiment with a seeding frequency of 10^{-6}. From a sample of 1×10^8 cells, 26 events (plotted in the figure) corresponding to BT-20 cells were selected by the automated analysis, and 10 events were in the manual gate. All selected events have forward (FSC) and side (SSC) light scatter characteristics of BT-20 cells. The manually selected analysis regions were the same for all experiments, while the automated algorithm optimized regions for each experiment. In four control samples not containing BT-20 cells, no events fell within the indicated gate when 4×10^8 cells were analyzed.

Fig. 2 shows that the number of recovered cells increased linearly with the number of cells seeded. The maximum expected number of BT-20 cells in the sample is the number sorted (or "seeded") into the tube containing only PBMCs. The collection of the sorted cells into the sample tube is not perfect, so the frequency of BT-20 cells in the samples is expected to be somewhat lower than the number seeded. Fig. 3 shows that the manual analysis method detected about 10% of the expected maximum events, and the automated method detected about 20% of expected events. Both analysis methods are optimized for specificity or selectivity (no events detected in the control sample) rather than sensitivity. Exclusion of normal cells is given very high weight in the analysis. This probably accounts for the rather low number of detected BT-20 cells.

To assist the interpretation of our results, we stained the BT-20 cells with the blue fluorescing dye CMAC before we seeded the BT-20 cells into the PBMCs. Histograms of blue (CMAC) fluorescence for cells detected as putative BT-20 cells by the genetic algorithm are shown in Fig. 4 *Left*. The histogram has a similar distribution for experiments at both the 10^{-5} and 10^{-6} seeding frequencies. A histogram of blue fluorescence from control cells is shown in Fig. 4 *Right*. Most control cells have almost no blue fluorescence, although a rare few have fluorescence comparable to BT-20 cells stained with CMAC. We used Kolmogorov–Smirnov (K–S) probabilities to quantify the significance of blue fluorescence on detected cells. The K–S probability is the probability that two cell populations, as characterized by their histograms, are the same (19). We compared the blue fluorescence histograms of cells detected in test samples by the genetic algorithm (e.g., as in Fig. 4 *Left*) with the blue fluorescence histogram of control cells shown in Fig. 4 *Right*. The K–S probabilities for experiments with a seeding frequency of 10^{-7} were 4%, 5%, and 27% for three experiments in which putative BT-20 cells were detected and undefined for the one experiment in which no rare cells were detected. To achieve more significance, one needs to detect more cells. For the experiments with seeding frequencies of 10^{-6} and 10^{-5}, the K–S probability dropped below 10^{-8} and 10^{-69}, respectively. When the four experiments with a seeding frequency of 10^{-7} were pooled (five detected events), the K–S probability was below 10^{-3}.

In a second set of experiments we sorted the BT-20 cells from the PBMCs onto a slide. The control samples (no seeded BT-20 cells) were chosen to select adequate sorting gates (positive for cytokeratin-PerCP, -PE, and -APC but negative for exclusion markers conjugated to FITC). Then $1.2–1.3 \times 10^8$ PBMCs with BT-20 cells seeded at frequencies of 10^{-5}, 10^{-6}, and 10^{-7} were run on a cell sorter. At each frequency we were able to selectively sort the BT-20 cells with only a very few irrelevant cells (see Table 1), as verified by fluorescence microscopy. In addition a few images of sorted cells were recorded with a CCD (charged-coupled devise) camera (Fig. 5).

