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CLINICAL STUDY

Advanced detection and measurement of cells on membrane from peripheral blood by laser scanning cytometry (LSC) in early stage breast cancer patients

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Abstract: *Background:* The aim of our study was the potential detection of circulating tumour cells (CTCs) in early stage breast cancer patients. Our approach was cell microfiltration through polycarbonate membrane as a concentration method suitable for CTC selection in peripheral blood. The isolated cells on membrane were further analysed by laser scanning cytometry.
Methods: Sixteen patients were enrolled in the study, of which 13 had early stage breast carcinoma and 3 patients had metastatic breast carcinoma. The analyses were performed from 9 ml of peripheral blood, in one patient blood was drawn twice. Blood samples were taken after adjuvant chemotherapy but prior to adjuvant radiotherapy. The control group consisted of 12 clinically healthy subjects.
Conclusions: In the control group 3 subjects out of 12 had 1 CTC, the mean CTC numbers being 0.25±0.45. In the early stage breast cancer patients 0–36 CTCs were detected (mean 13.9±12.9 CTCs. 10 patients out of 13 had more than 2 CTCs (62 %). The detection and measurement of cells on membrane is a simple and reproducible method of detection of CTCs in peripheral blood. Sensitivity of the method is 88.5 %. Detection of CTCs seems to be a promising method for the monitoring of adjuvant therapy in early stage breast cancer patients and for the identification of high risk patients in whom elevated numbers of CTCs are persisting following the termination of adjuvant therapy *(Tab. 1, Fig. 4, Ref. 35)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: breast cancer, circulating tumour cells, laser scanning cytometry, cell filtration.

Despite the advances in the therapy of early stage breast carcinomas, recurrent disease within 5 years occurs in 30 % of node negative, and in up to 60 % of node positive patients (1, 2). In case of positive lymph nodes about 40 % of patients may survive for 10 years or more (3, 4). The prognostic factors currently used in routine clinical practice do not discriminate between low and high risk subgroups of node positive and node negative breast cancer patients.

Recent findings suggest the independent haematogenic and lymphogenic route of dissemination of tumour cells (5). Tumour cells are released into the circulation in early stages of the disease (6, 7), although the characteristics of these circulating cells still remain unclear. Fehm et al (8) showed abnormal cytogenetic profile in circulating epithelial cells from peripheral blood (PB) in 25 of 31 patients.

To date no clear correlation has been shown between CTCs from PB and occult tumour cells (OTC) in bone marrow. Brugger et al (9) performed kinetic analysis of blood samples and showed two patterns of CTCs recruitment depending on the presence or absence of bone marrow micrometastases. Some papers showed correlations between CTCs and OTC, but predictive value of CTCs were low (10). However, the evidence of OTC in bone marrow is an independent prognostic factor in patients with carcinomas of breast (11, 12), lungs (13), and colon and rectum (14). O'Sullivan et al (15) demonstrated an increased metastatic potential of OTCs in patients with proven micrometastases in the bone marrow postoperationally, but not preoperationally. The detection of tumour cells in peripheral blood, in contrast to bone marrow punctions, is an elegant and easily reproducible method. The main drawback of measuring circulating tumour cells in peripheral blood (CTCs) is their low concentration, i.e., less than 1 CTC/ml of blood.

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To reach maximum sensitivity, a number of concentration methods have been developed which employ either immunomagnetic separation, density gradient centrifugation or microfiltration. Presently the former approach is the most widely used technique represented by the MACS system (Miltenyi Biotec Ltd., Bisley, UK) (16). Positive (anti-EpCAM) or negative (anti-CD45) CTCs selection methods may be used. Disadvantage of positive selection is variable expression of EpCAM on the tumour cell surface and disadvantage of negative selection is binding of CTCs on T cells and loss for further analysis. An advantage of immunomagnetic separation is its universal usability for further work with CTCs. This method can be used in combination with RT-PCR (17), LSC (18), or digital microscopy (19). These methods are capable of detecting one CTC per 5 ml of blood. At present the most widely used system (CellSearch, Immunicon Corporation, PA) combines immunomagnetic enrichment of the sample with digital microscopy (20).

