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Standardized quantification of circulating peripheral tumor cells from lung and breast cancer

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Abstract

Detection and quantitation of circulating tumor cells from solid epithelial tumors could become a valuable tool for therapy monitoring if the procedure can be standardized. In the present work we assessed the influence of preanalytical handling, storage and white blood cell isolation on analysis of a population of spiked tumor cell-line cells and intrinsically present epithelial cells in the peripheral blood of breast and lung cancer patients and the sensitivity of their detection. Sucrose density separation did not enrich epithelial cells, and even depleted them, leading to a gross underestimation of their numbers (3/13 positive, between 2.9 and 50 cells/mL) in comparison to red blood cell lysis (13/13 positive, between 77,200 and 800 cells/mL). Short-term storage of whole blood samples for up to 7 days had little influence on the number of epithelial cells recovered. The effectiveness of magnetic bead enrichment was dependent on the number of relevant cells and the volume used for enrichment. Red blood cell lysis and fluorochrome-labeled antibody staining in a no-wash procedure with subsequent laser scanning cytometry allowed the detection of circulating epithelial cells in 92% of breast and lung cancer patients. Two examples of how this method can be applied for the longitudinal analysis in individual patients are shown, with an increase in numbers preceding relapse and a decrease paralleling tumor reduction. The proposed simple and easy method allows close monitoring, which may help in real-time analysis of the response of solid tumors, especially their systemic component, to therapy and hopefully will contribute to more individually tailored therapy.

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Introduction

In patients with solid tumors, metastatic disease is the main reason for cancer mortality. It is caused by the systemic component of the tumor, represented by cells disseminated to the lymph nodes and/or circulating in peripheral blood. Circulating tumor cells are investigated by many researchers using highly sensitive methods to detect minimal residual tumor cells (1–3), such as flow cytometry, immunohistochemistry and polymerase chain reaction (PCR). The frequency of tumor cells among normal blood cells is assumed to be in the range 10^{-3}–10^{-7} (2). These circulating tumor cells may not only be a sign of tumor cell dissemination; increases and decreases in their number may also serve as a marker for timely and close monitoring of response to therapy (4). Of the requirements for analytical methods, handling of the probe is of utmost importance for reliable and reproducible results with respect to applicability, specificity and clinical impact of the results (5, 6). Therefore, in the present report different methods for cell preparation and enrichment of epithelial cells were compared for their efficiency and reproducibility in recovering such cells from peripheral blood samples. Expression of surface or intracellular antigens was used to immunologically discriminate epithelial cells normally not present in peripheral blood and bone marrow from normal hematological tissue (7). Laser scanning cytometry, which combines the speed of flow cytometry with the power to analyze every single positive event for its morphological properties, was used to detect and positively quantify stained events (8). The LSC® (laser scanning cytometer) allows analysis of up to 50,000 cells in half an hour. Using the unique relocation property of the LSC®, it was possible to reliably detect live tumor cells due to exclusive surface staining, to omit dead cells, which show intracellular staining, and to discriminate between unspecific fluorescence events and true cells; relocalization and reanalysis of defined cells will help to further clarify the nature of these cells.

Materials and methods

Blood samples were drawn from 210 tumor patients (150 patients with breast cancer and 60 patients with lung cancer) with informed consent according to the Ethics Committee approval and from 100 normal healthy donors aged between 17 and 72 years.

For spiking experiments, peripheral blood leukocytes and tumor cell-line cells were counted in a Cell Dyn (Abbott 3200; Wiesbaden, Germany). Different numbers of between 10^5 and 100 cell-line cells according to the requirements were each mixed with 1 mL of whole blood containing 6×10^6 leukocytes.

For comparison between Ficoll separation and red blood cell lysis, analysis was performed on blood from normal donors spiked with MCF-7 breast cancer cells and on blood samples from 13 patients with metastatic breast cancer.

For density gradient separation, 1 mL of whole blood was diluted 1:2 with phosphate-buffered saline (PBS), loaded carefully on top of the same volume of Ficoll-Paque PLUS (Amersham, Uppsala, Sweden) and spun for 20 min at $1500\times g$. The mononuclear cells were then isolated from the surface of the Ficoll solution, washed twice in PBS and re-diluted in 1 mL of PBS. The sediment, containing red blood cells and polymorphonuclear cells, was washed twice to remove sucrose using 10 mL of PBS and treated with red blood cell lysis as described below. A parallel sample of 1 mL of the same blood was subjected to red blood cells lysis using 10 mL of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 10 min in the cold. The white cell pellet was then spun down at $700\times g$ and re-diluted in 1 mL of PBS. Aliquots of 10 μL of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human epithelial antibody (HEA, Miltenyi, Bergisch-Gladbach, Germany) and in selected cases 5 μL of phycoerythrin (PE)-conjugated anti-CD45 were added to 100 μL of each cell suspension incubated for 15 min in the dark and readjusted to 1 mL, and 100 μL of this suspension (corresponding to 10 μL of the original blood sample) was used for measurements. A maximum of 6×10^4 leukocytes and a minimum of three epithelial antigen-positive cells could thus be expected per measurement.

For separation over magnetic columns (Miltenyi), samples from normal donors spiked with cell line cells and samples from patients with lung or breast cancer were used. The cells were treated according to the manufacturer's instructions. In short, the white cell pellet from 1–10 mL of blood after red cell lysis was resuspended in 400 μL of PBS and incubated with 30 μL of blocking reagent, 100 μL of HEA microbeads (Miltenyi) and 50 μL of FITC-conjugated mouse anti-human epithelial antibody for 30 min in the cold. The columns, provided by the company, were attached to the magnet, washed according to the manufacturer's instructions and the bead-coated cells were applied to the column. Negative cells were then eluted by rinsing with 5×500 μL of buffer and the columns were removed from the magnet. The cells retained in the columns were flushed out with 500 μL of additional buffer and were then used for measurements. For magnetic bead separation using CellSelect beads (beads coated with an anti-epithelial cell antibody) (Labsoft, Halle, Germany) the white cell pellet was diluted in 500 μL of PBS, 15 μL of magnetic beads and 15 μL of FITC-labeled HEA antibody in a 2-mL Eppendorf tube (Hamburg, Germany). After incubation of the sample with careful overhead mixing for 15 min, the tube was attached to the magnet provided by the company and incubated for another 20 min in the cold, with overhead mixing carried out three or four times. Cells carrying magnetic beads attached to the tube wall; the supernatant, devoid of labeled cells, was then carefully removed without touching the tube walls. The remnant cells containing fluorescence-labeled epithelial cells were diluted in 200 μL of buffer for subsequent measurements.

