Detection and Quantification of Small Numbers of Circulating Tumour Cells in Peripheral Blood Using Laser Scanning Cytometer (LSC®)

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The detection of circulating tumour cells disseminated from solid tumours requires extremely sensitive methods. Molecular genetic methods, which are most sensitive, are not applicable to solid tumours because no tumour-specific genetic markers are available. Detection of disseminated tumour cells by immunocytochemistry is time-consuming, whereas fluorimetry is fast and quantitative. The laser scanning cytometer (LSC®) provides an automated microscopic procedure for screening up to 5×10^4 cells in suitable time. Using this system together with an enrichment procedure which allows up to ten thousand-fold enrichment, we have quantified minimal numbers of tumour cells. In a model system, breast cancer cell line cells diluted into peripheral blood mimicked seeding of tumour cells into the periphery. After staining with fluorochrome-conjugated anti-epithelial antibody, slides were screened for positive events directly or after enrichment with antibody-coated magnetic beads. One positive cell was unequivocally detectable in 10^4 cells and 50 out of 60 tumour cells were reliably recovered from a 20 ml blood volume, equal to 1–2 cells per 10^7, after magnetic bead enrichment.

This method allows quantitation of tumour cells in peripheral blood and bone marrow in reasonable time and will, for the first time, enable extensive investigation of the seeding behaviour of tumours.

Key words: Solid tumours; Minimal residual disease; Laser scanning cytometry.

Abbreviations: FITC, fluorescein isothiocyanate; HEA, mouse anti-human epithelial antibody; LSC®, laser scanning cytometer; PCR, polymerase chain reaction; PBS, phosphate buffered saline; PMT, photomultiplier.

Introduction

In solid tumours, metastatic disease is the main reason for cancer mortality. It is caused by the systemic component represented by cells disseminated to the lymph nodes and/or circulating in peripheral blood. Some of the circulating tumour cells may eventually be trapped in remote compartments where they can re-grow. Such compartments are well known for some tumours, *e.g.* liver and bone marrow in breast cancer and in colon cancer. The frequency of tumour cells among normal bone marrow cells is, at most, in the 10^{-3} to 10^{-7} range. However, a special procedure is required to obtain samples for bone marrow diagnosis which cannot be repeated frequently for follow-up. Access is much easier to peripheral blood in which still fewer tumour cells are detectable. These circulating tumour cells have also become a major concern because they may contaminate the graft in the high dose chemotherapy/autologous stem cell transplant setting (1, 2). Therefore, highly sensitive systems are necessary to detect minimal residual tumour cells (3–5). To date, the most sensitive method is the polymerase chain reaction (PCR). In haematological malignancies, PCR amplification of tumour-associated gene sequences has shown a sensitivity superior to conventional techniques for the detection of minimal numbers of tumour cells. In solid tumours, however, PCR analysis has to be performed on normally expressed tissue-specific gene sequences which are assumed not to be present in blood and bone marrow. This raises a lot of questions with respect to applicability, specificity and clinical impact of the results (6, 7). On the other hand, tissue- and maturation-dependent expression of surface or intracellular antigens can be used to discriminate immunologically the epithelial cells normally not present in peripheral blood and bone marrow from normal haematological tissue (8). The frequency of 10^{-3} to 10^{-8} with which cells from solid tumours can be expected to appear in peripheral blood requires screening of a high number of negative cells in order to find one positive cell. This is extremely cumbersome if performed manually using the microscope. Image analysis may improve the accuracy and speed (9) but still requires several hours. Flow cytometry would be an extremely rapid method allowing screening of tens of thousands of cells in seconds (10), but without pre-selection the sensitivity of flow cytometry is only in the percentage range, and without subsequent sorting one can never be sure whether the rare events detected are actually tumour cells.

