Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases

W. Jeffrey Allard,[1] Jeri Matera,[1] M. Craig Miller,[1] Madeline Repollet,[1] Mark C. Connelly,[1] Chandra Rao,[1] Arjan G. J. Tibbe,[1] Jonathan W. Uhr,[2] and Leon W. M. M. Terstappen[1]

[1]Immunicon Corporation, Huntingdon Valley, Pennsylvania; and [2]Cancer Immunobiology Center and Departments of Microbiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas

ABSTRACT

Purpose: **The purpose of this study was to determine the accuracy, precision, and linearity of the CellSearch system and evaluate the number of circulating tumor cells (CTCs) per 7.5 mL of blood in healthy subjects, patients with nonmalignant diseases, and patients with a variety of metastatic carcinomas.**

Experimental Design: **The CellSearch system was used to enumerate CTCs in 7.5 mL of blood. Blood samples spiked with cells from tumor cell lines were used to establish analytical accuracy, reproducibility, and linearity. Prevalence of CTCs was determined in blood from 199 patients with nonmalignant diseases, 964 patients with metastatic carcinomas, and 145 healthy donors.**

Results: **Enumeration of spiked tumor cells was linear over the range of 5 to 1,142 cells, with an average recovery of ≥85% at each spike level. Only 1 of the 344 (0.3%) healthy and nonmalignant disease subjects had ≥2 CTCs per 7.5 mL of blood. In 2,183 blood samples from 964 metastatic carcinoma patients, CTCs ranged from 0 to 23,618 CTCs per 7.5 mL (mean, 60 ± 693 CTCs per 7.5 mL), and 36% (781 of 2,183) of the specimens had ≥2 CTCs. Detection of ≥2 CTCs occurred at the following rates: 57% (107 of 188) of prostate cancers, 37% (489 of 1,316) of breast cancers, 37% (20 of 53) of ovarian cancers, 30% (99 of 333) of colorectal cancers, 20% (34 of 168) of lung cancers, and 26% (32 of 125) of other cancers.**

Conclusions: **The CellSearch system can be standardized across multiple laboratories and may be used to determine the clinical utility of CTCs. CTCs are extremely rare in healthy subjects and patients with nonmalignant diseases but present in various metastatic carcinomas with a wide range of frequencies.**

INTRODUCTION

There are accumulating reports of the isolation and characterization of circulating tumor cells [CTCs (1–18)]. The findings that CTCs can be found in patients before the primary tumor is detected, CTCs are found in a significant proportion of patients when a carcinoma recurs, and CTCs persist in some patients after removal of the primary tumor have been the impetus for continued studies of these tumor cells. Evidence that CTCs are derived from clones in the primary tumor (16) suggests that they may reflect the tumor burden at all stages of tumor progression. Thus, in addition to a potential role in early diagnosis and prognostication, CTCs may play a major role in characterizing genetic and immunophenotypic changes with tumor progression, thereby helping to guide targeted therapy (17, 18). A particularly important attribute of a blood test is that it is safe and can be performed frequently, whereas repeated invasive procedures including bone marrow aspiration may provide limited patient compliance.

A major problem in the field is that different techniques have been used to isolate and characterize CTCs (1, 7, 19–23). It is not known to what extent these assays differ in sensitivity, reproducibility, and specificity for detection of CTCs in neoplastic and nonneoplastic diseases. Therefore, it is difficult to compare results from different laboratories without answers to the above-mentioned questions.

The purpose of the present study is to show that by the use of a cellular preservative to prevent CTC degradation and the CellSearch system consisting of semiautomated equipment and prepackaged kits for the isolation and identification of CTCs it is possible to obtain highly reproducible quantitative results from different laboratories and to prove that CTCs are rarely present in patients with nonneoplastic diseases.

Accuracy, precision, and linearity of the CellSearch system were determined, and the number of CTCs per 7.5 mL of blood in healthy subjects, patients with nonmalignant diseases, and patients with a variety of metastatic carcinomas was evaluated.

MATERIALS AND METHODS

Patients and Blood Collection. Blood was drawn at multiple geographically dispersed locations from healthy female volunteers, women with nonmalignant diseases, and patients with a variety of metastatic carcinomas into evacuated 10-mL blood draw tubes containing EDTA (Becton Dickinson, Frank-

Received 2/26/04; revised 6/4/04; accepted 7/6/04.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: Supplementary data for this article can be found at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org).
Requests for reprints: W. Jeffrey Allard, Immunicon Corporation, 3401 Masons Mill Road, Suite 100, Huntingdon Valley, PA 19006. Phone: 215-830-0777, ext. 193; E-mail: jallard@immunicon.com.

lin Lakes, NJ) or the CellSave Preservative Tube (Veridex LLC, Raritan, NJ). Specimens drawn into EDTA tubes had a cellular preservative manually added immediately after the blood draw (24, 25). The CellSave Preservative Tube is an evacuated blood draw tube containing EDTA as anticoagulant and a cellular preservative. Samples were maintained at room temperature and processed within 72 hours of blood collection. All patients were enrolled using institutional review board-approved protocols and provided informed consent. Blood was always drawn from cancer patients either before or a minimum of 7 days after the administration of intravenous therapy. Results of the control group were reported previously (26). The healthy volunteers had no known illness or fever at the time of draw, no history of malignant disease, and were ≥35 years old to provide a cohort age-matched with the cancer population. The women with nonmalignant diseases included those with benign breast diseases as well as other nonmalignant diseases. These subjects had no known history of cancer and were ≥25 years old.