For measurements, cells from the previous preparations were all applied in the same way to adhesion slides (Menzel Gläser, Braunschweig, Germany). After addition of 100 μL of cell suspension to the slides, live cells adhered to the slide surface after 10–15 min. Measurements were started when the cells had settled and took approximately 20–30 min, depending on cell density. Optimal measurement required a single cell suspension with a space of approximately two to three cell diameters between the cells. Adherent cells were measured using a laser scanning cytometer (LSC®; Compucyte Corporation, Cambridge, MA, USA). The cells could easily and unequivocally be contoured using forward scatter as a threshold parameter at 20× magnification. A defined area covered with 100 μL of cell suspension was used for analysis. Background fluorescence was determined dynamically to calculate both peak and integral fluorescence on a per-cell basis. This unique method corrects for variation in background fluorescence, thus achieving equivalent fluorescent calculation for all cells. FITC-HEA positive cell fluorescence was collected using a 530/30-nm bandpass filter and amplified using a photomultiplier (PMT). In some experiments the cells were subsequently spun down on slides fixed with 0.45% paraformaldehyde and stained with PE-labeled pan anti-cytokeratin-antibody (clone CAM5.2; Becton Dickinson, Heidelberg, Germany). Slides were reanalyzed and red fluorescence was collected using a 625/28 nm-bandpass filter and amplified using a second PMT. The green and red fluorescence overlap was compensated for using the WinCyte™ software (Compucyte Corporation, Cambridge, MA, USA) supplied with the LSC®. Values are displayed as scattergrams and histograms, as well as percentages and mean values of positive and negative cells calculated from the region comprised of single cells only. The LSC® enables the user to relocate cells contained within the positive population for visual examination through the microscope. In addition, a CCD camera attached to the microscope allows photo- and fluoromicrographs to be taken at the same time. This approach combines the power of rapid scanning of a high number of cells (Figure 1A) comparable to flow cytometry with the capability to reliably retrieve events with defined fluorescence intensities (Figure 1A,B). We have shown that this system is able to detect one positive cell among 100,000 negative events (8). Exclusive surface staining was taken as proof of cell viability.

Results

Isolation of the white cell fraction containing epithelial cells from whole blood

To determine the most reliable method for recovery of epithelial cells from peripheral blood, two different quantities of MCF-7 cells, 6000 and 60,000/mL, sufficient to give reliably measurable numbers of cells in a model system, were admixed with whole blood. Red blood cells, which interfere with analysis of white blood cells, were eliminated by two different methods, red blood cell lysis and separation over a sucrose (Ficoll) density gradient, and the recovery of epithelial cells was then compared. After staining for epithelial surface antigen, cell preparations were applied to adhesion slides and analyzed for positive events. A scheme of the procedure and one out of five typical experiments are shown in Figure 2A–C. For Ficoll density separation, between 13% and 60% of the admixed cells could be found in the mononuclear cell fraction of the interphase formed on top of the density gradient. On the other hand, 80–30% of MCF-7 cells sedimented with the red blood cells and polymorpho-

