

Detection and Analysis of Cancer Cells in Blood and Bone Marrow Using a Rare Event Imaging System¹

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ABSTRACT

An automated rare event detection system (Rare Event Imaging System) is described for the recognition of cancer cells that appear at low frequencies (1 in 1 million) in peripheral blood (PB) or bone marrow (BM). The instrumentation includes an automated fluorescence microscope (Nikon Microphot-FXA) with a cooled charge coupled device camera and a 60-MHz Pentium personal computer. Main features of the system are rapid analysis of large microscopic fields, including a total cell count, detection of fluorescently labeled cells, and a display of digitally stored images of the detected cells. Furthermore, the X,Y coordinates of each identified object are stored and can be recalled for morphological analysis of the cell using higher magnification or different fluorescent filter sets. The preparation of the blood or BM samples for automated analysis consists of lysis of the RBCs, attachment of sample cells onto adhesion slides, fixation, and fluorescent labeling with anticytokeratin antibodies. Cytokeratin-positive cells, however, were detected in 17% of the samples from healthy blood donors using this procedure (mean number, $\sim 7/10^6$ mononuclear cells in positive samples). To improve the specificity of the rare event detection, a double-labeling protocol combining intracellular cytokeratin with epithelial cell adhesion molecule (Ep-CAM) (breast, ovarian, colon, and lung carcinoma antigen) or disialo-ganglioside (GD2) antigen (small cell lung carcinoma, neuroblastoma, melanoma antigen) was developed. Examples of doubly labeled cultured cells and cancer cells from breast and small cell lung cancer patients are shown. Using the double-labeling protocol, no "positive" cells were seen in samples of healthy blood donors. Auto-

mated rare event detection (cytokeratin single-staining) was applied to 355 PB, BM, and stem cell (SC) samples from breast cancer patients before autologous BM transplantation. Cytokeratin-positive cells were found in 52% of BM, 35% of PB, and 27% of SC samples at frequencies of 1–1020 positive cells/ 10^6 mononuclear cells, thereby establishing the efficacy of the technique in the detection of rare cancer cells in hematopoietic tissue samples of cancer patients.

INTRODUCTION

Most human cancers are characterized by the aberrant expression of normal and/or mutated genes, and natural selection acts on cancer cells to cause a loss of growth control, angiogenesis, invasion, and metastasis (1). It is not understood how changes in the expression of specific genes cause later progression, but the identification and monitoring of the cells where these expression changes take place carry great diagnostic significance. Indeed, because most patients with epithelial malignancies die of disseminated disease, early detection of its presence is crucially important. This poses a serious challenge because even a very small number of malignant cells can cause a relapse of the disease: the presence of microscopic subclinical tumors outside the primary site or regional lymph nodes in a variety of epithelial cancers (breast, non-small cell lung cancer, esophageal, colon, and gastric) has been correlated with poor prognosis, presumably serving as a marker of metastatic potential or evidence of metastatic dissemination (2–10). Furthermore, such MRTs³ are also observed after high-dose chemotherapy and are thus survivors of an intense therapeutic barrage; their presence then implies *in vivo* resistance to therapy or an increased tumor burden.

The detection of residual tumor cells in autologous grafts from both BM and PB SCs after high-dose chemotherapy has been reported by many investigators (11) and was ascribed prognostic significance in both advanced and locally recurring breast cancer (12, 13). The presence of cancer cells in blood or BM is therefore being evaluated as an indicator for metastatic disease in patients with solid tumors, *e.g.*, carcinomas of the breast, lung, colorectum, and prostate (14, 15), and their quantitation is important in the evaluation of BM or peripheral SC preparations that serve as autologous transplants after high-dose chemotherapy. In addition, repeated determinations during a treatment period may help to monitor the degree of response to therapy, and the cancer cells in these readily accessible tissues may be used to further characterize the disease (*e.g.*, presence of markers associated with specific phenotypes).

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³ The abbreviations used are: MRT, minimal residual tumor; BM, bone marrow; CK+, cytokeratin-positive; DAPI, 4',6-diamidino-2-phenylindole; Ep-CAM, epithelial cell adhesion molecule; PB, peripheral blood; PBMC, PB mononuclear cell; SC, stem cell.

The detection of MRT cells relies on differential expression of normal or abnormal genes and the monitoring of their transcripts or products. Different methods have been used to do so (15–17), and they fall into three main categories: clonogenic, where the tumor cells are grown in culture and characterized; molecular, where the transcript levels of a marker gene are determined; and immunological, which monitors marker gene products with antibodies. Clonogenic assays are quite specific, but the sensitivity of the clonogenic approach is insufficient to score MRT contamination in hematopoietic samples (13, 18). The reverse transcription-PCR method to determine the presence of marker transcripts offers theoretically unparalleled sensitivity (up to 1 tumor cell in 10^7 or better), but this method is marred by high false-positive determinations and should be complemented with other methods for the confirmation of the positive hits, yet it does not allow direct visualization of the rare positive cells (19–22).