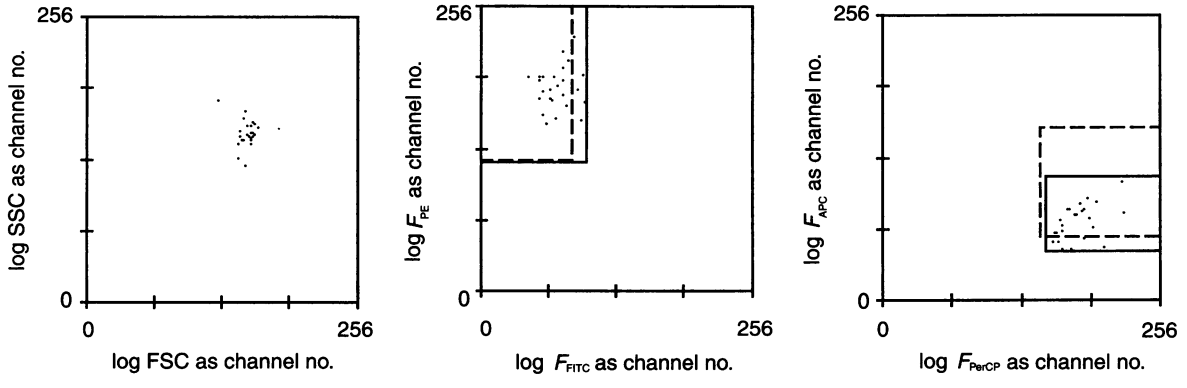

FIG. 1. Analysis of rare cells by four-color immunofluorescence. Cells analyzed by multiparameter flow cytometry were counted as meeting the criteria for BT-20 cells when they fell within the rectangular regions of 4-color space as indicated: dim for the FITC-labeled exclusion antibodies and positive for the three different cytokeratin markers tagged with PE, PerCP, and APC. Of 10^8 cells analyzed, only those meeting the selection criteria are displayed. The selection criterion required an event to be in both the rectangular regions in PE vs. FITC space and in APC vs. PerCP space. The dashed rectangular regions were determined by manual analysis of the data, and the solid regions were determined by the automated algorithm. Twenty-six events corresponding to BT-20 cells were selected by the automated analysis. *F*, fluorescence; FSC, forward light scatter characteristics of BT-20 cells; SSC, side light scatter characteristics of BT-20 cells.

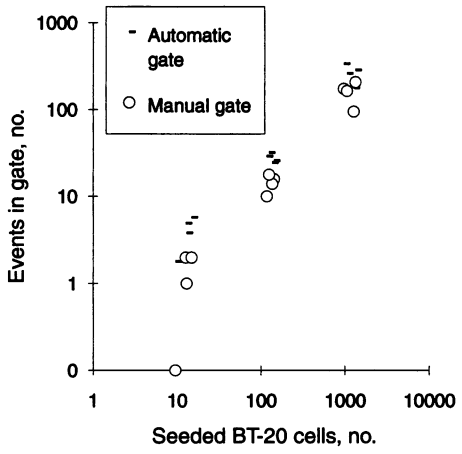

FIG. 2. Detected vs. expected number of BT-20 events. The maximum expected number of BT-20 cells in the sample is the number sorted into the tube containing only PBMCs. The graph shows results of four separate experiments at seeding frequencies of 10^{-5}, 10^{-6}, and 10^{-7} (1000, 100, and 10 expected events, respectively). The manual gate was kept constant for all experiments, but the automated gate was optimized for each experiment.

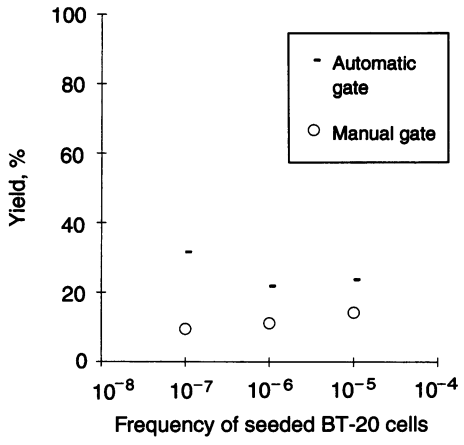

FIG. 3. Comparison of manual and automated analysis methods for yield of BT-20 cells. The yield is defined as the ratio of the number of detected events to the number of rare cells expected to be seeded into the sample. The graph plots the average yield of detected events vs. seeding frequency from the data in Fig. 2. The automated method is about twice as efficient as the manual method in detecting rare events due to BT-20 cells.

In summary, there are three independent facts that prove that we detected the seeded BT-20 cells at frequencies of 10^{-5}, 10^{-6}, and 10^{-7}. The number of detected events goes up linearly with the number of seeded cells, the detected events show higher blue fluorescence than control cells (as expected because the BT-20 cells were stained with CMAC before seeding), and the BT-20 cells can be selectively sorted.