The second most used concentration system is density gradient centrifugation. The main representant using for detection of CTCs in PB is OncoQuick assay (Greiner BioOne, Frickenhausen, Germany). This method is less accurate and sensitive than immunomagnetic separation (21).

A less commonly used concentration technique is microfiltration developed by Vona et al (22). Microfiltration employs a polycarbonate membrane with a pore size enabling the leukocytes and erythrocytes passing the membrane but trapping the CTCs (due to their size) on the membrane. The most suitable method for visualization of CTCs is LSC. LSC is used for morphological and cytometric analysis of the cells from the beginning of 90th (23). The use of LSC for analysing cells on membrane made it possible to simplify the preparation of CTCs and to use cytometric analysis of isolated cells (24).

In this report we describe an improvement in the measurement and standardisation of a technique suitable for clinical use in patients with breast cancer. We present our first preliminary results in patients with early stage breast carcinomas.

Methods

Patients: Clinical data of the patients are shown in Table 1.

Sample collection: Following informed consent, peripheral blood was drawn from patients with stage I and stage II breast carcinoma. All patients were post-operation and post-chemotherapy but before the start of adjuvant radiotherapy. Clinical data of the patients were obtained from their medical histories. Nine ml of blood was drawn from the antecubital vein into Vacutainer Na-citricum tubes (Becton Dickinson, San José, CA). Blood samples were also drawn from healthy volunteers which were used as a negative control. The samples were processed within 24 hours post-withdrawal.

Cell line: Human ovarian carcinoma cell line SKOV-3 was cultured in RPMI 1640 culture medium supplemented with 10 % fetal bovine serum as a monolayer cell culture in a humidified atmosphere of 5 % CO_2. For assays, both floating and adherent cells were collected. The floating cells were collected by centrifugation at 700 *g* for 3 min, whereas adherent cells were trypsinised prior to centrifugation. The cells were pooled and washed twice with cold PBS.

Tumour markers: CA 15-3 (Kryptor CA 15-3 Brahms) and TPS (Biotech AB, Sweden) were examined in patient sera following standard protocols according to the manufacturer's instructions. Thirty IU/ml CA 15-3, 80 IU/ml TPS were considered the cut-off values. CEA was examined using CEA-IRMA kits (Immunotech, Czech Republic). The cut-off value of CEA was set to 5 ng/ml.

Tab. 1. Clinical data of the 16 patients examined for circulating tumour cells.

Patient	Age	Histology	Grading	Staging	Surgery	Chemotherapy	ER (%)	PR (%)	HER-2	CEA (ng/ml)	CA15-3 (IU/ml)	TPS (IU/ml)	CTCs	Clusters
1	57	IDC	III	T1N1M0	QE+EA	4xFAC	0	0	2+	6.6	19.7	43	2	0
2	36	IDC	III	T1N0M0	QE+EA	4xFEC	0	60		0.4	14	57	12	2
3	81	IDC	III	T2N1M0	ME+EA	TMX	0	25	3+	1.4	16.1	78	11	0
4	44	IDC	II	T4N2M1	ME+EA	(*)	50	50	2+	0.9	17.3	136	0	0
5	50	IDC	III	T2N2M0R1	ME+EA	6xAC	0	10	NA	1.4	14.4	101	16	3
6	74	IDC	II	T1N1M0	QE+EA	TMX	75	25	1+	1.3	17.7	130	25	0
7	54	MEC	NA	T2N1M0	ME+EA	4xFEC	NA	NA	NA	0.7	18.2	37	11	3
8	54	IDC	II	T1N1M0	QE+EA	4xFAC	5	20	NA	0.8	17.9	35	26	4
9	74	IDC	I	T1N0M0	QE+EA	TMX	80	0	NA	1.6	17.4	20	31	1
10	73	IDC	II	T1N0M0	QE+EA	TMX	75	75	0	0.8	17.1	17	36	1
11	46	IDC	II	T1N0M0	QE+EA	4xCMF	0	10	1+	0.4	25.7	34	24	1
12	54	IDC	I	T1N0M0	LE	TMX	50	75	1+	1.2	18.2	226	0	0
13 a	46	IDC	II	T1N0M1	QE+EA	RT	75	75	1+	1.7	85.6	58	52	3
13 b	46	IDC	II	T1N0M1	QE+EA	3xFAC	75	75	1+	1	38.1	83	15	1
14	32	IDC	III	T1N0M0	QE+EA	4xTXT+EPI	0	0	3+	0.1	10	29	0	0
15	63	IDC	NA	T1N0M0	LE	WT	NA	NA	NA	NA	NA	NA	1	0
16	33	IDC	NA	T1N0M0	QE+EA	NA	NA	NA	NA	NA	NA	NA	2	0