Immunological methods are therefore the procedures of choice, whereby antibodies directed to characteristic cellular constituents are used to stain the cells of interest. One can distinguish approaches based on flow cytometry (23, 24) and on image cytometry (25–28). Flow cytometry allows the analysis of a large number of cells in a few minutes. Combined with an immunomagnetic separation technique, frequencies below one epithelial cell/ml blood can be detected (23). However, confirmation of the “positive events” by visual inspection of tumor cell morphology or other cell characteristics remains necessary. Rare cell enumeration can be performed manually under the microscope, but this is a laborious task and moreover, some positive events can easily be missed, especially when present at low frequencies. Therefore, attempts have been made to automate the microscopic detection and quantification of rare cells (23–27), and although still slower than flow cytometry, the performance of microscopic detection systems is improving. These methods offer the advantage that an image of each detected event can be stored in computer memory for later visual evaluation; the detected cells can also be relocated on the microscope slide at any time, if necessary, because the coordinates of all detected objects are known (26, 29).

We developed such an automated microscopic system (Rare Event Imaging System) for the detection and analysis of cancer cells in PB or BM preparations. Slides are automatically scanned at low magnification for the detection of tumor cells (positive events), which is based on cytokeratin/rhodamine labeling, and the total cell count, which is based on nuclear DAPI labeling. Cytokeratin, a cytoskeletal component of epithelial and carcinoma-derived cells, is the most widely used and best characterized marker of cancer cell contamination (8, 30–33). After the automated analysis, the user can review all positive events on the computer screen and manually confirm them using higher magnification. In addition, cells can be viewed with different fluorescence filters for multiple-marker analysis: for increased detection specificity and phenotype characterization of residual tumor cells, we established a double-labeling protocol that combines the labeling of cytokeratin with that of surface antigens reported to be expressed in specific types of cancers, *e.g.*, the Ep-CAM for breast, ovarian, colon, and lung carcinomas (34, 35) and the disialoganglioside GD2 for small cell lung carcinoma, neuroblastoma, glioma, and melanoma (36–38). In this

paper, we demonstrate the methodological power of the new automated rare event analyzer using model systems, and first results analyzing samples of patients with breast and small cell lung cancer.

MATERIALS AND METHODS

Collection of Blood and BM Specimens. Five to ten ml of blood or BM were drawn from control subjects or patients with a diagnosis of breast or small cell lung cancer into vacutainer tubes containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NY). All samples were obtained with informed consent from the subject or patient and were processed for microscopic analysis within 24 h of collection.

Cell Lines. The breast carcinoma cell line MCF-7 and the small cell lung cancer cell line SW2 were purchased from American Type Culture Collection and used to evaluate the staining protocol and to determine the sensitivity of the Rare Event Imaging System. Cell lines were maintained in DMEM (MCF-7) or RPMI 1640 (SW2) containing 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Sample Preparation for Microscopic Analysis. Blood or BM samples were mixed with two volumes of 0.17 M ammonium chloride, incubated at room temperature for 40 min, and centrifuged at $800 \times g$ for 10 min at room temperature. The cell pellet was then washed, resuspended in PBS, and the total number of living PBMCs or nucleated BM cells was counted using trypan blue dye exclusion. The cells were attached to adhesive slides (Paul Marienfeld GmbH & Co., KG, Bad Merzheim, Germany) at 37°C for 40 min, and the slides were then blocked with cell culture medium at 37°C for 20 min. The total number of cells applied per slide was 1.5×10^6 , and the adhesive area on these slides consists of three separate circles totaling 530 mm².

For the single labeling of cytokeratin, cells were fixed in ice-cold methanol for 5 min, rinsed in PBS, and incubated with a rabbit anticytokeratin antiserum directed against class I and II cytokeratins (Biomedical Technologies, Stoughton, MA) at 37°C for 1 h. Subsequently, slides were washed in PBS, incubated with rhodamine-conjugated antirabbit antibody (Jackson Immuno Research, West Grove, PA) at 37°C for 30 min, counterstained with 0.5 µg/ml DAPI (Molecular Probes, Eugene, OR) in PBS at room temperature for 10 min, and mounted in glycerol-gelatin (Sigma, St. Louis, MO). Processed slides were stored at room temperature and analyzed microscopically within a month.