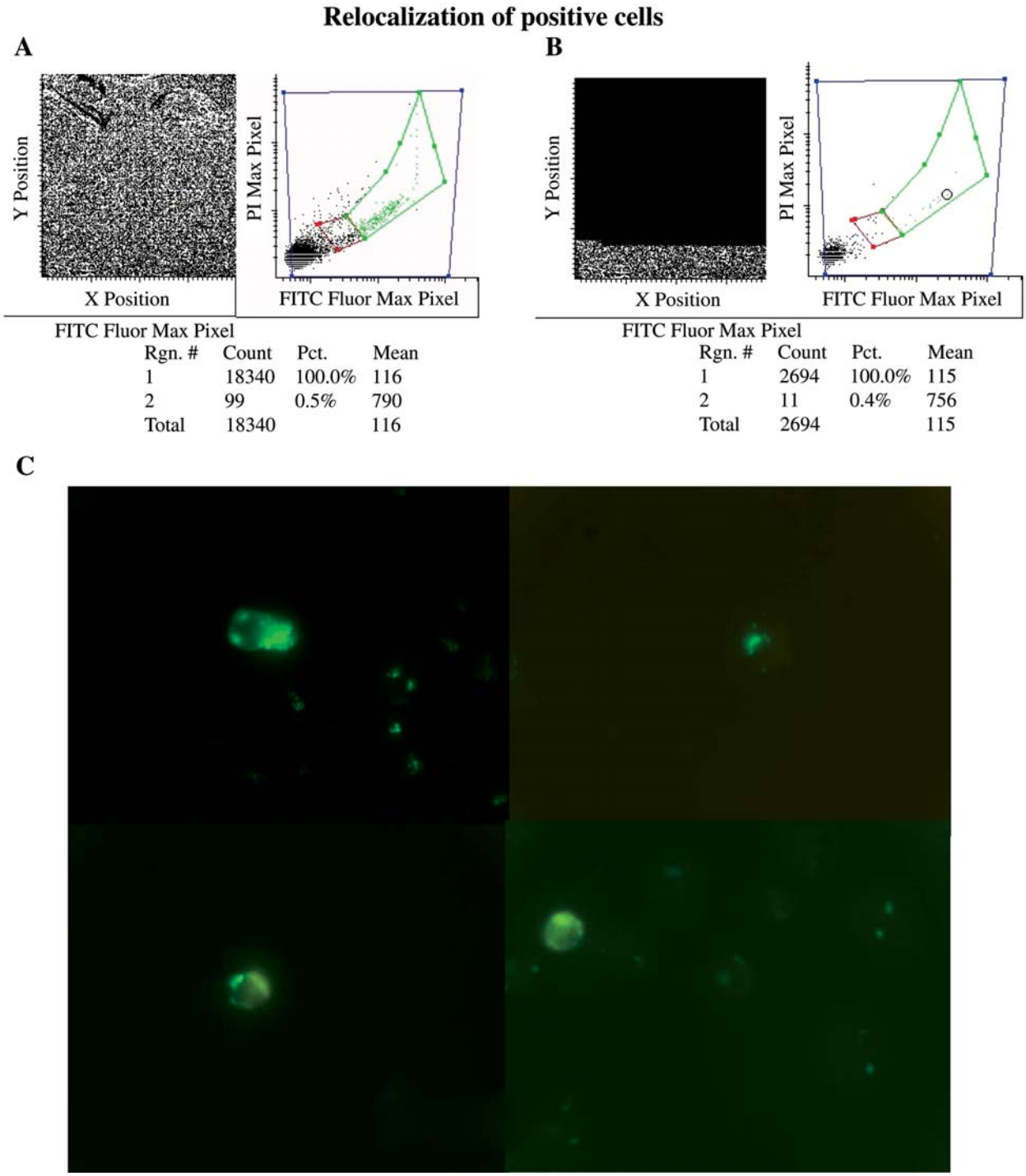


Figure 1 Display of the procedure for laser scanning cytometry. (A) A defined area on a slide is scanned and cells are recognized due to their scatter properties. Each dot on the left side of the plot represents a cell for which the x and y coordinates are stored. The fluorescence intensity of FITC-labeled anti-human epithelial antigen over each cell is measured and positive and background stained cells displayed in a red (y-axis) and green (x-axis) fluorescence dot plot. (B) Cells displaying fluorescence intensities over background can be gated and relocalized. The microscope stops at the x and y positions of the cell with a defined fluorescence intensity (circled cell) and allows the observer to inspect the cell. Only cells with visually detectable fluorescence (in the green window) are counted as positive (cells in the red window have fluorescence intensity above background but are not visible). (C) Examples of typical fluorescence staining, showing that only live cells with surface staining were counted.

nuclear cells and could be retrieved after red blood cell lysis of the sediment. Lysis of the spiked samples revealed between 97% and 100% retrieval of the spiked cells [in the experiment shown, 60 and 596 cells in the initial volume of 10 μL, corresponding to 6000 (100%) and 59,560 (99.3%) per mL] as already shown in a previous paper (8) (Figure 2B). Similar analysis was performed for the detection of epithelial cells in peripheral blood from three patients with metastatic breast cancer. After Ficoll separation, only