IDC – intraductal carcinoma, MEC – medullar carcinoma, QE – quadrantectomia, EA – exenteration of axilla, LE – lumpectomia, FAC – 5-fluorouracil+adriamycin+cyclophosphamide, FEC – 5-fluorouracil+epirubicine+cyclophosphamid, TMX – tamoxifen, AC – adriamycin+cyclophosphamide, CMF – cyclophosphamide+methotrexate+5-fluorouracil, TXT+EPI – taxotere+epirubicin, ER,PR – estrogen and progesteron receptors, CTCs – circulating tumour cells. NA – data not available, RT – radiotherapy, WT – without therapy, (*) – for data see Results

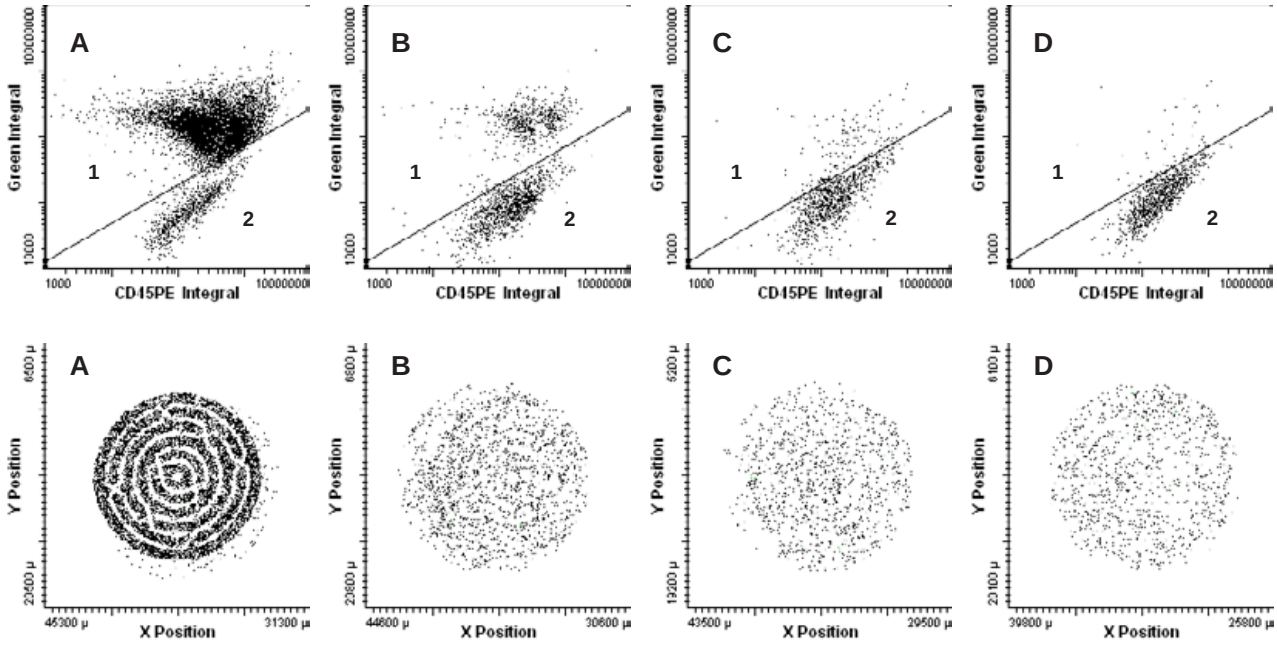

Fig. 1. Upper row: Region 1: CK+ cells in peripheral blood from blood donor 1. Dilutions: A – 1:10, B – 1:100, C – 1:1,000, D – 1:10,000. Region 2: CK – leukocytes. Lower row: Digital image of the membrane with trapped cells at the respective dilutions: A – 1:10, B – 1:100, C – 1:1,000, D – 1:10,000.