For the double labeling of cytokeratin and the cell surface antigens Ep-CAM or GD2, the cells were fixed in 1% paraformaldehyde in PBS (pH 7.4) at room temperature for 5 min, washed in PBS, and blocked with 20% human AB-serum (Nabi Diagnostics, Boca Raton, FL) in PBS at 37°C for 20 min. Subsequently, primary antibodies directed against the surface antigens Ep-CAM (monoclonal mouse KS1/4 antibody) or GD2 (monoclonal mouse 1418 antibody) were applied at 37°C for 1 h (both antibodies were kindly provided by Dr. Kin-Ming Lo, Lexigen Pharmaceuticals, Lexington, MA). Cells were then washed, fixed in ice-cold methanol for 5 min, blocked with 20% human AB-serum, and incubated with anticytokeratin antiserum at 37°C for 1 h. Secondary antibodies (FITC-conjugated anti-

mouse and rhodamine-conjugated antirabbit antibodies; Jackson Immuno Research) were mixed and applied at 37°C for 30 min, followed by counterstaining of the nuclei with 0.5 µg/ml DAPI in PBS. Doubly labeled cells were mounted in Gel/Mount (Biomedex, Foster City, CA). Slides were stored at 4°C and analyzed microscopically within a week.

Tumor Cell Dilutions for Determination of Sensitivity.

To determine the sensitivity of our method regarding the detection of CK+ cells, MCF-7 breast cancer cells were serially diluted in PBMC of a healthy blood donor. The dilutions tested were 1:10³, 1:10⁴, 1:10⁵, 1:2 × 10⁵, 1:5 × 10⁵, and 1:10⁶. Solutions were attached to adhesive slides and processed for cytokeratin labeling as described above. Up to eight adhesive slides were prepared and scanned per dilution. Samples were analyzed for the number of tumor cells per slide and related to the total cell count.

Automated Microscopic Detection of Tumor Cells and Total Cell Count. Slides were automatically scanned using a Rare Event Imaging System, developed by Georgia Instruments, Inc. (Roswell, GA). The system employs proprietary image processing algorithms to detect rare fluorescent events and determine the total number of cells analyzed. It is comprised of an advanced computer-controlled microscope (Nikon Microphot-FXA, Nikon, Japan) with autofocus, motorized X-, Y-, and Z-axis control, motorized filter selection, and electronic shuttering. Images are taken by an integrating, cooled CCD detector and processed in a 60-MHz Pentium imaging workstation.

In the first step, the slide is automatically scanned for the detection of positive events (e.g., CK+ cells) using the rhodamine filter set. The identification of positive events is based on fluorescence intensity and area. The (X,Y) coordinates of each positive event are stored into computer memory, and the image is archived. In the second step, the slide is scanned for the total number of DAPI-labeled nuclei per slide, representing the total cell count. The total scanned area per slide is 448 mm² (84% of the adhesive area) to avoid edge effects. At the end of the two scans, the number of positive events and the total cell count are given, and a gallery of images containing all positive events is displayed. The user can review the images and recall any of the events for further examination using the stored coordinates attached to each image. The field of interest can then be visualized using higher magnification and additional filter sets (e.g., fluorescein, or UV filter). Images of different fluorescent colors can be electronically overlaid for positive confirmation of the event and for phenotypic evaluation (multiple labeling). The total scanning time (two scans) for one slide is about 1 h. The two scans can be run independently, thereby offering the option of just screening for positive events and thus shortening the scanning time to 30 min/slide.

RESULTS

Evaluation of the Cell Deposition Procedure. One of the most critical steps during sample preparation is the deposition of the cells onto slides. A qualitative microscopic comparison of cell preparations attached to poly-L-lysine/PBS-coated slides (0.1%; Sigma, St. Louis), SectionLock Slides (Polysciences, Inc., Warrington, PA), and adhesive slides (Paul Marienfeld GmbH & Co.) revealed that the most homogeneous

Table 1 Efficiency of the cell deposition method^a

Sample type	Cell count/slide	Range (n)	Recovery
Normal PB	1,120,237 ± 93,372	733,833–1,470,633 (8)	89%
Cancer PB	811,400 ± 89,039 ^b	223,393–1,473,777 (17)	64%
Cancer BM	731,945 ± 72,906 ^b	157,110–1,459,414 (25)	58%
Cancer SCs	915,983 ± 95,806	76,745–1,631,660 (23)	73%

^a PB, BM, or SC samples from healthy subjects (normal) or cancer patients were prepared as described under "Materials and Methods", and 1.5 × 10⁶ cells were applied to each adhesive microscope slide. Cells were counted (based on DAPI labeling) on the number of slides indicated for each group (n), and results are expressed as mean ± SEM. For the calculation of recovery, note that the area scanned on each slide is 84% of the total adhesive area (see "Materials and Methods").

^b P < 0.05 versus normal PB by t-test.

cell monolayers (optimal cell density with minimal overlap) was obtained with the latter (not shown). To further validate our deposition technique for different types of samples, we compared the total number of cells as determined by the Rare Event Imaging System with the number of cells originally deposited onto the slides. Table 1 shows a high cell recovery (89%) for the PB of healthy blood donors, but a somewhat higher cell loss in samples from cancer patients (64, 58, and 73% recovery for PB, BM, and SC samples, respectively; P < 0.05 for PB and BM versus normal PB by t test).