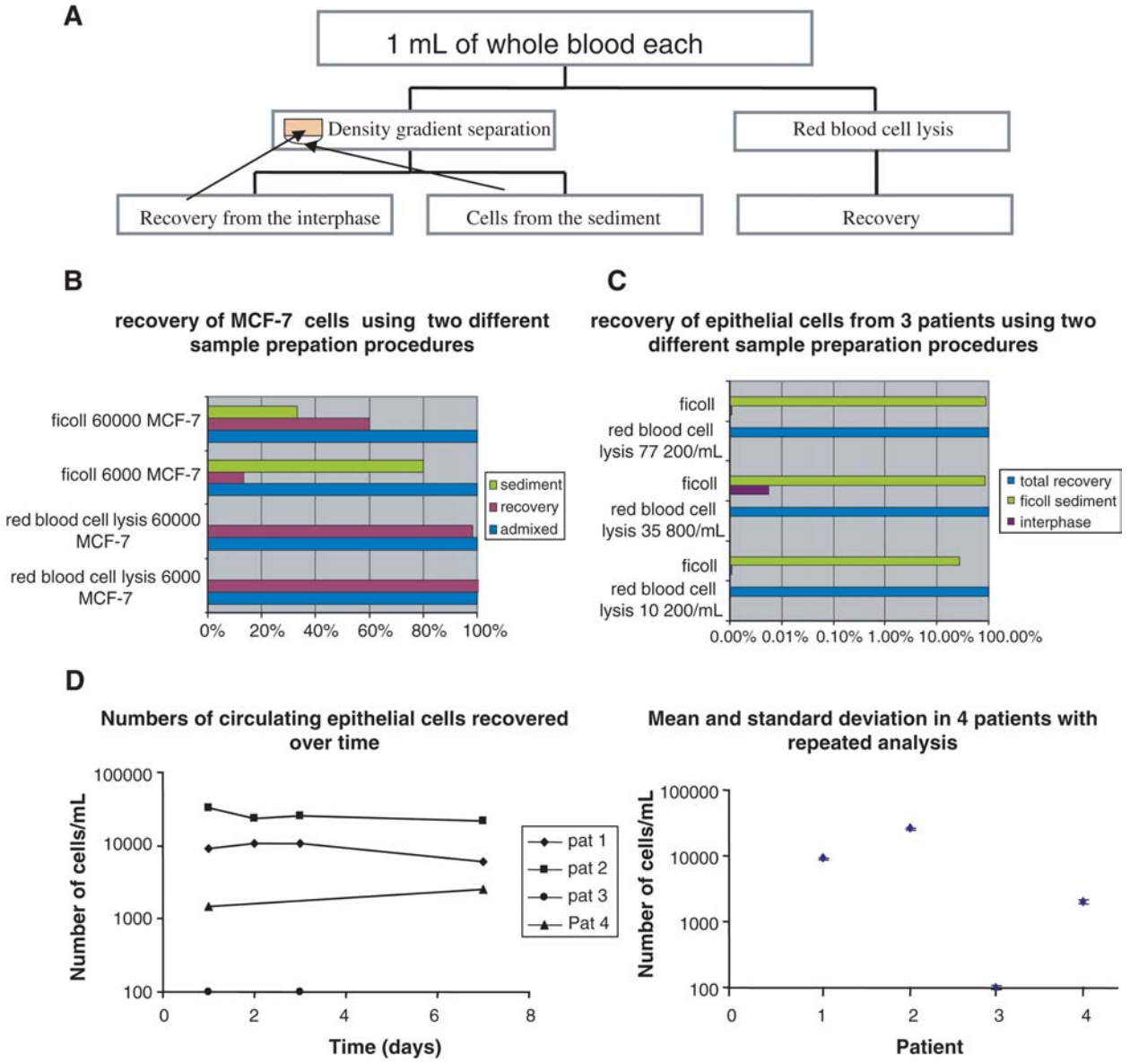


Figure 2 (A) Schema of the schedule for comparison of the two procedures applied for red blood cell elimination. (B) Results from admixture of epithelial breast cancer MCF-7 cells to whole blood and (C) from analysis of epithelial cells from three patients with malignant epithelial tumors (patients 1–3 in Table 1). From two different numbers of MCF-7 cells (6000 and 60,000/1 mL of whole blood) admixed with whole blood, almost all were retrieved using red blood cell lysis, whereas varying numbers were recovered from the interphase of the density gradient. In patient blood, although high numbers of epithelial antigen positive cells were detected after red blood cell lysis, only a minimal number was retrieved in one patient from the interphase, whereas the majority of these cells sediment with the red blood cells and granulocytes (note the different scales in B and C). (D) Variation in recovery after red blood cell lysis of disseminated epithelial antigen-positive cells from blood of four patients, with repeated preparation and measurement of epithelial cells from the same patient samples over a period of up to 7 days (left), and mean and standard deviation (right).

in one out of three patients were epithelial cells migrating with mononuclear cells detectable, and this was only a minor fraction of the cells observed using red blood cell lysis. In contrast, the number of epithelial cells retrieved from the sediment amounted to between 27% and 90% of the cells detected using the lysis method (Figure 2C). A further ten patients with breast cancer were analyzed with both methods. In the 13 patients, the number of epithelial cells detected directly after red blood cell lysis varied between 800 and 77,200/mL (mean 16,645 $\pm$ 20,886) using the lysis method (column 1, Table 1), whereas only in 3/13 cases (23%) were epithelial cells detectable in the mononuclear fraction from 1 mL of whole blood after Ficoll separation, ranging between 2.9 and 50/mL (column 2, Table 1). The remnant cells were detected in the sediment (column 3, Table 1). Thus, only a fraction of cells from an epithelial cell line admixed with whole blood migrated with the mononuclear cells; in patients, obviously, the major proportion of epithelial cells cannot be retrieved in the mononuclear fraction using density gradient separation. Therefore, in all

Table 1 Numbers of circulating epithelial cells detected by red blood cell lysis (direct) and after Ficoll separation in the interphase and among sedimented cells in 13 patients with breast cancer.

Direct positive cells/mL	Ficoll interphase positive cells/mL	Ficoll sediment positive cells/mL
77,200	0	69,480
35,800	28	31,146
10,200	0	2754
4200	0	1733
800	0	107
1800	50	433
9928	0	1534
8560	0	867
9360	0	6453
37,166	0	22,050
4600	2.9	3369
1200	0	886
15,568	0	5252

subsequent analyses we only used red blood cell lysis for the preparation of circulating epithelial tumor cells from peripheral blood.

Reproducibility of epithelial cell recovery over time

The reproducibility of this procedure was tested by repeatedly preparing and measuring epithelial cells from the same patient samples over a period of up to 7 days. Results from four patients with different cell numbers are given in Figure 2D, showing only minor deviations over 7 days.

Efficiency of epithelial cell recovery using magnetic bead enrichment

Without enrichment, laser scanning cytometry allows the reliable detection of one positive cell among 10^5 cells, corresponding to approximately 10 μL of whole blood. Enrichment procedures might increase sensitivity. Two methods of positive selection with antibody-coated ferritin particles and magnetic separation, one using HEA-beads from Miltenyi and separation over magnetic columns and one using CellSelect beads from Labsoft and magnetic attachment of the cells to the vial wall, were assessed. The number of cells retrieved with either the Miltenyi or the Labsoft procedure were compared for the addition of between 10^6 and 1000 MCF-7 cells to 10 mL of whole normal peripheral blood (corresponding to 10^5 and 100 MCF-7 cells per mL). The results are shown in Figure 3A. With increasing numbers of MCF-7 cells admixed with whole blood, the recovery decreased. Of 3000 cells admixed with 10 mL of blood (corresponding to 300 cells/mL or $3/10^5$ white blood cells), 93% were recovered with the Labsoft method; of 1000 cells admixed with 10 mL of blood (corresponding to 100 cells/mL or $1/10^5$ white blood cells) 27% were recovered with the Miltenyi method. These values decreased to 6% with the Labsoft method and to 0.2% with the Miltenyi method for admixture of 10^6 positive cells. There was excellent correlation of $r^2 = 0.99$ and $r^2 = 0.94$, respectively, between the number of admixed cells and the recovery for the two procedures, but with a negative exponential slope.