Cell Culture and Cell Spiking. The breast cancer cell line SKBR-3 was cultured in flasks containing RPMI 1640 supplemented with 10% fetal calf serum and subsequently harvested using trypsin. The cell suspensions were only used when their viability as assessed by trypan blue exclusion exceeded 90%. To determine the actual cell number, a 50-μL aliquot of the SKBR-3 cells was permeabilized and fluorescence labeled by adding 200 μL of PBS containing 0.05% saponin and 10 μL of anticytokeratin monoclonal antibody conjugated to phycoerythrin at a final concentration of 0.5 μg/mL. After a 15-minute incubation at room temperature, 200 μL of buffer and 20 μL of fluorescent beads (Beckman-Coulter, Inc., Miami, FL) containing approximately 20,000 total beads were added. Duplicate tubes containing beads only were run on a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) until 100% of the sample was aspirated. This provided an accurate estimate of the number of beads present in 20 μL. The experimental tubes were then tested in triplicate on the flow cytometer until 10,000 beads were counted in each tube. Using the known number of beads per unit volume, the concentration of cells was determined. For accuracy, linearity, and sensitivity experiments, the spiked cell numbers were estimated to be 4, 18, 71, 286, and 1,142, and for assay imprecision, the cell numbers were 58 and 319 in 7.5 mL of blood.

Sample Preparation for Isolation of Epithelial Cells from Blood. The CellSearch system (Veridex LLC) consists of a CellPrep system, the CellSearch Epithelial Cell Kit, and the CellSpotter Analyzer. The CellPrep system is a semiautomated sample preparation system (23, 27), and the CellSearch Epithelial Cell Kit consists of ferrofluids coated with epithelial cell-specific EpCAM antibodies (28) to immunomagnetically enrich epithelial cells; a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratins 8, 18, and 19; an antibody to CD45 conjugated to allophycocyanin; nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) to fluorescently label the cells; and buffers to wash, permeabilize, and resuspend the cells. Briefly, 7.5 mL of blood were mixed with 6 mL of buffer, centrifuged at 800 × *g* for 10 minutes, and then placed on the CellPrep system. After aspiration of the plasma and buffer layer by the instrument, ferrofluids were added. After the incubation period and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining reagents were then added in conjunction with a permeabilization buffer to fluorescence label the immunomagnetically labeled cells. After incubation on the system, the magnetic separation was repeated, and excess staining reagents were aspirated. In the final processing step, the cells were resuspended in the MagNest Cell Presentation Device (Veridex LLC). This device consists of a chamber and two magnets that orient the immunomagnetically labeled cells for analysis using the CellSpotter Analyzer.

Sample Analysis. The MagNest is placed on the CellSpotter Analyzer, a four-color semiautomated fluorescence microscope. Image frames covering the entire surface of the cartridge for each of the four fluorescent filter cubes are captured. The captured images containing objects that meet predetermined criteria are automatically presented in a web-enabled browser from which final selection of cells is made by the operator. The criteria for an object to be defined as a CTC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratin, and negative staining for CD45 (23, 27). Results of cell enumeration are always expressed as the number of cells per 7.5 mL of blood.

Accuracy, Sensitivity, and Linearity of Circulating Tumor Cell Detection. The following procedure was used to estimate the accuracy, sensitivity, and linearity of the CellSearch system. Five 7.5-mL aliquots of blood collected into CellSave Preservative Tubes from each of five healthy donors were prepared. The five aliquots from each donor were spiked with different numbers of SKBR-3 cells to produce separate tubes with approximately 1,142, 286, 71, 18, or 4 cells per 7.5 mL of blood. The 25 tubes were then processed and analyzed by a single operator according to the sample preparation and sample analysis protocols.

Assay Imprecision. Blood from a healthy donor was collected into CellSave tubes and dispensed into eight aliquots of 7.5 mL. A 50-μL volume containing 58 SKBR-3 cells was added to four of the tubes of blood, and a 50-μL volume containing 319 cells was added to the remaining four tubes. All eight tubes were mixed by repeated inversion and then allowed to sit overnight at room temperature without further mixing. On the following day, two of the tubes with 58 cells and two of the tubes with 319 cells were processed and analyzed in the morning, and the four remaining tubes were processed identically in the afternoon. This entire procedure was repeated each day for 20 days. Calculation of within-run and total imprecision was performed as per the National Committee for Clinical Laboratory Standards EP-5A guidelines (29).

Reproducibility of Circulating Tumor Cell Measurements between Duplicate Samples and Multiple Operators. A total of 477 samples were drawn in duplicate from patients with metastatic breast cancer and tested at one of seven different laboratory sites across the United States within 72 hours of the blood draw as described above. All duplicate tube measurements were performed by the same operator, and tubes were tested without pooling of the blood. In addition, the reproducibility of CTC analysis by different operators was measured. Results from 574 patient samples were enumerated by operators at one of six different laboratory sites, archived to CD-ROM disks, and sent to a central laboratory (Immunicon Corp., Hunt-

ingdon Valley, PA) for blinded review and enumeration of CTCs by trained operators.

RESULTS

Accuracy, Sensitivity, and Linearity of Circulating Tumor Cell Detection. Varying numbers of SKBR-3 cells were spiked into blood, and recovery was measured using the CellSearch system. The expected number of SKBR-3 cells spiked into the healthy donor samples (*i.e.*, 1,142, 286, 71, 18, or 4 cells) plotted against the actual number of SKBR-3 cells observed in the samples is shown in Fig. 1, and results are summarized in Table 1. Regression analysis of the number of observed tumor cells *versus* the number of expected tumor cells produced a slope of 0.85 [95% confidence interval (CI), 0.82–0.87], an intercept of 5.6 (95% CI, 1.8–9.5), and a correlation coefficient (R^2) of 0.99. As expected, the coefficient of variation (CV) increased as the number of cells spiked decreased, ranging from 4.7% at the 1,142-cell spike to 47.1% at the 4-cell spike. The average percentage of SKBR-3 cells recovered was ≥85% at each of the spiking levels, and in the samples spiked with only four cells, no fewer than two cells were detected in all five samples.