Preparation of cells for LSC: Blood samples were diluted 1:1 with physiological saline. For filtration, polycarbonate membranes were used (13 mm, Poretics polycarbonate track-etch-type, Genetics Research Instrumentation Ltd., Braintree, UK) with pore size of 8 µm. The membrane was placed in a standard filtration holder (Swines Millipore Ltd, Watford, UK). Whole blood samples were passed through the membrane manually applying slight pressure. Following filtration the membrane was removed from the holder, washed in physiological saline and fixed in methanol for 30 min. After fixation the membrane was placed on standard glass slide and attached to it by solacryl. Then the membrane was rehydrated with 100 µl saline. The cells on the membrane were then stained with phycoerythrin labelled CD45 (CD45-PE, Immunotech, France) (1:10) for 10 min at room temperature (RT). The sample was washed 5x with 1 ml of saline and pan-cytokeratin-FITC (CK-FITC, Miltenyi Biotec, Germany) (1:10) was added for 10 min at RT. Following the second staining the sample was washed 5x with saline and 7-AAD (7-aminoactinomycin D, Immunotech, France) (1:10) for 10 min. After the staining procedure was completed, the staining solution was discarded, the membrane was covered with a coverslip, and the sample was analysed by LSC. After gaining the LSC data the sample was conventionally restained with Giemsa (1:10) for 20 min and the selected cytokeratin positive (CK+) cells were relocalised.

LSC analysis: Membranes were analysed with a laser scanning cytometer (CompuCyte Inc., Cambridge, MA) using a WinCyte PC-based software. The cells were scanned using a 20x objective and argon laser 488 nm. The scanning area was set to a circle with a diameter of 13 mm including the membrane. Cell contouring was done through long red/7-AAD. Analysis of the events was performed on a XY scattergram (CK-FITC integral vs. CD45-PE integral). Following LSC analysis, CK+ cells were relocalised and a cell gallery was created. After Giemsa staining the cells were relocalised through a CCD camera and the 40x objective. After a comparative analysis of the two galleries we selected tumour cells meeting the cytomorphological criteria of tumour cells.

Statistical analysis: The levels of serum markers CEA, CA 15-3, and TPS were correlated with CTC numbers and the obtained data were evaluated by Pearson's correlation test.

Results

In the beginning we used the established cell line SKOV-3. Cells diluted in peripheral blood of healthy volunteers were filtered through membrane and stained with fluorochromes for LSC analysis. The technique was then validated to determine the sensitivity of the method. Blood was drawn from two volunteers into 4 tubes of 4.5 ml of blood each. To each of the tubes SKOV-3 cells were added at a dilution of 1:10, 1:100, 1:1.000 and 1:10.000 (Fig. 1). The measured data were plotted against the expected values (Fig. 2). We found that the sensitivity was 88.5 % and the linearity was 99 %. Negative control measurements were performed in 12 healthy volunteers. In 3 of them 1 CTC was found, while the other control subjects were free of CTCs.