Sensitivity of the Detection Method. To explore the sensitivity of our Rare Event Imaging System, we prepared PBMC samples that had been spiked with breast cancer cells (MCF-7) and processed for cytokeratin labeling. The brightly stained epithelial MCF-7 cells could easily be distinguished from the mesenchymal background of the WBCs (Fig. 1A). The sensitivity of detection of CK+ cells was tested with increasing tumor cell dilutions (MCF-7/PBMC) as described in "Materials and Methods." Cancer cells in expected quantities could be detected up to the most diluted samples tested, 1 MCF-7 cell/10⁶ PBMCs (Table 2; expected and observed curves not statistically different; χ² test).

Double Labeling of Tumor Cells. To increase the specificity of rare event detection and to be able to further characterize the cancer cells identified, we developed a staining protocol that allows the detection of intracellular cytokeratin and a cell surface marker simultaneously. The double-labeling procedure consists of two sequential steps: first fixing the cell surface and labeling for Ep-CAM or GD2, and second, permeabilizing the cells and staining for intracellular cytokeratin. The double-labeling protocol was optimized in the cancer cell lines MCF-7 (breast cancer) and SW2 (small cell lung cancer). Fig. 1B shows SW2 cells labeled with the anti-GD2 antibody and the anticytokeratin antiserum. The sequential fixation preserved the antigenic sites of both proteins with regard to their cellular localization, as demonstrated in the optical sections taken with a confocal laser scanning microscope (Fig. 1B). The cells clearly showed cytokeratin in the cytoplasm (red) and GD2 at the cell surface (green). The expression levels of both proteins was quite heterogeneous within the cell population. A similar result was obtained when MCF-7 cells were doubly labeled with the anti-Ep-CAM antibody and the anticytokeratin antiserum (data not shown). Control experiments in which one of the primary anti-

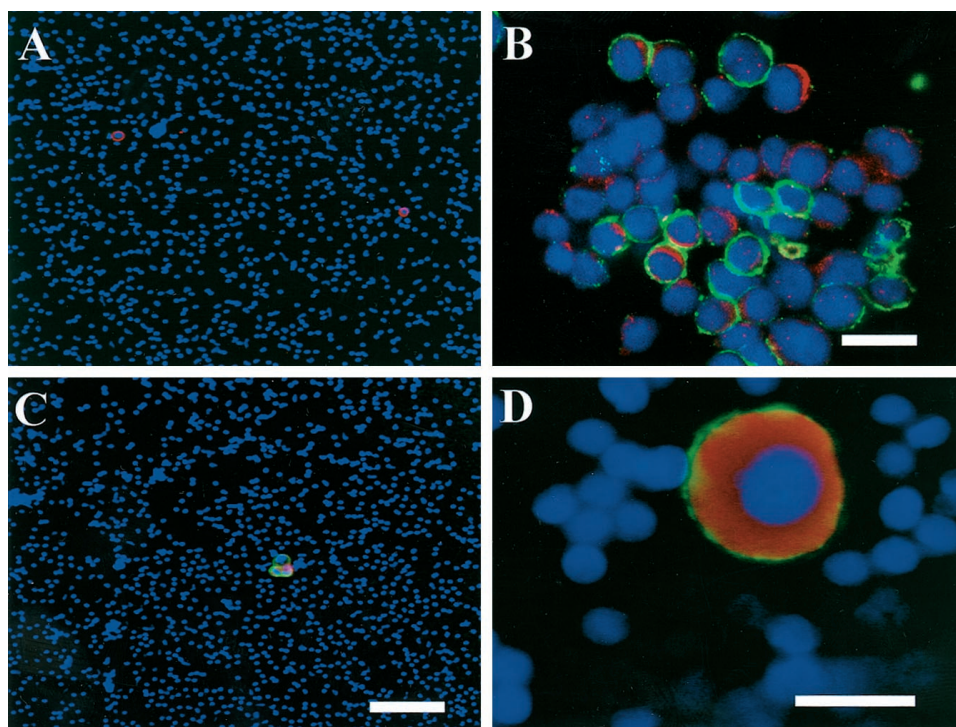


Fig. 1 Visualization of breast and small cell lung cancer cells using single cytochrome c, double Ep-CAM/cytochrome c, or double GD2/cytochrome c labeling. MCF-7 breast cancer cells were mixed with PBMCs, attached to adhesive slides, and labeled for cytochrome c alone (A) or doubly labeled for Ep-CAM/green and cytochrome c/red (C and D). SW2 small cell lung cancer cells were attached to adhesive slides and doubly labeled for GD2/green and cytochrome c/red (B). All nuclei were counterstained with DAPI/blue. Bars, 100 μ m (A and C) and 25 μ m (B and D).