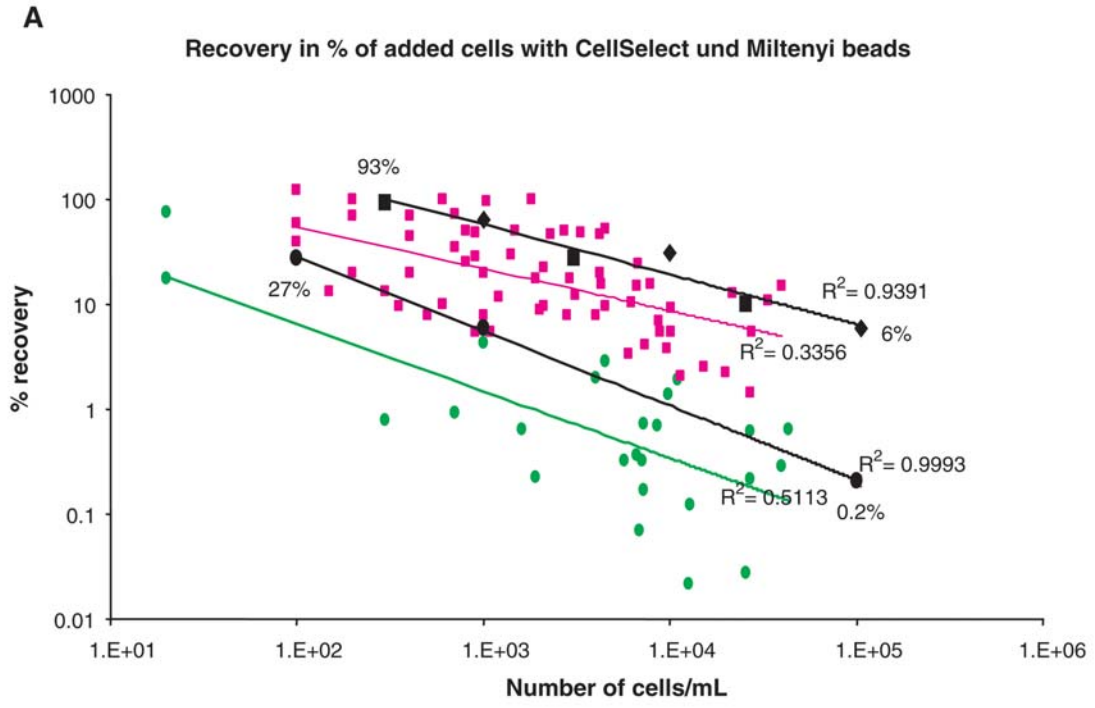
The same was true for patient cells, for which only the directly determined cell numbers from the samples after red blood cell lysis could be compared with the numbers from magnetic separation. Analyzing 66 patients using the Labsoft method and 22 patients using the Miltenyi method (10 patients were analyzed with both methods) (Figure 3A) we found the same phenomenon of increasing loss for higher numbers of directly detected epithelial cells. In both methods there was correlation between the directly analyzed cell numbers and the numbers obtained after magnetic bead enrichment. This was comparable to the result for MCF-7 cells, although shifted to a lower level and with lower correlation coefficients of 0.33 and 0.51 for the Labsoft Miltenyi procedures, respectively. In both methods the cells missing from the number directly determined could almost completely be retrieved from the supernatant in the Labsoft procedure and in the eluate from the columns in the Miltenyi method. Thus, although not bound by the magnet, these cells were not lost.

Influence of blood volume on efficiency of recovery with magnetic beads

The factors responsible for the poor retention of magnetically labeled cells were further analyzed for the Labsoft method. Blood volumes of between 0.1 and 7 mL from the same patients diagnosed with breast cancer were used for separation. Data from 17 patients are shown in Figure 3B,C. Again, if no enrichment procedure was used, one to two positive cells could be reliably detected in up to 10^5 cells or 10 μL of whole blood, respectively, which corresponds to the first data point in Figure 3B and the first line in the list; numbers of positive cells retrieved are calculated as cells/mL for better comparison. In Figure 3C, values from magnetically enriched samples were normalized as a percentage of the directly determined value; the standard deviation is also indicated. In patient 8, only one cell was detectable in the non-enriched sample corresponding to 100 cells/mL of blood. Even if a more reliable cell number of 80 cells were detected in the cell sample enriched from 1 mL of blood, this did not change the final result. In one patient no cells were detectable either with or without enrichment. Recovery of positive cells was lower in the enriched than in the non-enriched samples from all but one patient, and decreased below 20% of the number obtained without magnetic separation when using 1 mL or higher volumes of whole blood for separation (Figure 3C). A comparable extensive investigation was not performed for the Miltenyi separation.

Specificity of staining using the HEA-125 antibody

To determine whether the cells detected by surface antigen staining with the HEA-125 antibody were the same cells as detected by pan-cytokeratin staining, some slides were reanalyzed for cytokeratin staining.



						Number of cells/mL											
Analyzed volume	pat 1	pat 2	pat 3	pat 4	pat 5	pat 6	pat 7	pat 8	pat 9	pat 10	pat 11	pat 12	pat 13	pat 14	pat 15	pat 16	pat 17
0.01	35200	1200	9500	10200	400	1700	11300	100	2000	2300	0	8900	3400	600	2500	700	2000
0.1	26076	1376	5170									6600	3200	700	2500	600	2800
1	18230	1080	1108	1760	180	150	940	80	60	320	0						
7	2308	1045	139.6		6	30	50				0	0	0				