Out of the 16 patients with breast cancer, 13 were in stages I and II. In patient No. 13, initially classified as T1N0M0 due to a persistently elevated CA 15-3 level, re-examination by MRI confirmed multiple small liver metastases. The patient was further treated with chemotherapy. Before chemotherapy was started, 52 CTCs were detected. In the patient samples, cell doublets and

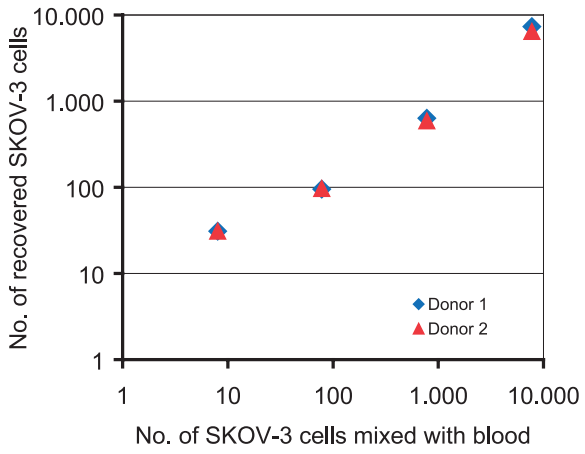

Fig. 2. Diagram of detected and expected numbers of SKOV-3 cells in two clinically healthy blood donors. y=0.8858x–6.2688, r^2=0.9935.

clusters of tumour cells were found in addition to single CK+ cells (Fig. 3). The patient received 3 courses with FAC (for details see Table 1) after which both the marker levels and the number of CTCs decreased (15 CTCs and 1 cluster). Patient No. 4 was initially classified as stage IV. After neoadjuvant chemotherapy with Taxotere + epirubicin followed by surgery, the patient further received 5 courses of chemotherapy with CMF and hormonal therapy with zoladex + tamoxifen. In this patient metastases were found in the liver and skin, then she received combination therapy with herceptin+navelbin in a weekly regimen. She underwent 25 courses resulting in disappearance of the metastases. In her peripheral blood we failed to detect any CTCs. Patient No. 5 had her first recurrence in the ipsilateral axilla but did not receive chemotherapy.

As shown in Table 1, in the 13 early stage patients (of them 8 were node negative and 5 were node positive) we detected 0 to 36 CTCs (mean: 13.9±12.9 CTCs). Five patients were negative for CTC examination (≤2 CTCs), with a mean number of 1±1 CTC, and 8 patients were positive for CTC detection (62 %) with a mean number of 22±9.6 CTCs. Of the eight node negative patients with a mean of 13.25±15 CTCs, 4 (50 %) had more than 2 CTCs. Of the 5 node positive patients with a mean of 15.0±10.3 CTCs, 4 (80 %) had more than 2 CTCs. In 4 patients with positive CTCs in whom blood sample for CTC detection was taken after adjuvant chemotherapy the mean CTC number was 18.3 ± 7.8. In 4 patients with CTC positivity, with respect to their age (over 70 years) and ER and/or PgR positivity, adjuvant chemotherapy was not given. Following radiotherapy they were treated with tamoxifen for 5 years. These patients had higher CTC numbers than the patients after adjuvant chemotherapy (mean: 25.8± 10.8).

In the early stage patients a 62 % positivity for CTCs in peripheral blood was detected but only one patient had elevated CEA levels (8 %), no patient had elevated CA 15-3 (0 %), and two had elevated TPS levels (15 %). Comparison of CTC numbers and CA 15-3 levels showed an significant positive correlation (r^2 = 0.4077) (Fig. 4A), an insignificant negative correlation between TPS and CTCs (r^2 = 0.1575) (Fig. 4B) and no correlation was found between CEA and CTCs (Fig. 4C).

Discussion

Many authors use for the recognition of the CTCs mAb against EpCAM receptor. Pantel et al (25) proved that expres-