With laser scanning cytometry there is now a method available, which combines the speed of flow cytometry with the power to analyse every single positive event for its morphological properties. The laser scanning cytometer (LSC®) allows analysis of up to 50000 cells in 30 min. In order to test the limit of detection, we have prepared an artificial dilution series of tumour cells in normal peripheral blood and determined the number of intact cells carrying surface epithelial antigens. Together with an enrichment step with magnetic beads and the unique relocation property of the

LSC®, it was possible to reliably detect one live tumour cell per 10^7 cells, to discriminate between nonspecific fluorescence events and true cells and to define the nature of such cells.

Materials and Methods

Peripheral blood was mixed in different proportions with tumour cells. Tumour cells were from a breast cancer cell line SK Br2 (kindly provided by Dr. E. Schulze, Miltenyi, Bergisch-Gladbach, Germany). Peripheral blood leukocytes as well as the tumour cells were counted in a Cell Dyn 3200 (Abbott Laboratories, Abbott Park, IL, USA). In the first dilution step, 6×10^5 tumour cells were mixed with 100 µl whole blood containing 6×10^5 leukocytes. The next dilution steps were 6×10^4 tumour cells and 6×10^3 tumour cells each added to 100 µl blood. In the subsequent dilution steps 6×10^3 and 6×10^2 tumour cells were added to 1 ml blood and finally 6×10^2 and 60 tumour cells to 10 ml or 20 ml blood, the latter resulting in a final dilution of five cells per 10^7 normal cells. For the 100 µl samples, 10 µl of antibody were directly added to the whole blood and incubated for 15 min in the dark. Thereafter, red cells were lysed using ammonium chloride lysis method. For the samples with higher volume, staining was performed after the lysis buffer was added to the cells. The pellets containing white blood cells together with the tumour cells were washed once in phosphate buffered saline (PBS) and resuspended in about 500 µl. The cells were then counted, readjusted to $5\times10^{6-7}$ cells per 100 µl and incubated with 10 µl of fluorescein isothiocyanate (FITC) conjugated mouse anti-human epithelial antibody (HEA, Miltenyi, Bergisch-Gladbach, Germany) for 15 min in the dark. The samples were thereafter washed once in PBS.

For separation on magnetic columns (Miltenyi, Bergisch-Gladbach, Germany) the cells were treated according to the manufacturers instructions. In short, after red cell lysis 400 µl of up to 5×10^7 cells of the same dilution series as before were incubated with 100 µl of blocking reagent for 30 min in the cold. The cells were then treated with the appropriate volume (100 µl) of HEA microbeads (Milteny, Bergisch-Gladbach, Germany), incubated for the indicated time and washed in tenfold volume of labelling buffer. The columns, provided by the company, were attached to the magnet, washed according to the instructions and the bead-coated cells applied to the column. Negative cells were then eluted by rinsing with 5×500 µl of buffer and the columns removed from the magnet. The cells retained in the columns were then flushed out with additional buffer, spun down and again stained with the FITC-conjugated antibody as above.

For the measurements the cells were applied to adhesion slides (Menzel Gläser, Omnilabo, Breda, Netherlands). After addition of 100 µl of cell suspension to the slides, live cells became adherent to the slide surface after 10–15 min. Measurements were started when the cells had settled and took about 20–30 min depending on cell density. For optimal measurements it was imperative to have a single-cell suspension with about 2–3 cell diameters space between the cells. The 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 thresholding parameter at twenty-fold magnification. According to the expected percentage of positive cells, between 3000 and 50 000 cells were run. 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 and makes the fluorescent calculation equivalent for all cells. The

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 to the slides and dipped into PBS containing 1 µg/ml propidium iodide. In these cases threshold contouring was based on red nuclear fluorescence. 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® and displayed as scattergrams and histograms, and percentages and mean values of FITC-positive and negative cells were calculated from the region comprised of single cells only. The LSC® enables the user to relocate cells from the positive and the negative region for visual examination with the microscope. In addition, a CCD camera attached to the microscope allows taking photo- and fluoromicrographs at the same time. More detailed morphology could be determined in conventional panoptic staining.