The analytical lower limit of detection cannot be measured directly for this assay due to the difficulty of spiking low numbers of cells accurately and reproducibly. However, the minimum average number of CTCs required to be present in a blood sample to ensure recovery of at least one CTC can be estimated using the Poisson distribution (refs. 30 and 31; see supplementary data). Results of this analysis demonstrate that to detect one CTC with an assay recovery of 85%, a 7.5-mL blood sample would have to contain, on average, 1.2 ± 0.4 CTCs.

Assay Imprecision. The reproducibility of cellular enumeration was measured using a single stock of SKBR-3 cells spiked into blood from healthy donors at levels of 58 cells per 7.5 mL and 319 cells per 7.5 mL. Duplicate samples at each level were tested twice per day for 20 days. The within run CVs for the 319-cell spike and 58-cell spike were 8.2% and 15.4%, respectively. Similar results were found for total imprecision, with CVs of 9.4% and 15.8% for the 319- and 58-cell spikes, respectively. This level of variability is only slightly higher than the minimum variability predicted by statistics for counting rare events. A more detailed description of the limitations in counting rare events is provided separately (see supplementary data).

Table 1 Method accuracy measured by recovery of SKBR-3 tumor cells spiked into 7.5 mL blood of five healthy donors

Expected CTC count	Observed CTC count			% Recovery		
	Average	SD	95% CI	Average	95% CI	%CV
4	4	2	1–11	110	25–275	47
18	22	5	14–33	122	78–183	22
71	70	8	55–88	99	77–124	11
286	247	5	216–277	86	76–97	2
1142	971	46	910–1032	85	80–90	5

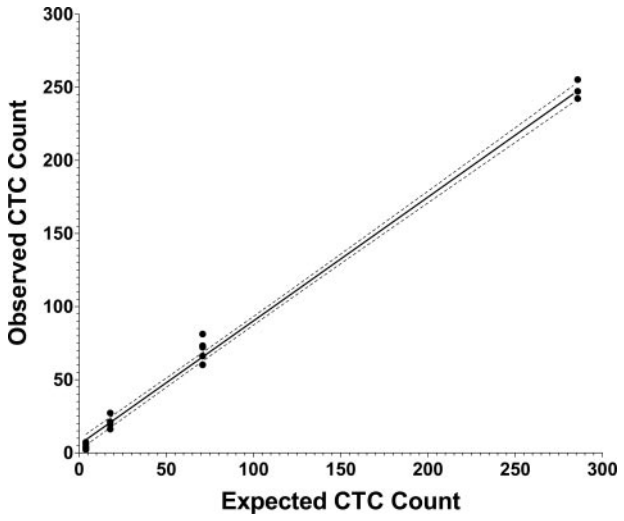

Fig. 1 Recovery of known numbers of spiked SKBR-3 cells from whole blood. SKBR-3 cells (1,142, 286, 71, 18, and 4 cells) were spiked into 7.5 mL of blood from five healthy donors on each of 5 days. The number of cells spiked is plotted *versus* the observed number of cells recovered.

Reproducibility of Circulating Tumor Cell Counts across Duplicate Tubes and Multiple Operators. To measure the variability associated with the analysis of CTCs, the browser images obtained from 574 blood specimens from women with metastatic breast cancer analyzed by operators at six different laboratory sites were archived and sent to a central laboratory (Immunicon Corp.), where image analysis was repeated. The site CTC results were compared with the central laboratory CTC results. Fig. 2*A* shows the comparison of these results, and regression analysis demonstrated a slope of 1.01 (95% CI, 1.00–1.02), an intercept of 0.49 (95% CI, −1.430 to 0.441), and a correlation coefficient (R^2) of 0.994. In Fig. 2*B*, the data from only samples with an average CTC count from the site and the central laboratory of <50 (n = 505) are demonstrated using a Bland-Altman plot. The error of each CTC measurement is represented by the difference in the CTC count obtained at the site and the central laboratory divided by the average of both CTC counts. This is represented as a percentage in the figure, and as expected, the error is greater at the lower CTC counts. This suggests that the criteria for selection of CTCs from the images presented in the CellSpotter browser can be taught effectively and that results can be reproduced across multiple laboratories.

To measure the variability in CTC counts obtained from duplicate blood tubes, a total of 477 samples were drawn in duplicate from patients with metastatic breast cancer and processed on the same day at one of seven laboratory sites. Fig. 3*A* shows the correlation between the CTC counts obtained from both tubes. Regression analysis gave a slope of 1.06 (95% CI, 1.054–1.082), an intercept of 0.64 (95% CI, −2.370 to 0.675), and a correlation coefficient (R^2) of 0.975. In the 298 samples with 0 or 1 CTC in tube 1, 29 (9.7%) had ≥2 CTCs in tube 2; conversely, in the 293 samples with 0 or 1 CTC in tube 2, 24 (8.2%) had ≥2 CTCs in tube 1. Thus, approximately 9% of all samples were discrepant across the analytical lower limit of 2 cells. In Fig. 3*B*, the data from all samples with an average duplicate CTC count of <50 (n = 426) are demonstrated using a Bland-Altman plot. The error of each CTC measurement is