Table 2 Sensitivity of rare event detection^a

Cells added per 10 ⁶ PBMCs	Total no. of cells detected	Total cell count	Cells detected per 10 ⁶ PBMCs
1000	1789	1.94 \times 10 ⁶	922
100	169	1.79 \times 10 ⁶	95
10	27	2.35 \times 10 ⁶	12
5	38	5.16 \times 10 ⁶	7
2	11	3.94 \times 10 ⁶	3
1	13	6.13 \times 10 ⁶	2

^a MCF-7 cells were serially diluted in PBMC and labeled for cytochrome c on adhesion slides. Two to eight slides for each dilution were analyzed for CK+ cells and total number of cells. Observed cell counts (last column) do not differ statistically from the expected count (first column) (χ^2 test).

bodies was omitted but both secondary antibodies were applied did not reveal any cross-reactivity between the two detection systems (data not shown).

To further validate the staining protocol, we labeled PBMCs that had been spiked with MCF-7 or SW-2 cells. The goal was to obtain a bright fluorescent signal of the cancer cells and a low background signal from the surrounding PBMCs. The two most important factors to achieve this goal were found to be the sequential application of the primary antibodies and two blocking steps (20% human AB-serum in PBS) before the incubation with the primary antibodies. As shown in Fig. 1C, the doubly labeled MCF-7 cells could clearly be distinguished from the surrounding PBMCs. At higher magnification, we were also able to confirm the intracellular cytochrome c labeling and the surface staining of Ep-CAM (Fig. 1D). Similar results were obtained with PB-

MCs spiked with SW-2 cells and doubly labeled for GD2 and cytochrome c (data not shown).

We also applied the double-labeling protocol to PB and BM samples of cancer patients. Fig. 3B shows an example of a GD2/CK+ cell from the PB of a patient with small cell lung cancer. Fig. 3C shows an Ep-CAM/CK+ cell from the BM of a breast cancer patient. In this example, the cancer cell is not only bigger than the surrounding BM cells, but it also exhibits the distinct localization of the individual stains: cytochrome c (red) is cytoplasmic, whereas Ep-CAM (green) is concentrated toward the cell periphery, at the cell membrane.

Detection of CK+ and Doubly Positive Cells in Normal Blood Samples. To evaluate the specificity of the single- and double-staining protocols, we analyzed blood samples from healthy donors. We compared the number of "positive" cells using the single cytochrome c or the double cytochrome c/Ep-CAM or cytochrome c/GD2 labeling methods. As seen in Table 3, 16–18% of the PB samples scored positive for cytochrome c using any of the protocols, with the number of CK+ cells ranging from 1 to 26 labeled cells/10⁶ WBCs. In contrast, when the samples were processed with the double-labeling protocol, positivity was almost completely eliminated from the samples of healthy subjects (a single doubly positive cell was observed in a total of 77 PB samples).

Evaluation of Spatial and Temporal Variations in Sample Collection. To assess a possible heterogeneity in the distribution of CK+ cells in different areas of the BM, paired BM samples from the right and the left iliac crests of the same patient were taken and analyzed. Of 24 pairs, 21 showed concordant results (Fisher's exact test) with regard to cytochrome c positivity (Fig. 2A). We also tested for temporal fluctuations in

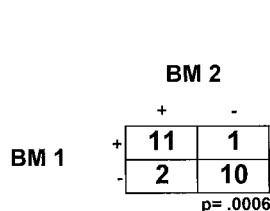
Table 3 Positive cells in blood samples from healthy blood donors^a

Marker(s)	Total samples	CK+ labeled			DBL+ labeled ^b	
		Positive samples	CK+/10 ⁶ (all samples)	CK+/10 ⁶ (CK+ samples)	Positive samples	DBL+/10 ⁶ (DBL+ sample)
CK	57	10 (17%)	1.18 ± 0.53	7.28 ± 2.59		
CK/Ep-CAM	43	7 (16%)	0.46 ± 0.21	2.85 ± 0.81	1 (2.3%)	1.4
CK/GD2	34	6 (18%)	0.78 ± 0.44	4.41 ± 1.98	0 (0.0%)	0

^a Blood samples from healthy blood donors were labeled for cytokeratin alone or doubly labeled for CK/KSA or CK/GD2 (see “Materials and Methods”). Positive samples are those containing CK+ cells (in single labeling) or doubly labeled cells (in double labeling). Numbers of positive cells in each category are expressed per 10⁶ cells analyzed and are given as mean ± SEM (except for the single positive cell in one sample containing 7.14 × 10⁵ cells in the CK/Ep-CAM group).

^b DBL+, doubly labeled.

A: Spatial (BM) heterogeneity



B: Temporal (SC) heterogeneity

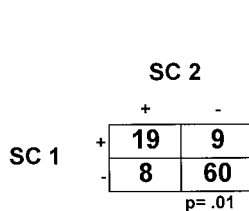


Fig. 2 Assessment of the spatial and temporal sampling heterogeneity. A, twenty-four paired BM samples (BM 1 and BM 2) were taken from both iliac crests of the same patients and analyzed for CK+ and total cells. B, ninety-six paired SC samples (SC 1 and SC 2) were drawn from the same patients at consecutive days without therapeutic intervention and analyzed for CK+ and total cells. Statistical analysis was performed by Fisher’s exact test.

the occurrence of CK+ cells in PB samples. Two SC samples from each of 96 patients were taken at consecutive days but without therapeutic intervention. Paired samples showed a statistically significant concordance with regard to cytokeratin positivity (Fig. 2B).