Results

In order to mimic the situation as closely as possible to that of circulating tumour cells in peripheral blood, cell line cells were admixed to whole blood. No additional separation step was performed apart from red blood cell lysis, and washing steps were reduced to the absolute minimum in order to reduce cell loss. The dilution series was repeated independently three times. The tumour cells were distinguished from the remnant blood leukocytes by their property to bind anti-epithelial antibody tagged with the green fluorescent dye FITC. The green fluorescence of positive cells was either displayed as the maximal fluorescence intensity measured per cell or as total fluorescence integrated over each cell. The gate for the negative population was set using normal leukocytes without admixture of tumour cells, and cut-off level for the green fluorescence of the positive population was set excluding all normal blood cells and easily defined as shown in Figure 1. The four low-fluorescing dots detected in the control dot plot in the positive gate were visually identified as nonspecifically fluorescing particles. No positive cells were detectable without admixture of cell line cells. In the 50% (histogram not shown), the 10%, the 1% and the 0.1% admixture of tumour cells, positive cells could be readily detected and correctly determined (Figure 1) in direct measurements. The positive population varied between 48 and 52% in the 50% admixture, between 10 and 14% in the 10% admixture, between 0.8% and 2% in the 1% admixture and between 0.1% and 0.2% in the 0.1% admixture. Direct determination of the proportion of positive cells became difficult at the 0.01% level, being too high in one case with 0.1% and in a second case with 0.03%, but this was due to difficulties in adding a correct number of tumour cells to the normal cells at this dilution. The correlation between expected and observed values in this range is shown in Figure 2, with a high correlation coefficient of 0.98.

At a 10^{-5} dilution, as expected from the fact that only up to 5×10^4 cells were measured per slide, no positive
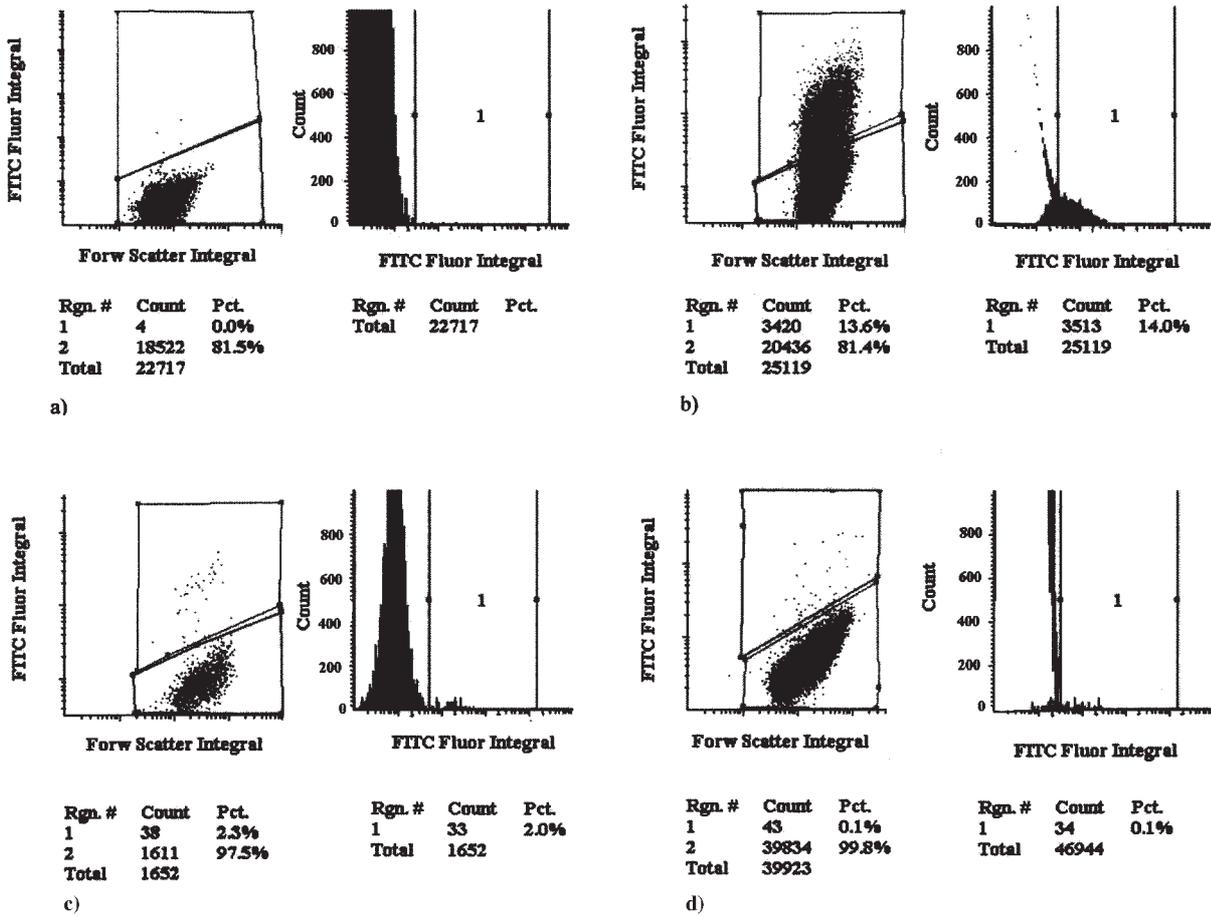
Fig. 1 Scattergrams and histograms of a) a normal blood sample: size (forward scatter) *vs.* FITC-fluorescence intensity of HEA-staining and FITC-HEA frequency distribution histogram; b) a 1:10 dilution of SKBr2 breast cancer cell line cells in normal peripheral blood, c) a 1:100 and d) 1:1000 dilution of tumour cells in peripheral blood.