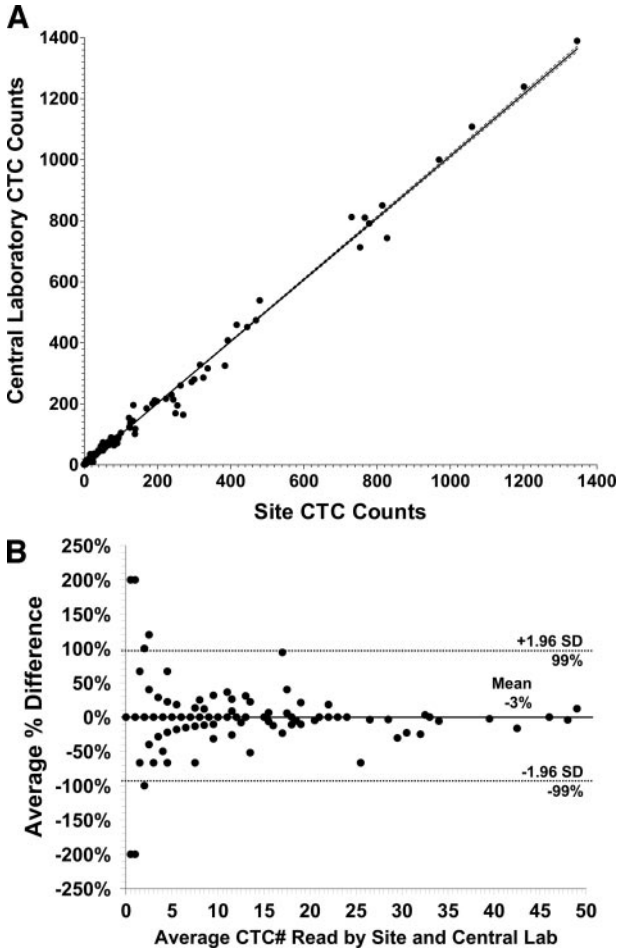

Fig. 2 Comparison of CTC enumeration for 577 samples by different operators. CTC counts obtained by the site operators were correlated with CTC counts derived by operators from the central laboratory. Regression analysis provided the equation $y = 0.49x + 1.01$, with a correlation coefficient R^2 of 0.994. *B* shows the Bland-Altman Plot of these data. The average percentage of difference is defined as (site CTC count − central laboratory CTC count)/average CTC count of site and central laboratory.

represented by the difference in the CTC count between both tubes divided by the average of both CTC counts. The reproducibility of enumeration of low numbers of CTCs from duplicate blood samples was remarkable and approached the theoretical limit of rare cell counting (see supplementary data).

Circulating Tumor Cells in Healthy Subjects and Patients with Nonmalignant Diseases. CTCs were measured in samples from 72 healthy premenopausal women and 73 healthy postmenopausal women. Only eight of these samples (5.5%) had 1 CTC per 7.5 mL of blood, and no samples were found to contain ≥2 CTCs per 7.5 mL of blood (Table 2). Similarly, only 14 of the 199 women (7.5%) with benign breast diseases (including fibrocystic disease, fibroadenoma, ductal hyperplasia, and microcalcifications) or other nonmalignant diseases (including diabetes, arthritis, asthma, thyroid abnormalities, and hypercholesterolemia) had 7.5-mL blood samples containing 1 CTC. One patient with hyperthyroid disorder had a 7.5-mL blood sample that contained 3 CTCs, and none contained ≥5 CTCs per 7.5 mL of blood (Table 2). The mean number of CTCs in healthy women, women with benign breast diseases, and women with other nonmalignant diseases was 0.1 ± 0.2 CTC per 7.5 mL of blood. The mean plus twice the SD for the entire population of healthy subjects was 0.5, and the 95% CI was 0 to 1 CTC, justifying an upper reference limit of 1 CTC per 7.5 mL of blood. Based on these results, we consider detection of ≥2 CTCs per 7.5 mL of blood to be abnormal. Using this upper limit, none of the healthy women and only one woman with a nonmalignant disease were found to have elevated CTCs, giving a specificity of 99.7%.

Circulating Tumor Cells in Patients with Metastatic Carcinomas. CTCs were enumerated in 2,183 blood samples from 964 patients with various metastatic carcinomas. The presence of a nucleus, expression of cytokeratin, cellular morphology, and a lack of CD45 expression were the required characteristics of CTCs. Morphologically, CTCs within and between