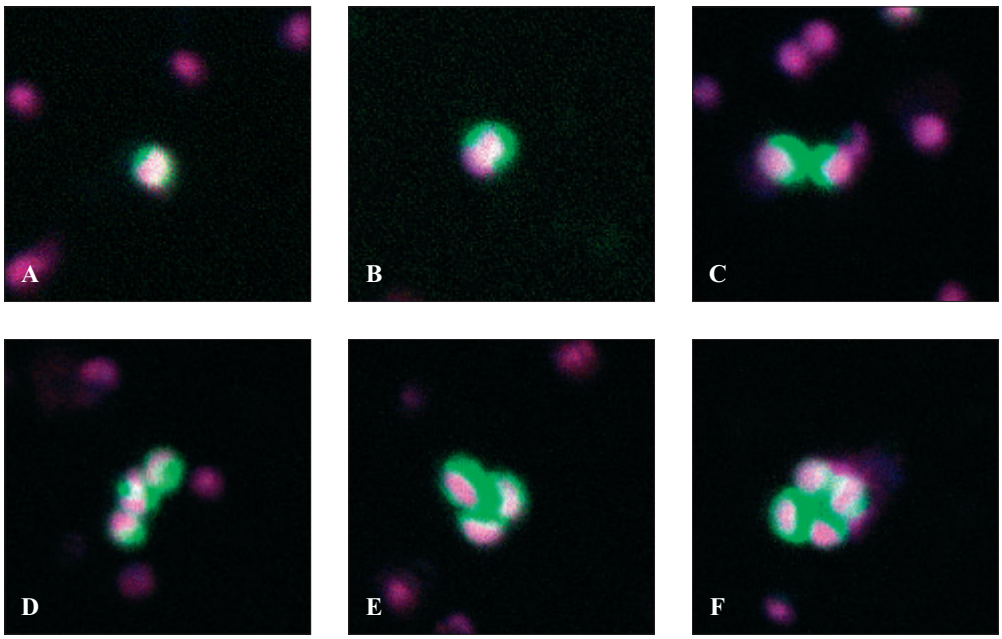

Fig. 3. Gallery of stained patient tumor cells by fluorochromes 7AAD& pan-CYTOKERATIN-FITC&CD45-PE. A, B – single tumor cells, C – double tumor cells (cluster), D, E – triple tumor cells (cluster), F – quadruplet tumor cells (cluster)

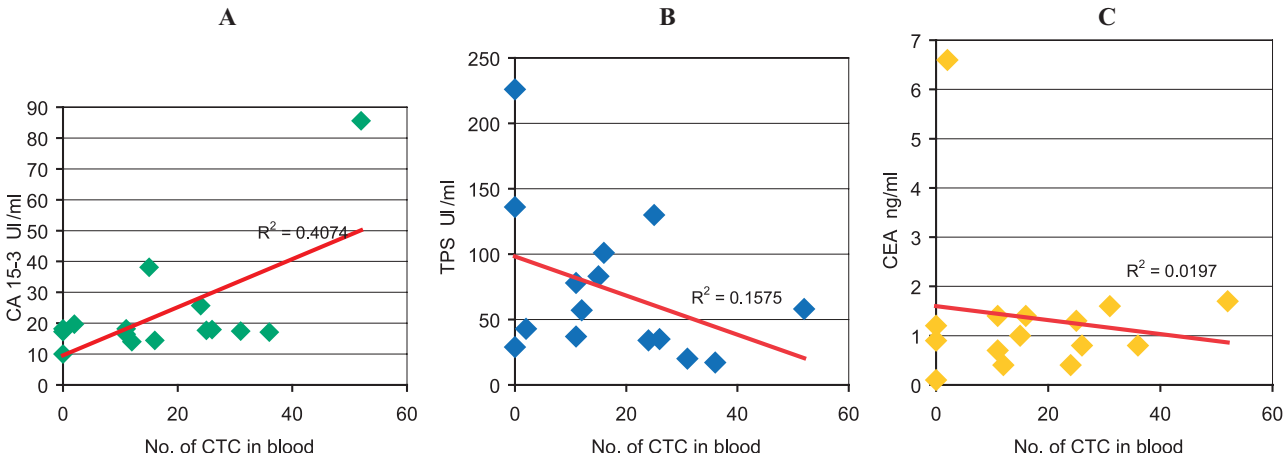

Fig. 4. A – correlation between CA 15-3 and CTCs, B – correlation between TPS and CTCs, and C – correlation between CEA and CTCs.

sion of EpCAM on OTC in bone marrow of breast cancer patients is 64.5 % and can lead to increase of false negative outcomes. It seems that using pan-CK for detection of CTCs is more suitable. We used two markers, namely CD45-PE and pan-CK-FITC, for selection of the CTCs, because the employment of CK-FITC only yielded an elevated percentage of false positivity (26).