a)

Rgn. #	Count	Pct.
1	4	0.0%
2	18522	81.5%
Total	22717	

Rgn. #	Count	Pct.
Total	22717	

b)

Rgn. #	Count	Pct.
1	3420	13.6%
2	20436	81.4%
Total	25119	

Rgn. #	Count	Pct.
1	3513	14.0%
Total	25119	

c)

Rgn. #	Count	Pct.
1	38	2.3%
2	1611	97.5%
Total	1652	

Rgn. #	Count	Pct.
1	33	2.0%
Total	1652	

d)

Rgn. #	Count	Pct.
1	43	0.1%
2	39834	99.8%
Total	39923	

Rgn. #	Count	Pct.
1	34	0.1%
Total	46944	

$R^2 = 0.9859$
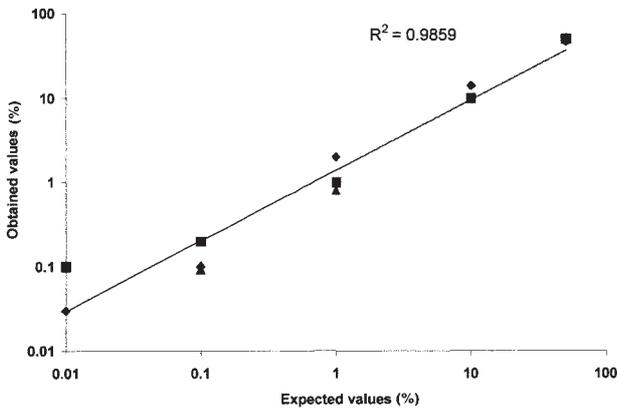
Fig. 2 Correlation between expected and obtained values of positive cells for different dilutions of SKBr2 tumour cells in peripheral blood. Squares, triangles and diamonds: results from three different independent dilution experiments. Coefficient of correlation was 0.98.

Tab. 1 Initial dilution and enrichment of cell line cells added to normal peripheral blood.