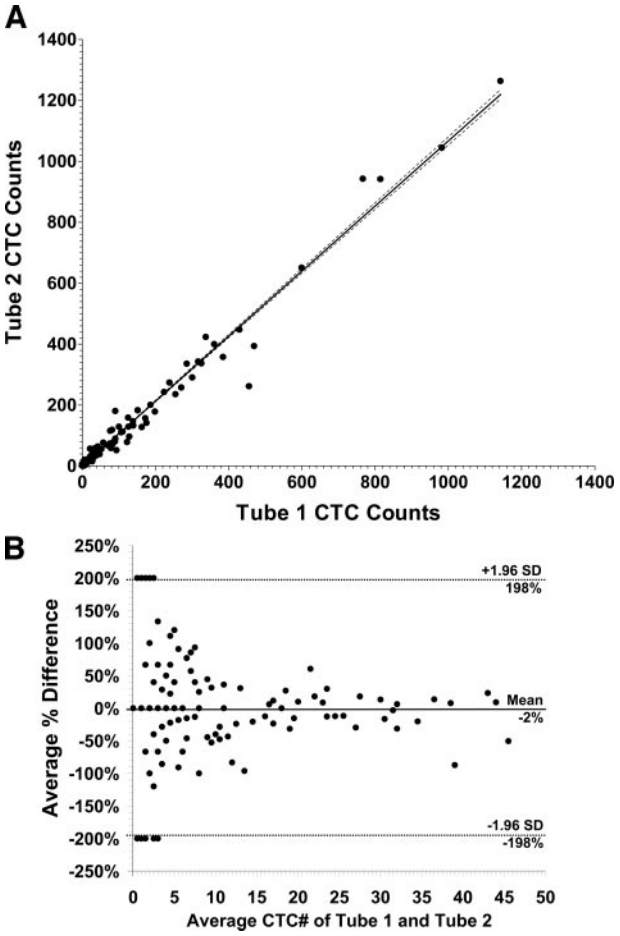

Fig. 3 Reproducibility of CTC preparation and analysis from 477 samples drawn in duplicate from metastatic breast cancer patients. *A* shows the correlation between the CTC counts obtained from both tubes. Regression analysis provided the equation $y = 0.64x + 1.06$, with a correlation coefficient R^2 of 0.975. *B* shows the Bland-Altman Plot of this data. The average percentage of difference is defined as (tube 1 CTC count − tube 2 CTC count)/average CTC count of tube 1 and tube 2.

Table 2 Summary of CTC counts in 7.5 mL of blood from patients with various types of carcinomas

	No. of patients	No. of specimens	Mean ± SD	Median with ≥ 2	No. (%) ≥ 2	No. (%) ≥ 5	No. (%) ≥ 10	No. (%) ≥ 50
Subject								
Healthy	145	145	0.1 ± 0.2	N/A	0 (0)	0 (0)	0 (0)	0 (0)
Nonmalignant	199	199	0.1 ± 0.3	3	1 (1)	0 (0)	0 (0)	0 (0)
Metastatic cancer type								
Prostate cancer	123	188	75 ± 333	13	107 (57)	77 (41)	61 (32)	27 (14)
Unknown cancer	11	27	16 ± 35	13	14 (52)	13 (48)	8 (30)	2 (7)
Ovarian cancer	29	53	6 ± 16	9	20 (38)	12 (23)	9 (17)	1 (2)
Breast cancer	422	1316	84 ± 885	10	489 (37)	340 (26)	256 (19)	129 (10)
Gastric cancer	9	13	24 ± 83	3	4 (31)	1 (8)	1 (8)	1 (8)
Colorectal cancer	196	333	4 ± 11	5	99 (30)	56 (17)	30 (9)	5 (2)
Bladder cancer	7	7	42 ± 107	146	2 (29)	2 (29)	1 (14)	1 (14)
Renal cancer	11	12	1 ± 1	2	3 (25)	0 (0)	0 (0)	0 (0)
Lung cancer	99	168	30 ± 178	9	34 (20)	24 (14)	16 (10)	10 (6)
Pancreatic cancer	16	21	2 ± 6	3.5	4 (19)	1 (5)	1 (5)	0 (0)
Assorted other cancer	41	45	1 ± 4	11	5 (11)	4 (9)	3 (7)	0 (0)
Combined cancers	964	2183	61 ± 696	9	781 (36)	530 (24)	386 (18)	176 (8)

NOTE. The group labeled unknown are patients with metastatic disease with unknown primary tumor; the assorted others are carcinomas with <5 specimens.

Abbreviation: N/A, not applicable.

carcinoma patients demonstrated broad heterogeneity. Fig. 4*A* shows a gallery of CTC images from different patients with characteristic round to oval shape, an intact nucleus, and cytokeratin staining throughout the cytoplasm. Cellular sizes varied over a wide range from <4 μm to >30 μm, even among CTCs from the same patient. Although less frequent, doublets, clusters, and irregular shapes, including elongated cells and multinucleated CTCs, were present (Fig. 4*B*). Other objects that stained positively for cytokeratin and lacked CD45 staining were commonly detected in these samples (Fig. 4*C*). These objects may represent CTCs undergoing apoptosis and fragments subsequent to cellular apoptosis. Nuclear size and nuclear to cytoplasmic ratio varied widely and therefore were not used for cellular identification. Cells were classified as CTCs in controls and patient samples if they showed features consistent with those illustrated in Fig. 4*A* and *B*. Objects with features shown in Fig. 4*C* were not classified as CTCs in this study. In Table 2, the number of blood specimens tested for CTCs from healthy donors, patients with nonmalignant diseases, and patients with metastatic carcinomas of various origins is provided. For some patients, multiple blood draws were analyzed on different occasions. The number and percentage of blood specimens with ≥2, ≥5, ≥10, and ≥50 CTCs in 7.5 mL of blood are provided. The patient groups in Table 2 are sorted according to the frequency of patients with ≥2 CTCs. The median number of CTCs of those patients with ≥2 CTCs is also shown in the table. Fig. 5 is a scatterplot comparing CTC counts from healthy donors and patients with nonmalignant diseases with CTC results from patients with metastatic prostate, breast, lung, ovarian, colorectal, pancreatic, and other cancers.