Detection of CK+ Cells in Cancer Patient Blood and BM Samples. To demonstrate the power of the Rare Event Imaging System, we analyzed 355 PB, BM, and SC samples from patients with breast cancer before autologous BM transplantation but after high-dose chemotherapy using the single cytokeratin labeling method. An example of two CK+ cells from the PB of a breast cancer patient is shown in Fig. 3A. The positive cells showed clear cytoplasmic labeling, whereas the surrounding blood cells were not stained. We found CK+ cells in 52% of the BM, 34% of the PB, and 27% of the SC samples (Table 4). The frequency of CK+ cells in the positive samples varied from 1/10⁶ to 1020/10⁶. However, many PB samples from normal subjects displayed a small number of CK+ cells, and these were found to be false-positive cells based on the double-labeling experiments (Table 3). Therefore, to declare definite positivity in PB samples from cancer patients, we set a cutoff point at the mean number of CK+ cells plus two times the SD as observed in the control samples, i.e., 9/10⁶. Applying this threshold, we still found a higher degree of cytokeratin positivity in BM (40%) compared to PB (24%) or SC preparations (12%; Table 4). Furthermore, patients with stage IV disease were found to be cytokeratin positive in a significantly higher percentage than patients with stages II/III disease in all types of samples analyzed (Table 4). We are presently analyzing clinical samples from breast and small cell lung cancer patients using the double-labeling protocol; the results will be published elsewhere.

DISCUSSION

We developed an automated analysis system for the detection of cells of interest that occur at low frequencies (rare events) using dual- or multiple-marker analysis. The preparation procedure for the microscopic analysis of blood or BM samples was optimized for automation and includes lysis of RBCs, deposition of mononuclear cells onto adhesive sides, and immunofluorescent labeling of the sample. Slides are then examined at low magnification under a fluorescence microscope fitted with a motorized stage, and all of the fluorescent events are imaged and catalogued in a computer database for ulterior retrieval.

For the automated detection of rare events in PB or BM, it was critical to use a preparation method with minimal cell loss during sample processing. Simple lysis of erythrocytes was preferred over Ficoll-based isolation methods to ensure maximal recovery of rare cancer cells. Our cell preparation/adhesion procedure yielded a homogeneous cell monolayer, with a recovery comparable to that obtained in cytopsin preparations performed with specially designed buckets and high centrifugal forces, where minimal cell loss has been reported (26). In contrast, regular cytopsin preparations can result in a loss of up to 2/3 of the cells (39). Information on cell number is unavailable for most studies using microscopic rare event detection because these fail to record the total number of cells actually being analyzed on the slides (32, 40). Rather, those papers relate the number of positive events to the total number of cells processed, assuming a complete recovery. This introduces a bias: not only did we find that cells are indeed inevitably lost during preparation, but the recovery can vary greatly between samples of a given type (see “Range,” Table 1) as well as according to the type of sample. It is not clear why cell loss was