Initial concentration (%)	Enrichment	Final concentration (%)
0.000001	18000	0.9
0.00001	12000	1.2
0.0001	8000	8.0
0.01	500	5.0

cells were directly measurable. However, at this and lower dilutions enrichment on the magnetic columns with depletion of normal cells resulted in measurable numbers of positive cells. Enrichment was 500-fold in the 10^{-4} dilution, resulting in 5% positive cells, 8000-fold in the 10^{-5} dilution, resulting in 8% positive cells, 12000-fold in the 10^{-6} dilution, resulting in 1.2% positive cells, and 18000-fold in the 10^{-7} dilution still resulting in 0.9% positive cells (Table 1). Even at this dilution, 50 of the 60 cells added could be retrieved. At these low concentrations visual verification became essential since not all positive events were tumour cells. Figure 3 shows an example how the relocation works. In Figure 3a all particles, contoured in the forward scatter as cells, are shown with their location on the x- and y-axis. In Figure 3b a gate is set over the positive events. For relocation, the microscope walks along the events in the positive fluorescence intensity gate. It stops at each positive event (example shown in Figure 3c), allowing
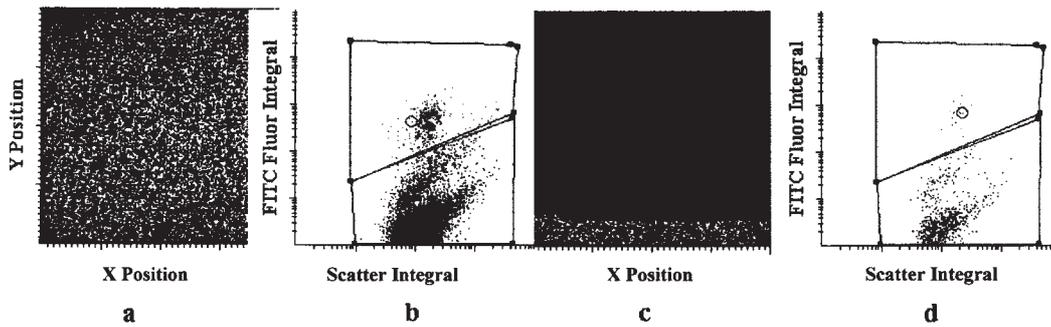
Fig. 3   Relocalisation of cells. a) The first dot plot shows all cells with their localisation on the x- and y-axis. b) A gate is set over the positive events. c) The scanning will then proceed from the x, y position from d) one event in the positive gate to the next (circled the cell under consideration) allowing the observer to visualise every event in the transmitted light and incident fluorescence.
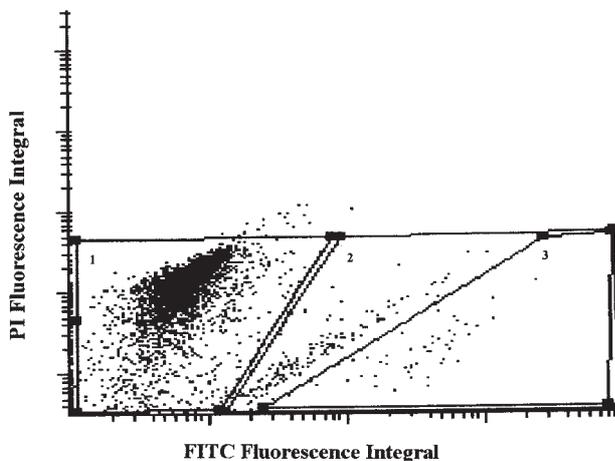
Fig. 4   Example of a sample in red *vs.* green fluorescence display. Events in gate 1 are negative cells. Events in gate 2 were identified as nonspecifically fluorescing particles. Events in gate 3 are positive cells, most of them tumour cells.