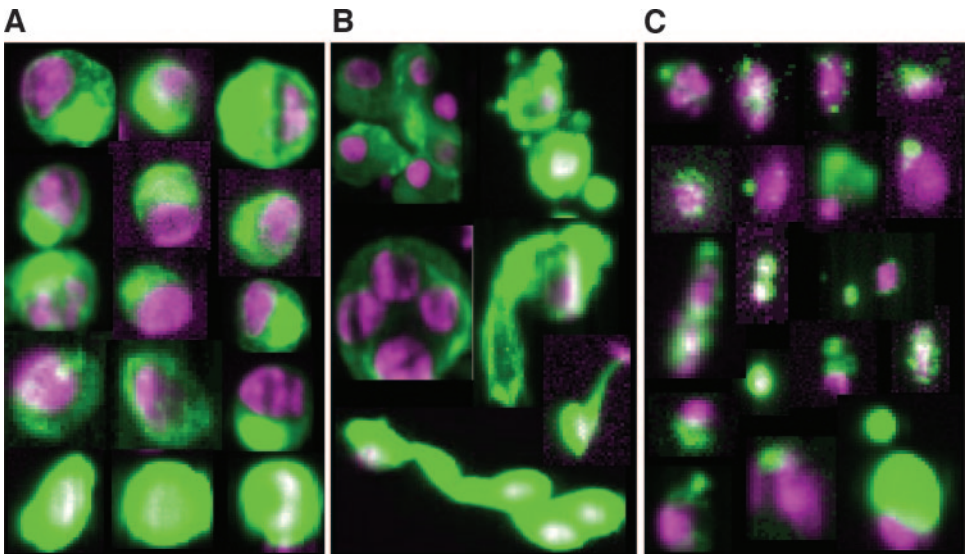

Fig. 4 Gallery of CTC images from the CellSpotter Analyzer obtained from 7.5 mL of blood from cancer patients. *A* shows examples of typical intact CTCs, *B* shows examples of intact CTCs present as clusters or with odd shapes that are present less frequently, and *C* provides examples of CTC fragments and apoptotic CTCs. Images presented in *C* were not included in the CTC counts but are frequently observed in CTC analysis of carcinoma patients.

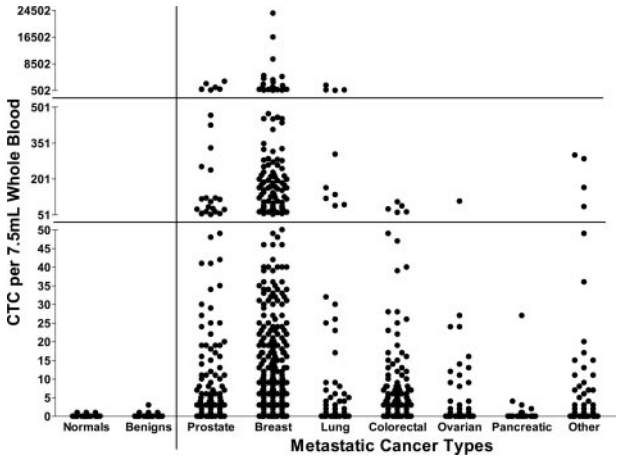

Fig. 5 Prevalence of CTCs in 7.5 mL of blood of 145 women donors, 199 women with nonmalignant diseases, 188 samples from 123 metastatic prostate cancer patients, 1,316 samples from 422 metastatic breast cancer patients, 333 samples from 196 metastatic colorectal cancer patients, 168 samples from 99 metastatic lung cancer patients, 53 samples from 29 metastatic ovarian cancer patients, 21 samples from 16 metastatic pancreatic cancer patients, and 104 samples from 79 patients with other metastatic cancers.

DISCUSSION

The analytical performance of the CellSearch system for the isolation and enumeration of CTCs in 7.5 mL of blood was evaluated. Recovery of cells of the SKBR-3 tumor cell line spiked into 7.5 mL of blood using the CellSearch system was greater than 85% and linear over the range of CTCs routinely detected in the majority of metastatic carcinoma patients. The reproducibility of the assay measured at two cell spike levels provided a total CV of 9.4% at 319 cells and 15.8% at 58 cells.

The heterogeneity of EpCAM expression on the surface of CTCs can cause variation in the ability to recover and/or detect these cells both in cell lines and in patient samples. Variation in the selection efficiency caused by varying EpCAM antigen density on CTCs was reduced by using a method that increases the magnetic load of cells expressing low antigen densities (32). Even with these improvements in the assay, the analytical performance achieved with the CellSearch system using blood samples spiked with tumor cells, as well as the reproducible identification of CTCs across multiple laboratories in patient samples, cannot avoid the sampling error inherent to rare event detection and random distribution (see supplementary data). Even at very low numbers of spiked tumor cells, the precision of CTC enumeration was very close to the predicted minimum based on a Poisson distribution of the data. In addition to limitations imposed by sampling error, the volume of blood that can be sampled is low. We sampled a 7.5-mL volume of blood, which represents ~0.15% of the total blood volume in an average patient. The sensitivity of the CellSearch system is thus limited by both statistical considerations and the blood volume that can be tested. Given the theoretical limitations, the reproducibility of duplicate measurements from the same patient was surprisingly high (Fig. 4).

Criteria used to define what is and what is not considered a tumor cell have been an issue for immunohistochemical techniques, in which reproducibility across laboratories is poor despite attempts to standardize such criteria (33). Here we have demonstrated that the parameters used to define a tumor cell can be reproduced across multiple laboratories (Fig. 3). The myriad of morphologic findings in our survey of peripheral blood samples from patients with various carcinomas is consistent with previously published reports. A high proportion of clustered tumor cells was found in peripheral blood from 32 colon cancer patients using immunomagnetic enrichment and immunocytochemistry with an antibody to cytokeratins 7 and 8 (9). Similar results were reported in peripheral blood samples from 6 of 19 CTC-positive breast cancer patients (10). Whereas we found occasional doublets and clusters, this was not a common finding in our survey. The finding of tumor cell fragments was consistent with other studies that found apparent enucleated cellular fragments in the blood of patients with prostate carcinoma (11, 12, 34). These fragments stained with antibodies to cytokeratin and prostate-specific membrane antigen, but not with the nuclear dye DAPI, and the irregular shapes and sizes reported in their population were similar to our findings.