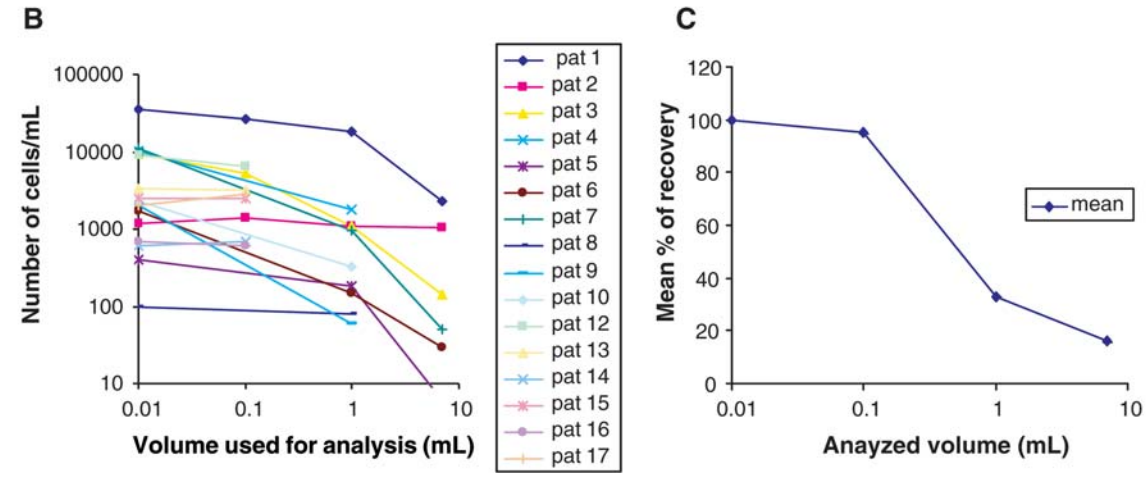


Figure 3 (A) Recovery of epithelial antigen-positive cells from blood using magnetic bead enrichment depends on the initial concentration of these cells in blood: squares and diamonds, enrichment of epithelial cells using CellSelect beads (Labsoft); circles, positive enrichment with HEA beads (Miltenyi). Black squares and diamonds, comparison of the recovery of cell-line cells from two independent dilution series with the original numbers added. Lilac squares: numbers of epithelial cells from 66 patient samples recovered with CellSelect beads as compared to numbers determined by red blood cell lysis. Black circles, comparison of the recovery of cell-line cells with the numbers originally added. Green circles: numbers of epithelial cells from 22 patient samples recovered with HEA beads as compared to numbers determined by red blood cell lysis. Regression lines: least-squares regression. (B) Dependence of the recovery of epithelial-antigen positive cells by magnetic bead enrichment using the Labsoft procedure on the blood volume used for enrichment. Numbers of cells/mL for 17 patients for whom cells were analyzed from the same blood sample (0.01 mL is the non-enriched sample with only red blood cell lysis) using different volumes of blood for enrichment, as indicated. (C) Mean percentage and standard deviation of recovery for different blood volumes used for enrichment.

As shown in Figure 4, all cells stained for both antigens and no cells stained for either antibody alone. These results could be repeated in all patients analyzed so far.

Sensitivity of detection of epithelial cells

Subsequently, 100 consecutive patients with breast or lung cancer in different stages of disease and treat-

ment (Table 2) and 100 normal healthy subjects were analyzed for circulating epithelial cells in their peripheral blood. Epithelial cells were detectable in 92% of cancer patients (Table 2) and no cells were detectable in only eight patients (three newly diagnosed breast cancer patients, one newly diagnosed lung cancer patient, one lung cancer patient undergoing chemoradiotherapy, and two lung and one breast cancer patients in complete remission). There was a highly significant difference between patients with metastasized disease and in complete remission ($p = 0.004$ in breast cancer and $p = 0.005$ in lung cancer). On the other hand, in 97% of normal donors no circulating epithelial cells were detected, whereas in three normal individuals circulating epithelial cells were found. These individuals are under further monitoring.

Influence of therapy on the number of circulating tumor cells

Having shown that it was possible to reliably and reproducibly detect circulating epithelial cells in tumor patients, the approach was used to investigate to what extent these cells reflect the response of solid tumors to a chemotherapeutic regimen. Two typical examples of longitudinal monitoring of circulating epithelial cells are shown, one in a breast cancer patient under neoadjuvant therapy and one in a patient with an adeno-cystoid metastasis of a parotid tumor into the lung under adjuvant chemotherapy. The latter patient, who was free of detectable tumor and metastases at the start of monitoring, was treated with five cycles of chemotherapy. The initially very high numbers of circulating epithelial cells before onset of therapy decreased during three cycles of chemotherapy, but subsequently continuously increased again after therapy was discontinued, preceding clinical relapse by at least 2 months (Figure 5A). Figure 5B shows the results for a patient with breast cancer treated with neoadjuvant chemotherapy to reduce the tumor size. In this patient a considerable tumor mass was present at the beginning of therapy. Circulating tumor cells decreased after the first cycle of chemotherapy but then showed a steep and rapid increase. Since tumor was still present although its size had decreased, this may have been the result of massive dissemination of tumor cells into the circulation due to tumor disintegration. The same phenomenon subsequently occurred repeatedly, with continuous decreases in cell numbers on repeated cycles of chemotherapy. Surgery finally revealed no remnant tumor mass. More patients are now monitored under adjuvant and palliative chemotherapy to study whether the behavior of circulating tumor cells could be an early indicator of therapy response and imminent relapse.

Discussion

Cells from epithelial tissue are not normally found in the circulation, but have been reported to be present in patients with malignant epithelial tumors (9–12), the most frequent of which are lung, breast and colon carcinoma. It has long been assumed that tumor cells are shed into the circulation and are the origin of metastasis formation (13). Therefore, numerous attempts have been made to trace these epithelial cells, assumed to be tumor cells, by flow cytometry (1, 14), immunohistochemistry (15–18), molecular genetics approaches (19–21) and laser scanning cytometry (8) techniques, as well as in combination with enrichment techniques (1, 14, 22–24). Whether such cells already represent micrometastases has been questioned (25) and the prognostic relevance of detection of such cells is still not firmly established (3, 5, 6, 12, 26). Due to the different approaches and the discrepancy of the results, standardized methods are urgently required (27–30).