the investigator to look at the cell, which is circled in the fluorescence intensity gate (Figure 3d), in incident fluorescent light. Relocation revealed four different situations. 1. Particles fluorescing nonspecifically in the red and the green channel (Figure 4); they could be excluded using compensated red *vs.* green fluorescence intensity (gate 2 in Figure 4). Live tumour cells were restricted to the region of high green fluorescence (gate 3 in Figure 4). 2. Some of these positive cells were, however, enriched skin epithelial cells. They express human epithelial antigen like the tumour cells but were easily visually distinguishable due to their pyknotic nuclei, their wide cytoplasm and their intracellular staining (Figure 5a). 3. True tumour cells were identified by their typical ring or cap-formed staining and their high nuclei to cytoplasm ratio (Figures 5b and c). 4. Visually excluded were also fragmented or dead cells with cytoplasmic staining or particles without an intact nucleus. Morphological restaining is shown in Figure 6. An aggregate of four tumour cells is shown, each with different staining pattern: one cell appearing evenly stained which may be a dying cell, one cell with a cap and two showing only dots of staining. Panoptically, the dying cell shows cytoplasmic effusions. Additionally to the four fluorescent cells, a fifth cell appears, a polymorph nucleated granulocyte which does not fluoresce at all. Thus only relocalisation of positive events and subsequent morphological analysis allowed to determine the true live tumour cells.

Several control studies in breast cancer patients using this method are now under way.