The number of epithelial cells in the blood of healthy subjects and patients with a variety of nonmalignant diseases was remarkably low, as shown in Table 2 and Fig. 5. Only 1 of 344 samples tested contained >1 CTC. In contrast, the number of CTCs in our survey of metastatic cancer patients ranged from 0 to 23,618. A significant fraction of the specimens tested from these patients with metastatic carcinomas had no detectable CTCs. It is unlikely that treatment is responsible for the lack of detectable CTCs because the breast cancer cohort included 177 metastatic breast cancer patients starting a first or new line of therapy, and in 52 of these patients (29%), no CTCs were detected in 7.5 mL of blood. Intermittent release of cells from the metastatic sites is unlikely because we have shown previously that CTC counts obtained from the same patients at different time points during a 24-hour period yielded similar counts (13). The presence of CTCs and the mean number of CTCs varied widely in samples from different carcinomas. The highest proportion of positive specimens was seen in patients with metastatic prostate cancer, followed by patients with metastatic cancer of unknown origin, ovarian cancer, and breast cancer (Table 2). Differences in vascularization of the tumors, sites of metastasis, and aggressiveness of the tumor are factors that may contribute to these differences. Notably, the median number of CTCs in gastrointestinal carcinomas was relatively low, suggesting that filtration via the portal circulation may be effective in prevention of tumor cell dissemination.

Several studies have suggested a potential clinical utility for CTCs. One recent study measured CTCs in blood from patients before prostate biopsy using reverse transcription-polymerase chain reaction for prostate-specific antigen (PSA) (35). PSA was expressed in blood from 80% of these patients, and 18 of 22 patients (81.8%) with a finding of prostate cancer on biopsy were positive in the assay, whereas 54 of 62 patients (87.1%) with a negative finding on biopsy provided a negative result. In a similar study, Ellis *et al.* (36) found 54% of bone marrow specimens and 26% of peripheral blood specimens were positive for PSA expression before prostatectomy. Detection of PSA decreased at a median of 4 months after prostatectomy to 33% in bone marrow and 9% in

peripheral blood. Taken together, these data suggest that CTCs in blood and possibly in bone marrow may provide a useful tool for early detection of cancer and may provide prognostic utility in the postsurgery setting. Preliminary studies using the CellSearch system in metastatic prostate and breast cancer suggested that the presence of CTCs is associated with shorter overall survival (37, 38). This association has been confirmed for women with metastatic breast cancer in a multicenter, prospective clinical trial (26). In this study, a finding of <5 CTCs in 7.5 mL of peripheral blood before initiation of a new line of therapy and 3 to 4 weeks after initiation of therapy was associated with significantly longer progression-free survival and overall survival as compared with those patients with ≥ 5 CTCs in 7.5 mL of peripheral blood. These data demonstrate that CTC measurement is a useful prognostic tool for stratification of patients into different treatment groups and for monitoring response to therapy in the metastatic setting.

Our data demonstrate that the CellSearch system provides an accurate and reproducible assay that can count CTCs reliably across laboratories, despite low numbers of cells and a wide range of morphologic heterogeneity. The numbers of epithelial cells in blood from subjects without known cancer is very low and almost never exceeds 1 cell per 7.5 mL of blood. In contrast, ≥ 2 CTCs were detected in 7.5 mL of blood in 36% of the specimens from patients with various types of carcinomas. These data suggest that CTC measurement may have clinical utility in all cancers of epithelial origin.

ACKNOWLEDGMENTS

The authors would like to thank G. Dittonno, M. Gianotti, and J. Rowand for processing patient samples. We also thank J. Loebel for procurement of blood samples from healthy subjects and patients with nonmalignant diseases.

REFERENCES

1. Racila E, Euhus D, Weiss AJ, et al. Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci USA 1998;95:4589–94.

2. Gomella LG, Raj GV, Moreno JG. Reverse transcriptase polymerase chain reaction for prostate specific antigen in the management of prostate cancer. J Urol 1997;158:326–37.

3. Ghossein RA, Bhattacharya S, Rosai J. Molecular detection of micrometastases and circulating tumor cells in solid tumors. Clin Cancer Res 1999;5:1950–60.

4. Jiang WG, Martin TA, Mansel RE. Molecular detection of micrometastasis in breast cancer. Crit Rev Oncol Hematol 2002;43:13–31.

5. Gaforio JJ, Serrano MJ, Sanchez-Rovira P, et al. Detection of breast cancer cells in the peripheral blood is positively correlated with estrogen-receptor status and predicts for poor prognosis. Int J Cancer 2003; 107:984–90.

6. Brugger W, Bross K, Glatt M, et al. Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. Blood 1994;83:636–40.

7. Ross AA, Cooper BW, Lazarus HM, et al. Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunohistochemical and clonogenic assay techniques. Blood 1993;82:2605–10.

8. Moss TJ, To LB, Pantel K. Evaluation of grafts for occult tumor cells. J Hematother 1994;3:163–4.

9. Molnar B, Ladanyi A, Tanko L, Sréter L, Tulassay Z. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. Clin Cancer Res 2001;7:4080–5.

10. Witzig TE, Bossy B, Kimlinger T, et al. Detection of circulating cytokeratin-positive cells in the blood of breast cancer patients using immunomagnetic enrichment and digital microscopy. Clin Cancer Res 2002;8:1085–91.