As a first step in sensitivity testing, cancer cells are usually added to white blood cells at defined ratios (24, 31–34). However, this does not reflect conditions in patient samples. The artificial tumor cells need to be added to whole blood and not to already isolated blood mononuclear cells to allow control of the preanalytical phase, since this phase of sample preparation can be crucial (35–37). We therefore compared red blood cell lysis with density gradient isolation for tracing MCF-7 cells admixed with whole normal peripheral blood and showed that almost 100% of the added cells were retrieved after red blood cell lysis, whereas varying fractions of added cells were found on top of the density gradient together with the mononuclear cells. These results are comparable to the results of Benez et al. (38) who were able to detect 90% of cells recovered from a density gradient sediment. Epithelial antigen-positive cells from blood of breast cancer patients in 10 of 13 cases could not be recovered from the mononuclear fraction, even if a considerable number was detected upon red blood cell lysis. In contrast, between 90% and 25% of the directly detected cells were found among the sedimented cells. Thus, epithelial (tumor) cells in most patients obviously do not have the same properties as mononuclear cells. The low number of epithelial cells detected by density gradient separation corresponds to the low frequency and number detected by other researchers (5, 12, 32), but obviously does not reflect the actual number of epithelial cells present. Sabile et al. (11), using RT-PCR subsequent to the isolation procedure, found density gradient centrifugation superior, but RT-PCR may be impaired by hemoglobin release. The complete numerical recovery of artificially added tumor cells and the viability of the cells retrieved, as shown by exclusive surface staining, demonstrate that the lysis method is superior when cytofluorimetry is used. It is, therefore, highly questionable whether density gradient isolation of circulating epithelial antigen-positive cells is appropriate for quantitative analysis of the systemic component of solid epithelial tumors, since loss of epithelial cells may be high and results from artificially admixed cells may be misleading. Cells recovered from the top of the gradient may be cycling cells, important for early relapse, since their detection obviously correlates with clinical progress (12), but these cells may be only the tip of the iceberg.

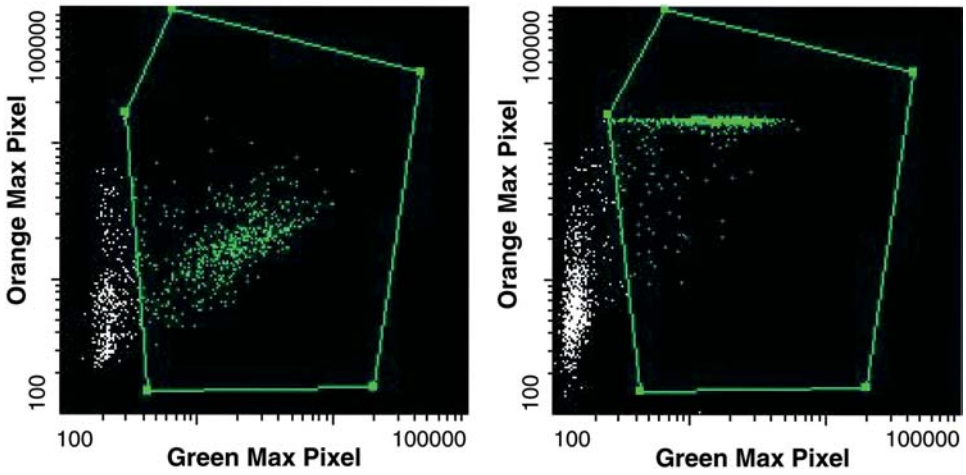


Figure 4 Comparison of the staining pattern for epithelial cells from a patient with an epithelial tumor with FITC-labeled human anti-human epithelial antigen (left) with that of cells co-stained with PE-labeled anti-cytokeratin.

The enrichment capacity of magnetic beads was assessed for artificially and intrinsically present epithelial cells over a range of four decades. With higher cell numbers, an exponential decrease in recovery occurred for the two methods tested. Comparable results have been reported by the group of Uhr (22),

Table 2 Numbers of circulating epithelial cells detected in 100 breast and lung cancer patients in different stages of disease, as indicated.

Breast cancer				
New diagnosis (17 patients)	After surgery (14 patients)	During neoadjuvant therapy (16 patients)	Metastasized (8 patients)	Complete remission (8 patients)
600	4190	3100	5340	3300
9200	3200	53,200	12,600	0
3760	4930	32,000	4000	2242
3300	6600	1800	7600	900
0	3584	27,200	3000	200
20,720	680	10,000	4000	400
600	2100	36,520	12,580	2800
31,200	6000	7200	2400	1336
23,600	23,600	22,200		
3600	2000	6600		
14,200	6000	9000		
800	24,288	53,200		
0	3584	4400		
1800	78,832	43,000		
6400		57,400		
0		6600		
53,000				
Lung cancer				
New diagnosis (14 patients)	After surgery (5 patients)	During chemoradio therapy (7 patients)	Metastasized (3 patients)	Complete remission (8 patients)
7800	20	34,620	8800	300
15,000	3600	0	10,000	0
19,150	7300	3200	9400	4900
300	6028	62,000		0
200	6536	2800		100
1000		30,040		600
19,360		3600		8800
11,592				200
0				
422,928				
2100				
18,312				
6408				
10,400				