Discussion

In solid tumours, surgical resection of the tumour mass, including unaffected margins, hopefully removes most of the tumour load from the patient. Still, dependent on the malignant cells and their stage of maturation, frequently this does not lead to complete eradication of the tumour. This is due to a systemic component, tumour cells circulating in peripheral blood which may represent the metastatic ability of the tumour. Frequently tumour cells are also present in the apheresis product collected for autologous stem cell transplantation (1). Whether there is a correlation between the detection of tumour cells and the time to relapse is still a matter of debate (8, 10, 12, 13). Since the frequency of circulating tumour cells is low, a sensitive and quantitative detection system is required. PCR is assumed to be the most sensitive method, but it needs a genetic marker, is difficult to quantify, and, with respect to epithelial cells, it is charged with a high rate of false positives (11). Specificity is high if the amplified sequence is a typical mutation carried only by the tumour cells, but this is the case only for a minority of tumour types, primarily haematological malignancies (14). Amplifying mRNA specific for the tissue the tumour is originating from, is a method preferentially applied to the detection of occult disease in solid tumours (15). Such an approach is hampered by the fact that these mRNAs are also present in normal tissue and the presence of normal cells may impair specificity as well as sensitivity (16). In addition, illegitimate transcription
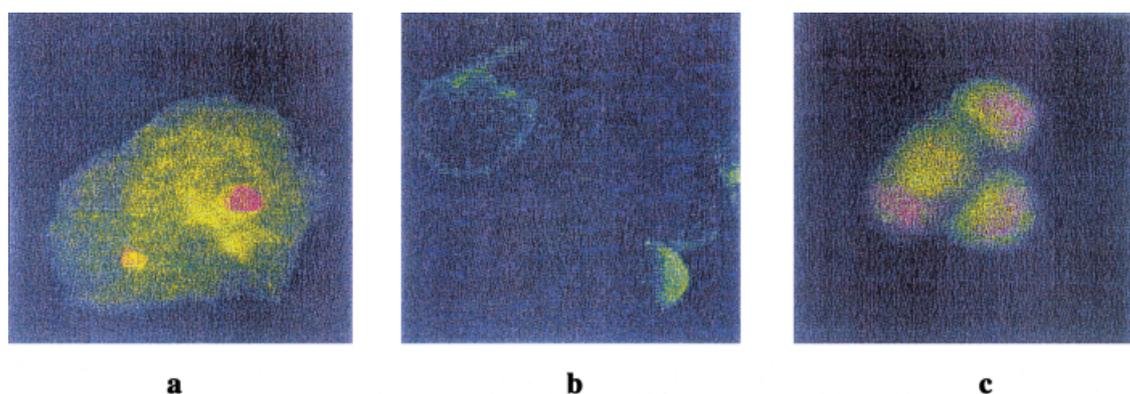
Fig. 5 Micro-fluorographs of a) typical skin epidermal cells of flat appearance with a small condensed nucleus and even HEA-staining, b) tumour cells stained with FITC-HEA showing rim-like fluorescence without propidium iodide staining, and c) a large nucleus with propidium iodide staining.
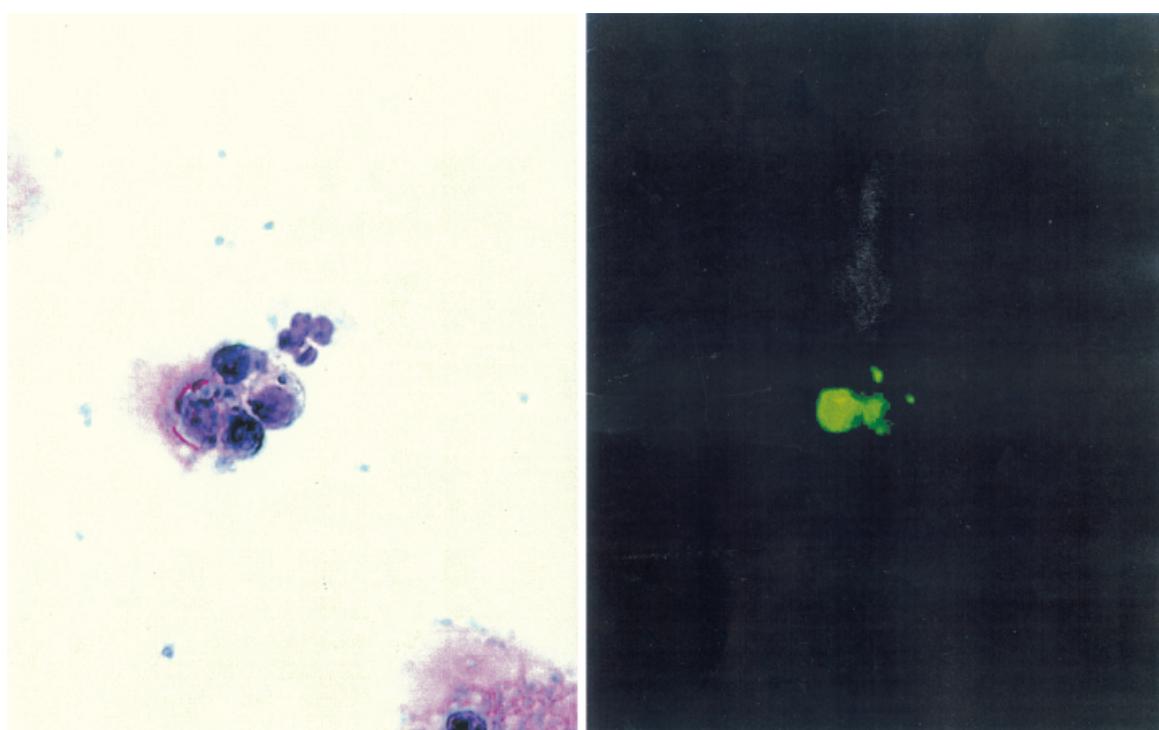
Fig. 6 Relocalisation of an aggregate of four tumour cells showing different characteristic forms of fluorescence staining: one with even, one with formed cap and two with dotted staining. The same cells in panoptic staining reveal one staining cell with cytoplasmic effusion which may be a dying cell and an additional granulocyte which is negative in fluorescence staining.

of such genes in tissues, where they are usually silent, has to be considered as a source of false-positive results (11).

Immunocytological analysis using either immunocytochemistry or immunofluorescence is one of the next most sensitive methods. The specificity and the sensitivity of detection of cancer cells is, however, prone to the pitfalls of variable antigen expression in tumour cells (17) and the technical problems of immunochemical staining (18). More importantly, evaluation of immunocytochemistry is subjective and very time-consuming, and therefore not amenable to routine diagnosis.

High numbers of cells can be analysed in reasonable time by flow cytometry (10, 19). This has been facilitated by immunomagnetic enrichment of cells positive for epithelial surface antigens (20), *e.g.* in epithelial tumours. However, flow cytometry measures fluorescent events, the true-positive cells being frequently confounded by admixture of nonspecific fluorescing events which may mimic cells. As long as the positive events cannot be controlled visually, it is difficult to separate true tumour cells from nonspecific events.