However, the number of events gated by the genetic algorithm at 10^{-7} is higher than expected from the yields at 10^{-6} and 10^{-5}. This is confirmed by a plot of the CMAC histogram of the gated events (Fig. 4), which shows that there is a background of cells with no CMAC. There is a background of false positives because the genetic algorithm exploits the differences between the control sample and the test sample to

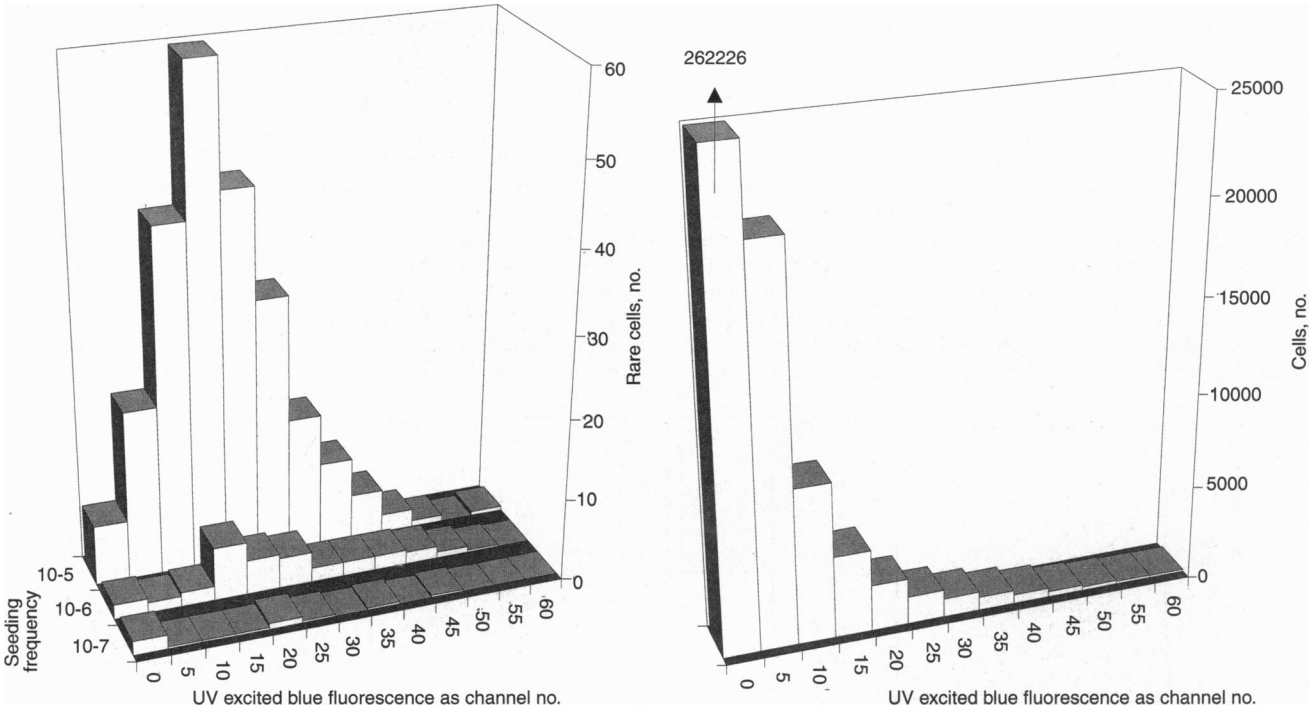

FIG. 4. (*Left*) Histogram of blue UV-excited fluorescence intensity for events identified as BT-20 cells by the automated analysis method. UV-excited/blue-fluorescing CMAC is an independent marker of the BT-20 cells in this model experiment. The graph plots the number of events (rare cells) vs. fluorescence intensity only for cells identified by the automated analysis gate as having characteristics of BT-20 cells. Histograms for experiments at seeding frequencies of 10^{-5}, 10^{-6}, and 10^{-7} are shown. The distribution of fluorescence intensities is characteristic of the CMAC staining properties of BT-20 cells. (*Right*) Histogram of blue UV-excited fluorescence intensity (autofluorescence) for cells in a control sample without BT-20 cells. The histogram contains all events from a sample of 10^8 cells that were above threshold values in forward scatter and either the APC or PerCP fluorescence channels. Since the sample was not stained with the CMAC dye, the blue fluorescence signal is due to autofluorescence. Most control cells have little or no blue fluorescence, and the fluorescence histogram is drastically different from that for CMAC-stained BT-20 cells. Also note the first channel of the histogram is off scale with 262,226 events, and there is a large scale difference on the vertical axis compared with *Left*.