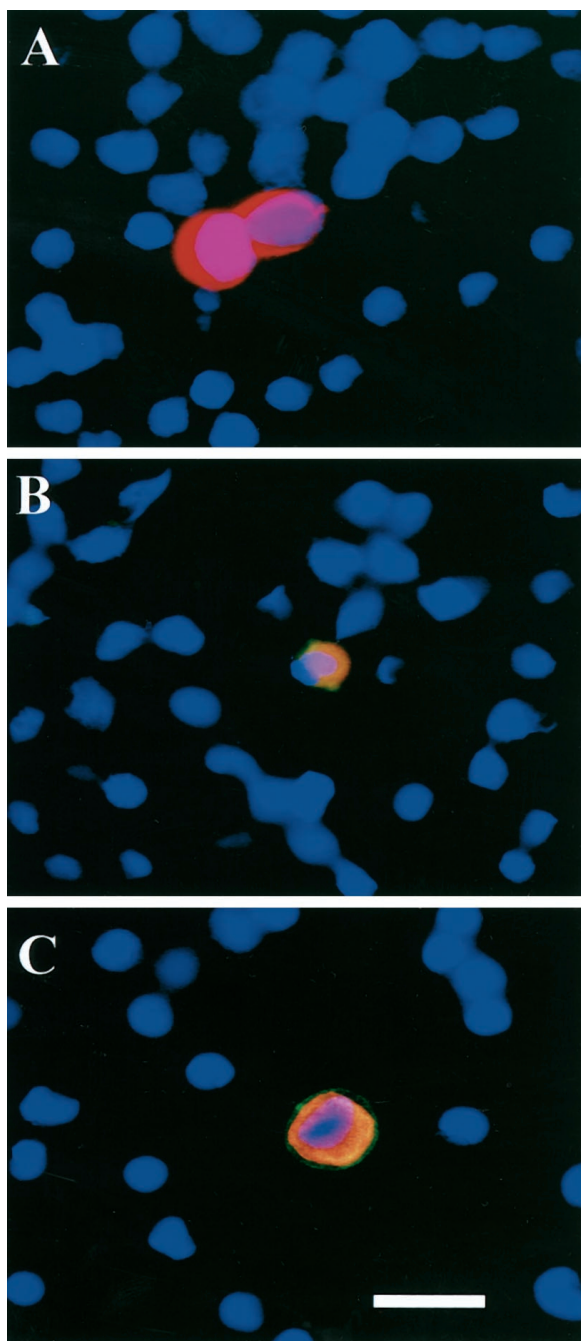


Fig. 3 Detection of cancer cells in hematopoietic tissue samples from cancer patients. PB (A and B) and BM (C) samples were prepared from patients with breast (A and C) or small cell lung cancer (B). The specimens were labeled for cytokeratin alone (A), doubly labeled for GD2/cytokeratin (green/red; B), or doubly labeled for Ep-CAM/cytokeratin (green/red; C). All nuclei were counterstained with DAPI. Bar, 20 μ m.

higher in PB samples from cancer patients compared to like samples from normal subjects; there are numerous reports that malignant cells express abnormal levels of a variety of adhesion molecules (including cadherins, integrins, selectins, laminins,

Table 4 Detection of cytokeratin-positive cells in hematopoietic tissues of breast cancer patients before autologous BM transplantation^a

	Total samples	CK+ samples (all)		CK+ samples (≥ 9 CK+/10 ⁶ PBMCs)	
		Count	%	Count	%
BM samples	63	33	52	25	40
Stages II/III	20	7	35	5	25
Stage IV	43	26	60	20	46
PB samples	59	20	34	14	24
Stages II/III	13	2	15	2	15
Stage IV	46	18	39	12	26
SC samples	233	64	27	29	12
Stages II/III	49	11	22	4	8
Stage IV	184	53	29	25	14

^a BM, PB, and SC samples from a total of 156 patients were analyzed for cytokeratin-positive cells and total cell count. Note that there were multiple samples from some patients, whereas for others, only one kind of sample could be analyzed. "CK+ samples (all):" number of samples with at least one CK+ cell; "CK+ samples (≥ 9 CK+/10⁶ PBMCs):" number of samples with nine or more CK+ cells per 10⁶ PBMCs (mean + 2 SD of CK+ cells in normal PB; Table 3). The highest numbers of CK+ cells per sample were 504/10⁶ for BM, 371/10⁶ for PB, and 1020/10⁶ for SC.

members of the immunoglobulin superfamily, variant isoforms of the transmembrane glycoprotein CD44, and others; see Refs. 41–47), and differences in cell-matrix interactions have been reported specifically between leukocytes (T lymphocytes and dendritic cells) and tumor cells (invasive melanoma; Ref. 48). Although we are presently optimizing the cell deposition procedure and attempting to minimize variability, we nonetheless conclude in view of these observations, that the determination of the total number of cells actually included in the analysis (*i.e.*, on the slides) is imperative in microscopic rare event detection.

Experiments whereby PB samples are mixed with defined numbers of breast cancer cells show that the detection sensitivity of our Rare Event Imaging System is at least 1 CK+ cell/1 million PBMCs (Table 2), which should be adequate for clinical applications. The brightly stained CK+ MCF-7 cells are easily distinguished from the dark background of negative cells. A similar detection sensitivity has been shown for other automated image analysis systems (26, 27, 49) and for manual microscopic analysis (50). A further increase in detection sensitivity could be achieved by analyzing more cells (*e.g.*, 10–20 million/sample). However, although such large numbers of cells can readily be obtained with regular blood or BM sampling methods, their microscopic analysis would be very time consuming.

Speed is therefore a fundamental parameter for the evaluation of automated rare event analysis systems. The system described herein takes about 1 h to scan 1 million cells for positive events (*e.g.*, CK positivity) and for the total cell count. This is similar to the fastest automated microscope-based cell analysis systems described by others (26, 49), and it makes the processing of large numbers of cells reasonable. By comparison, the commercially available Laser Scanning Cytometer (CompuCyte Corp., Cambridge, MA) can only scan between 1000–2000 cells/min (Ref. 29 and technical information from Compy-

Cyte Corporation, Cambridge, MA). We are presently developing a much faster system by using a more sensitive CCD camera and faster computer, which will bring down the processing time to a few minutes per million cells, comparable to flow cytometry (23) yet retaining the possibility to observe each positive event at higher magnification or with different optics, for morphological confirmation.