Therefore, we have combined the new technique of laser scanning cytometry, a method which enables automated relocalisation of events with defined fluores-

cence intensity and visual identification, with immuno-magnetic bead enrichment in a model system for the detection of very rare tumour cells. Tumour cells were added to whole blood and not, as in other model systems (3, 9), to the already isolated white blood cells. Thus, loss of tumour cells due to isolation and washing procedures could be taken into account. We chose not to perform any other separation step than red cell lysis and reduce washing steps to a minimum. The anti-epithelial antibody could be added either directly to the cell pellet or the cells were reacted with anti-epithelial microbeads separated over magnetic columns and then stained with the antibody. Realistic numbers of cells to be measured per slide were in the 5×10^4 range. Thus it was possible to detect one tumour cell in 10^4 normal leukocytes without previous enrichment. For this and the subsequent dilutions it turned out to be more time-saving to perform an enrichment step than to measure higher numbers of cells. We could show that even out of 60 cells diluted in 20 ml blood, 50 cells could reliably be retrieved. Thus, at these low concentrations the recovery from the magnetic columns was still 80%, although the purity was only 0.9%. We observed, however, that using enrichment steps, not only tumour cells were enriched but also normal skin epidermal cells were now detectable, as well as a number of nonspecific particles. Thus, out of the positive events only half to one third were true cells. Some of the nonspecific events could already be separated from the tumour cells by their property of emitting fluorescence in the red and in the green emission range, but skin epithelial cells, reacting specifically with the antibody, could only be distinguished by morphological analysis. In order to obtain a true value of the present live tumour cells, all positive events were relocated individually and reanalysed. Only cells with surface staining were included in the analysis. Dead cells with intracellular nuclear or cytoplasmic staining were excluded by morphology. Fluorescent particles ingested by macrophages or nonspecific fluorescence of eosinophilic grains in granulocytes thus could also be excluded. Nonspecific staining of other haematological cells was not observed when staining live cells even after immunomagnetic enrichment. May Grünwald-Giemsa staining for morphology also allowed exclusion of such cells.

Using this combination of methods, we were thus able to standardise the analysis of rare tumour cells with respect to the analysed volume, staining specificity and cell morphology and to exclude all troubles which hitherto have been responsible for the inconsistency of results. The procedure is fast and robust, yielding results the same day the samples are received, and it allows quantitative determination of the number of tumour cells present in the circulation.

For the determination of minimal residual disease in solid tumours, immunologic analysis may be preferable to PCR analysis (6, 7, 11, 15), not only because the amplified tissue-specific gene sequences can have been erroneously induced (21), but also because quantification is difficult. Immunological evaluation of rare cells, on the other hand, should only be performed in conjunction with morphological analysis, which is easily possible in LSC® in contrast to flow cytometry (22). It is known, that tumour cells are heterogeneous with respect to antigen expression and metastatic cells can loose their original antigenicity (23). Therefore it may be necessary to use a cocktail of antibodies for the detection of tumour cells (24). Using this approach, we have now started analysing patient samples. We were able to detect circulating epithelial cells in 80% of breast cancer patients at different stages of disease but so far none in 50 healthy normal donors. Additionally, we have started to combine the analysis with methods such as FISH (15) and *in situ* PCR (25) for *e.g. Her2/neu* and adhesion markers in individual cells to further distinguish tumour cells with metastatic properties. This will, hopefully, help to clarify the extent to which circulating and contaminating tumour cells are responsible for relapse. In addition, our approach is quantitative. This allows monitoring of the number of circulating tumour cells in response to adjuvant therapy.

To further analyse the stage of dormancy in the same cells, we have started combining proliferation markers with immunofluorescence using the relocation property of the LSC. Longitudinal studies are now under way to solve the question how circulating tumour cells behave under different conditions such as adjuvant chemotherapy or peripheral stem cell mobilisation (26).

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