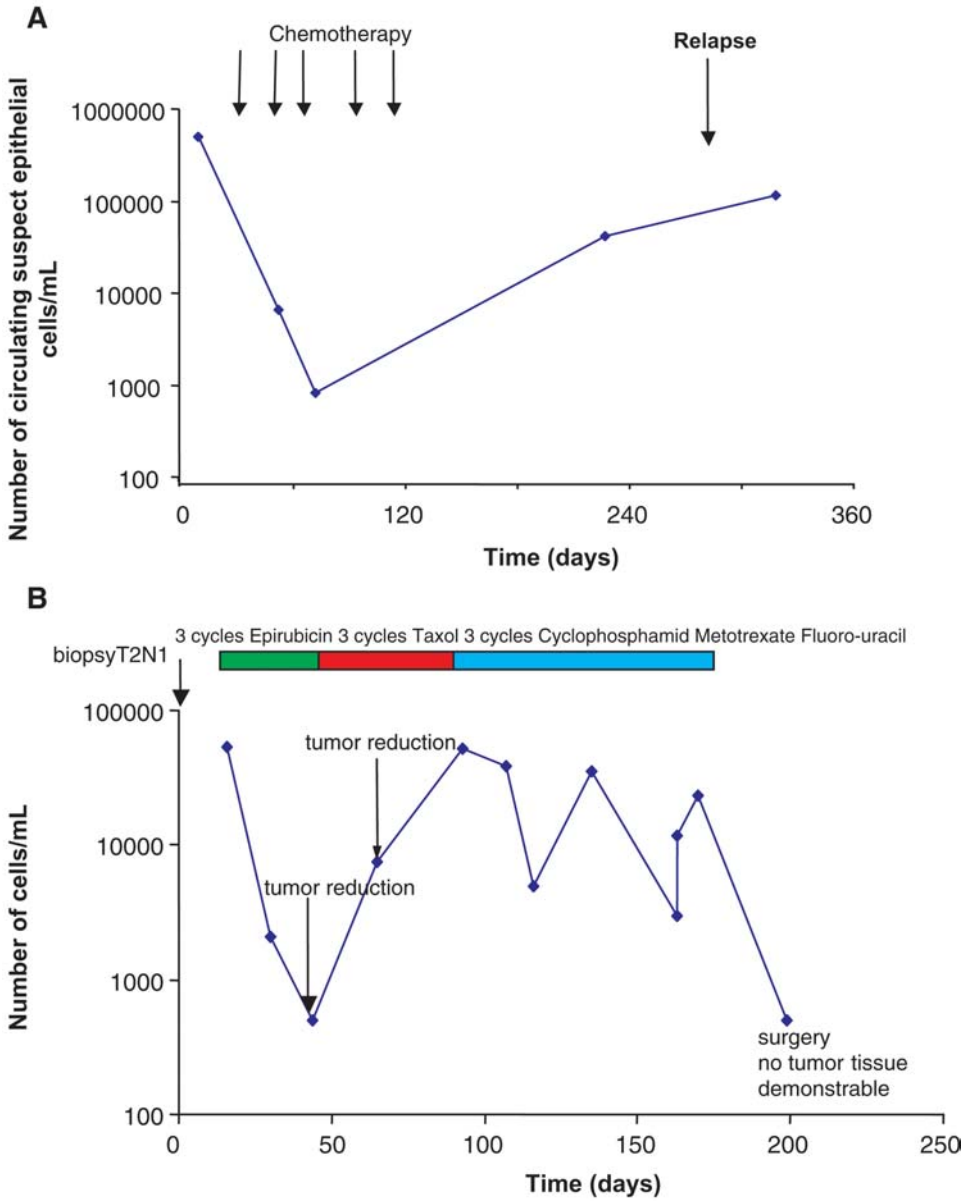


Figure 5 Changes in the number of epithelial cells during the course of disease. (A) Patient with an adeno-cystoid malignancy of the lung. No remnant tumor mass or metastases were detectable at the onset of chemotherapy, whereas a considerable number of epithelial cells were found in the circulation. This number decreased during chemotherapy, but increased again when chemotherapy was discontinued, and this rise in cell numbers preceded manifest relapse by at least 2 months. (B) Behavior of the number of circulating epithelial cells in a patient with breast cancer during neoadjuvant chemotherapy. Numbers of circulating cells decreased during the first course of chemotherapy, but then increased again, probably due to the disintegration of tumor masses, with massive seeding of tumor cells into the circulation. Such cells were eliminated during repeated chemotherapy cycles and no remnant tumor mass was detectable at the time of surgery.

one of the few groups that omitted density gradient separation, although they did not analyze the recovered cell numbers over a wider range. The recovery for magnetic bead separation was, however, not only influenced by the blood load of epithelial cells, but also by the volume used for analysis. Thus, recovery for the same patient sample decreased with increasing blood volume used for analysis. Therefore, in samples with low numbers of epithelial cells, it would not be helpful to increase the blood volume to increase recovery. Surprisingly, the optimal volume of whole blood for complete recovery of magnetically separated cells was as low as 100 μL. The number of cells analyzable in laser scanning cytometry in a timely fashion is restricted to 100,000 cells and corresponds to between 10 and 20 μL of whole blood; this number can be increased to up to 10^6 cells when enriching epithelial cells from 100–200 μL using the Labsoft procedure without loss of information. Comparable methodological evaluation, to the best of our knowledge, has not yet been performed for other enrichment procedures.

Lack of standardization of these procedures may, on one hand, be the reason for the difficulty in comparing results from different laboratories. On the other hand, loss of epithelial cells during the preparation

procedures may differ not only between patients, but also in the same patient even from the same sample (24), and an improved density gradient may not even improve the results (39). In contrast to the variation in the recovered cell numbers in duplicate or triplicate analysis found by others (24) and in attempts to standardize results (40, 41), we found highly reproducible results in the same patients, even over several days. Therefore, we recommend red blood cell lysis for optimal recovery of all white cells in blood, direct staining of surface antigen in a no-wash procedure and magnetic bead enrichment if no epithelial cells are directly detectable using 100 μL of blood for enrichment.

We are now able to perform longitudinal investigations during therapy in patients with solid tumors. Two typical such analyses show that in tumor patients the decrease and reincrease in circulating epithelial cells in response to chemotherapy may correlate with the behavior of the tumor. These responses may also be a marker indicating resistance of a fraction of the cells to chemotherapy and/or increasing growth potential. This will be further investigated by simultaneous cell cycle and apoptosis analysis of these cells. Whether increasing numbers of circulating epithelial cells are due to growing metastases increasingly seeding cells into circulation or to increased growth potential of the circulating cells themselves with a higher metastatic potential needs to be further investigated. Such questions can be approached now using the proposed simple and highly standardized method. Thus, the analysis of circulating tumor cells may be used as an early indicator of therapy response and imminent relapse.

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