The specificity of the detection of cancer cells in blood or BM preparations can only be as good as the marker and antibodies used in the procedure. The most widely used marker is cytokeratin, a cytoskeletal component of epithelial and carcinoma-derived cells (8, 30–33). Although it has been validated as a valuable marker for breast, prostate, gastric, and colorectal cancer in a large number of clinical studies, cytokeratin is not a tumor cell-specific marker and can result in false-positive staining of epidermal cells or weak cytoplasmic staining of phagocytic cells that contain cytokeratin debris or dye particles (30). In these cases, as mentioned above, definitive microscopic confirmation of the malignant cytology of the immunostained cells is crucial. Another source of false-positive events is cross-reactive staining of the epithelial or cancer cell marker with blood or BM cells; *e.g.*, mucin-like epithelial membrane markers are able to cross-react with hematopoietic cells. Indeed, we found that cytokeratin antibodies can label PBMCs from healthy blood donors (Table 4): 17% of the PB samples from normal blood donors exhibited cytokeratin positivity, albeit at a low level (mean, 1.18 CK+/10⁶ cells). It is not clear whether these CK+ cells in “normal” samples represent benign epithelial cells, cross-reacting hematopoietic cells, or cancer cells disseminated from an undiagnosed primary carcinoma.

To improve the specificity of the rare event detection, a double-labeling protocol was developed for the simultaneous detection of cytokeratin and the epithelial surface markers, Ep-CAM and GD2. This procedure dramatically reduced the positivity of “normal” samples, with only one doubly labeled cell among the 77 samples tested (compounded of CK/Ep-CAM and CK/GD2; Table 3), suggesting that the few CK+ cells detected in normal samples were not of cancer origin. In addition to the mere detection of cancer cells in blood or BM samples, efforts have been made to further characterize the phenotype of rare tumor cells, *e.g.*, with respect to their aggressiveness, cell cycle stage, or growth behavior (40, 51–53). Protocols for multiple marker analysis, combining cytokeratin labeling with growth factor receptors or proliferation-associated antigens to analyze breast cancer samples (52), or combining cytokeratin labeling with prostate specific antigen to analyze prostate carcinoma (53) have been developed. Also, in gastric cancer patients, cells that were doubly positive for cytokeratin and the urokinase plasminogen activator receptor correlated with high metastatic potential (51). A variety of possible additional (cancer-specific) markers have been described, *e.g.*, glycoproteins (39), gangliosides (54), cell adhesion molecules (55, 56), and others (57). The sensitivity and specificity of the cancer cell detection method and the quality of tumor characterization we will be able to make will all improve as new markers become available. Extensive double-labeling studies of cancer patients’ hematopoietic samples are under way in our laboratory to explore the biological properties of MRT cells.

Automated rare event detection using single cytokeratin

labeling was applied to 355 BM, PB, and SC samples from patients with breast cancer before autologous BM transplantation (Table 4). BM showed the highest percentage of CK+ samples (52%), followed by PB (34%) and SC preparations (27%). Furthermore, samples from patients with stage IV disease contained CK+ cells at a higher frequency than patients with stage II/III disease. Similar gradations have been reported by others (12, 32, 39, 58). These numbers are in general accordance with previous studies using various rare event detection procedures (12, 30, 39, 54, 59), although some authors report a higher proportion of cancer patients with BM or PB contamination than observed here, possibly due to chemotherapy treatment received by most of the patients in our cohort shortly before sample collection (33). However, given the large proportion of low-level CK+ samples observed in PB samples from healthy blood donors (Table 3), we set a cutoff in the frequency of CK+ cells required to declare a sample contaminated (mean + 2 SD of CK+ cell contamination in samples from healthy subjects, *i.e.*, 9 CK+ cells/10⁶ cells). Using this cutoff, 40%, 24%, and 12% of the samples were declared contaminated with cancer cells in BM, PB, and SC samples, respectively. Upcoming double-labeling studies will not be subject to this false-positive problem or require that a cutoff background level of contamination be used.

Analyses of paired BM samples of at least 10⁶ cells for spatial sampling heterogeneity showed a good concordance within pairs (Fig. 2). Pantel *et al.* (30) had found that sampling from two sides of the iliac crest is necessary to obtain reliable results. However, it is not clear whether in that study the mere doubling of the number of cells analyzed improved the result or whether true heterogeneity was captured. Our results indicate that it is sufficient to analyze only one BM sample of at least 10⁶ cells taken from either side of the iliac crest. Analyses of the temporal sampling heterogeneity revealed that there was little fluctuation with regard to cytokeratin positivity in SC preparations within the constraints of our experimental protocol, indicating a good repeatability of the method.

In summary, an automated microscopic method was developed for the detection of cancer cells in blood or BM samples. It was demonstrated that the system is capable of the detection of rare events (1 per 1 million) in a reasonable time and double-labeling protocols result in a drastic reduction of false-positive determinations. This allows the use of the system in a clinical setting to monitor cancer treatment and to determine recurrences. Furthermore, detected cancer cells can be characterized phenotypically for different markers.

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