Table 1. Summary of sorting experiments with BT-20 cells

Parameter	Exp. 1	Exp. 2	Exp. 3
Frequency of rare cells	10^{-5}	10^{-6}	10^{-7}
Cells analyzed, no.	1.3×10^8	1.2×10^8	1.2×10^8
Rare cells expected, no.	1300	120	12
Sort decisions, no.	154	27	23
Rare cells by UV microscopy, no.	127	17	5
Yield, %	10	14	40

At three different frequencies of rare cells, the number of recovered events are shown.

the maximum extent. To further investigate this, we divided the combined four control files (no seeded BT-20 cells) into two parts—a "fictive" control set and a "fictive" test set. Each event of the four control files had an 80% chance of being assigned to the fictive control set and a 20% chance of being assigned to the fictive test set; the ratio 4:1 reflected the ratio of the number of events in the four combined control sets and each of the seeded data sets. The gates found by the genetic algorithm contained on the average two events in the fictive test set and always 0 in the fictive control set. The standard deviation was one event. The limit of detection using the genetic algorithm is thus five events (mean plus 3 times the standard deviation).

The number of detected events using the genetic algorithm exceeded the limit of detection in all experiments with a seeding frequency of 10^{-6} and 10^{-5} and also in the pooled experiment with a seeding frequency of 10^{-7}, in which a total of 4×10^8 cells were analyzed. When 1×10^8 cells were in the data set, only one of the experiments with a seeding frequency of 10^{-7} detected more than five events. It should be noted that this limit of detection is caused by the formulation of the detection problem as an optimization problem. The genetic algorithm will, regardless of biological significance, find differences between a control set and a test set. It is the responsibility of the experimenter to make sure that the difference correlates with biological significance.

DISCUSSION

Detection of rare cells down to a frequency of 10^{-7} is possible if 4×10^8 PBMCs are analyzed. For that test, one will need $\approx$200 ml of blood or 2–20 ml of bone marrow. Since 200 ml of blood will often be impractical, the limiting factor in achieving lower limits of detection will be sample size and not methodology.

For each rare-event application, an appropriate staining method has to be chosen. The basic approach should be to stain the subpopulation of the cells one does not want to detect with one color (the exclusion color) and stain the rare cells with one, two, or three of the remaining colors. Measuring more than three or four colors does not help discrimination since aberrant positive values are often correlated (such as autofluorescence). Adding more exclusion antibodies will help, but they can all be tagged with the same dye.

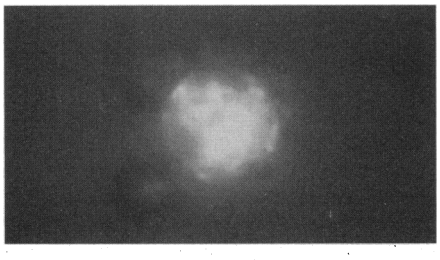

FIG. 5. Image of a BT-20 breast cancer cell sorted from a sample containing 10^8 PBMCs. BT-20 cells were distinguished visually by the characteristic blue color of the CMAC dye.

Not removing bursts from the data can give two kinds of errors (13). First, bursts can perturb the control files. In two control files, spurious events were found in the manual gates if bursts were not removed. Also, the yield of the genetic algorithm did decrease if bursts were not removed. Second, bursts can perturb the test data. In one of the experiments at a seeding frequency of 10^{-7}, the genetic algorithm found a gate that enclosed a large number of false positives (as judged by the absence of blue fluorescence and by the fact that more events were detected than seeded). The false positives were eliminated by the burst-removal algorithm (13).

Our study has established a method to detect rare cells at a level of 10^{-7}. It is suitable for diagnostic applications, like detecting micrometastases in bone marrow, CD34 stem cell preparations, and potentially, peripheral blood. Our study serves as a starting point for clinical studies.

Our study also is a first step in showing feasibility for purging cell populations contaminated with rare tumor cells by fluorescence-activated cell sorting. In this study we minimized the number of false positives, of which there were potentially more than 10^8. By use of purging, the potential number of false positives (the residual or metastasized cancer cells) will be orders of magnitude lower.

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