11. Wang Z, Eisenberger MA, Carducci MA, et al. Identification and characterization of circulating prostate carcinoma cells. Cancer (Phila) 2000;88:2787–95.

12. Méhes G, Witt A, Kubista E, Ambros PF. Circulating breast cancer cells are frequently apoptotic. Am J Pathol 2001;159:17–20.

13. Moreno JG, O'Hara SM, Gross S, et al. Changes in circulating carcinoma cells in metastatic prostate cancer patients correlates with disease status. Urology 2001;58:386–92.

14. Soria JC, Gauthier LR, Raymond E, et al. Molecular detection of telomerase positive circulating epithelial cells in metastatic breast cancer patients. Clin Cancer Res 1999;5:971–5.

15. Pierga JY, Bonneton C, Vincent-Salomon A, et al. Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast patients. Clin Cancer Res 2004;10:1392–400.

16. Fehm T, Sagalowsky A, Clifford E, et al. Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. Clin Cancer Res 2002;8:2073–84.

17. Hayes DF, Walker TM, Singh B, et al. Monitoring expression of HER-2 on circulating epithelial cells in patients with advanced breast cancer. Int J Oncol 2002;21:1111–8.

18. O'Hara SM, Moreno JG, Zweitzig DR, et al. Multigene reverse transcription-PCR profiling of circulating tumor cells in hormone-refractory prostate cancer. Clin Chem 2004;50:826–35.

19. Naume B, Borgen E, Beiske K, et al. Immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood. J Hematother 1997;6:103–14.

20. Hardingham JE, Kotasek D, Farmer B, et al. Immunobead-PCR: a technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. Cancer Res 1993; 53:3455–8.

21. Zigeuner RE, Riesenberg R, Pohla H, Hofstetter A, Oberneder R. Immunomagnetic cell enrichment detects more disseminated cancer cells than immunocytochemistry in vitro. J Urol 2000;164:1834–7.

22. Bilkenroth U, Taubert H, Riemann D, et al. Detection and enrichment of disseminated renal carcinoma cells from peripheral blood by immunomagnetic cell separation. Int J Cancer 2001;92:577–82.

23. Kagan M, Howard D, Bendele T, et al. A sample preparation and analysis system for identification of circulating tumor cells. J Clin Ligand Assay 2002;25:104–10.

24. Rao C, Rutner H, Hermann M, Russell T, Terstappen LWMM. Preservation of blood specimens to prevent breakdown of circulating tumor cells. Proc Am Assoc Cancer Res 2002;43:734.

25. Allard WJ, Hayes DF, Repollet MI, et al. A cellular preservative improves the specificity and yield of circulating tumor cells in carcinoma patients [abstract A3482]. Proc Am Soc Clin Oncol 2003;22:866.

26. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.

27. Kagan M, Howard D, Bendele T, et al. Circulating tumor cells as cancer markers, a sample preparation and analysis system. In: Diamandis EP, Fritsche HA, Lilja H, Chan DW, Schwartz M, editors. Tumor markers: physiology, pathobiology, technology and clinical applications. Washington, DC: AACC Press; 2002. p. 495–8.

28. Momburg F, Moldenhauer G, Hammerling GJ, Moller P. Immunohistochemical study of the expression of a M_r 34,000 human epithelium specific surface glycoprotein in normal and malignant tissues. Cancer Res 1987;47:2883–91.

29. National Committee for Clinical Laboratory Standards. Evaluation of precision performance of clinical chemistry devices; Approved guideline. National Committee for Clinical Laboratory Standards document EP-5A; ISBN 1-56238-368X; 1999.

30. Motulsky H. The confidence interval of counted variables. In: Intuitive biostatistics. New York: Oxford University Press; 1995. p. 245–9.

31. Box GEP, Hunter WG, Hunter JS. Significance tests and confidence intervals for means, variances, proportions, and frequencies. In: Bradley RA, Hunter JS, Kendall DG, Watson GS, editors. Statistics for experimenters. New York: John Wiley and Sons; 1978. p. 137–45.

32. Liberti PA, Rao CG, Terstappen LWMM. Optimization of ferrofluids and protocols for the enrichment of breast tumor cells in blood. J Magnetism Magnetic Materials 2001;225:301–7.

33. Borgen E, Naume B, Nesland JM, et al. Standardization of the immunocytochemical detection of cancer cells in BM and blood. I. Establishment of objective criteria for the evaluation of immunostained cells. Cytotherapy 1999;5:377–88.

34. Larson CJ, Moreno JG, Pienta KJ, et al. Apoptosis of circulating tumor cells in prostate cancer patients. Cytometry. In press 2004.

35. Gao CL, Rawal SK, Sun L, et al. Diagnostic potential of prostate-specific antigen expressing epithelial cells in blood of prostate cancer patients. Clin Cancer Res 2003;9:2545–50.

36. Ellis WJ, Pfitzenmaier J, Colli J, et al. Detection and isolation of prostate cancer cells from peripheral blood and bone marrow. Urology 2003;61:277–81.

37. Miller MC, Gross S, Allard WJ, et al. Circulating tumor cells (CTCs) predict survival in patients with metastatic prostate cancer [abstract A5270]. Proc Am Assoc Cancer Res 2004;45:1215.

38. Cristofanilli M, Reuben JM, Fritsche HA, et al. Circulating tumor cells (CTCs): a novel prognostic factor for newly diagnosed metastatic breast cancer (MBC) [abstract A1349]. Proc Am Assoc Cancer Res 2003;44:1349.

Clinical Cancer Research

Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases

W. Jeffrey Allard, Jeri Matera, M. Craig Miller, et al.

Clin Cancer Res 2004;10:6897-6904.