To increase the specificity of detection, M30-FITC may be used instead of pan-CK-FITC (27). M30 is a monoclonal antibody capable of recognizing neoepitopes in cytokeratin 18 which are exposed during apoptosis of tumour cells, but not of healthy or necrotic cells. A disadvantage of the employment of M30--FITC is the detection of only apoptotic but not viable cells. The ADAMCOM technique can be also used for the detection of viable cells, i.e. cells on the membrane, without fixation in methanol, are stained with PE-labelled CD45 and FITC-labelled EpCAM in the presence of 7-AAD followed by LSC analysis. Viable cells will incorporate 7-AAD only to a minimum extent, while necrotic and apoptotic cells will show strong positivity for 7-AAD (data not shown).

The advantage of cell separation by microfiltration, as compared to immunomagnetic separation, is in the possibility of direct staining of the separated cells by different antibodies labelled with different fluorochromes immediately after separation, e.g. the staining of sarcomas with anti-GD2, or melanomas with S100. In the case of immunomagnetic separation, besides the fluorochrome-labelled antibody, it is necessary to prepare also the immunomagnetic grain for the separation of circulating cells.

CTCs can be detected also in other types of carcinomas. Allard et al found CTCs in peripheral blood of patients with metastatic carcinoma of prostate, breast, ovary, colon and rectum, and lungs. Kahn et al (28) detected CTCs in patients with nonmetastatic breast carcinoma, in node positive patients in 47 %, and in node negative patients in 39 % of cases, respectively.

The detection of CTCs in peripheral blood is promising in the monitoring of effects of chemotherapy. Hayes et al (29) found that in patients with CTCs the decrease below 5 circulating cells after one course of chemotherapy was a strong predictor having effect on overall survival and progression free survival. Cristofanilli et al (30) proved predictive value of the CTCs and clinically significant cut off value of 5CTCs in 7.5 ml in metastatic breast cancer patients.

Despite the limited number of thus far examined patients, our results revealed interesting data. Sixty-two per cent of patients with early stages of carcinoma had more than 2 CTCs in their circulation. Eighty per cent of node positive patients, while 50 % of node negative patients had CTCs in peripheral blood. Patients with positive CTCs and after chemotherapy had fewer circulating cells than patients after surgery without adjuvant chemotherapy. One patient with metastatic carcinoma after immunochemotherapy was free of CTCs in peripheral blood. In this patient metastases disappeared from parenchymatous organs after therapy, however an early progression of the diseases in her brain was seen. The monitoring of CTC numbers and the expression of HER-2 or of other receptors for CTCs might be a perspective method of assessment of response of the disease to immunotherapy by monoclonal antibodies (31, 32).

Comparisons of CTCs and serum tumour markers (CEA, CA 15-3, TPS) showed insignificant correlations besides correlation between CTCs and CA 15-3. A positive correlation between CTCs and CA 15-3 might indicate a poorer prognosis with increasing CTC numbers, although no elevated CA 15-3 levels were found in patients with early stages of the disease. Muller et al observed significant correlation between CA 15-3 and CTCs in group of metastatic breast cancer patients. Interesting was the correlation between TPS and CTCs: with increasing TPS levels we detected a decreasing number of CTCs. This fact can be related to previous chemotherapy. Chemotherapy in a sensitive tissue induced apoptosis resulting in cleavage of the cytoskeleton. Cytokeratin fragments are released into the extracellular space and can be related to the negative correlation between TPS and CTCs (33–35).

In our group of patients elevated numbers of CTCs were found in patients who did not receive adjuvant chemotherapy

but did receive tamoxifen. CTC detection seems to be a promising method for the monitoring of efficacy of adjuvant therapy in early stage breast cancer patients and for the identification of high risk patients in whom elevated numbers of CTCs are persisting following the termination of adjuvant therapy.

These preliminary results should be verified in a larger group of patients with early stage breast carcinoma.

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