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Rating of Isolated Disseminated Tumor Cells in Bone Marrow in Comparison with Other Factors of Prognosis in Breast Carcinoma

ST. LEINUNG[a*], P. WÜRL[a], A. SCHÖNFELDER[b], C.-L. WEISS[c], I. RÖDER[d] and M. SCHÖNFELDER[a]

[a]*Chirurgische Klinik I (Director: Prof. Dr. Schönfelder), Universität Leipzig,* [b]*Zentrum für Traumatologie mit Brandverletztenzentrum, Fachbereich Plastische und Handchirurgie/Branntverletztenzentrum (Head physician: Dr. Raff), Städtisches Klinikum St. Georg, Leipzig,* [c]*Chirurgische Abteilung (Head physician: Dr. Steuber), St. Elisabeth Krankenhaus, Leipzig and* [d]*Institut für Medizinische Informatik, Statistik und Epidemiologie (Director: Prof. Dr. Löffler), Universität Leipzig*

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Background: Despite the use of radical loco-regional therapeutic methods and although conventional methods of diagnosis give no indication of metastases at the time of operation, distant metastases develop in approximately 50 percent of the carcinoma patients within 5 years. While with the R0 resection of solid tumors local relapses are mainly a matter of concern for the operating surgeon, distant metastases can be traced back to systemic dissemination of tumor cells at the time of operation.

Aims: The goal of our prospective six year continuous study is to compare the rating of the IDT BM with established prognosis factors and to reach conclusions for the practice. *Methods:* A prospective study is represented in which 197 patients suffering from breast carcinoma were analyzed for prognostic relevance of detected isolated disseminated tumor cells in the bone marrow (IDT BM). The patients were operated between 1993–1997 and subsequently observed until 1999.

The monoclonal antibodies CK II and A45-B/B3 were used with the immuno-cytochemical standard method for detecting IDT BM. For the purpose of cell cultivation, the cells were marked with the HEA 125 antibody and separated by means of magnetic cell sorting (MACS). In this investigation, only the presence of isolated disseminated tumor cells detected by the *Results*: A45-B/B3 antibody proved to be an independent prognostic factor for survival time. The risk of an earlier death increased with the detection of IDT BM at least by a factor of two. The detection of IDT BM also represented an independent prognostic factor for the time until advancement of the tumor. The risk of an earlier relapse of the tumor increased with the detection of disseminated tumor cells in the bone marrow containing the A45-B/B3 antibody by at least a factor of four.

Conclusion: A generally acknowledged standardization of the method is desirable. Due to the importance of the independent prognostic IDT BM factor, this method of ascertaining the pathological stage should be established at institutions of higher learning.

Keywords: breast carcinoma, isolated disseminated tumor cells, bone marrow

* Contact Address: Dr. med. Steffen Leinung, Chirurgische Klinik I, Universität Leipzig, Liebigstraße 20 a, 04103 Leipzig. Tel.: 0049 341 97 17 084, e-mail: leinungs@medizin.uni-leipzig.de

INTRODUCTION

Despite the use of radical loco-regional therapeutic methods through surgery and radiation therapy and although conventional methods give no indication of metastases at the time of operation, distant metastases develop in approximately 50 percent of the carcinoma patients within 5 years [13]. While local relapses with the R0 resection of solid tumors are mostly a matter of concern for the operating surgeon, distant metastases can be traced back to systemic dissemination of tumor cells at the time of operation [15].

After increased testing of various detection methods of this "minimal residual disease" the generally accepted term *isolated disseminated tumor cells in bone marrow* (IDT BM) was coined [13]. The UICC took this matter into account by introducing a M1(i) stage [28]. Immunocytochemical analysis has been in development for more than ten years by various working groups and has also been validated in terms of its clinical relevance [16, 17, 18]. At present, this analysis is regarded as the standard method for detecting occult early dissemination of solid tumor cells [13, 15]. Large-scale microscopic screening of large amounts of cytological preparations can be simplified by an automatic analysis of the marked cells with the help of an image analysis system which has been developed by several firms and is in clinical testing [15].

An increasing number of publications have engaged themselves with the application of the PCR method for detecting isolated disseminated tumor cells of epithelial tumors [29, 30]. However the data thus far available do not suggest that the sensitivity of the PCR is any higher than that of the immune cytological technique. Moreover, using the PCR analysis means loosing the possibility of characterizing morphology and phenotype. In addition, the prognostic significance of disseminated tumor cells identified by the PCR is not yet sufficiently backed by relevant data [13]. Using this complicated and expensive method as a screening method is almost out of the question today because of financial pressures. An alternative to the analysis of large volumes of samples is tumor cells concentration by "magnetic cell sorting" methods [13]. While in several studies the evidenced occurrence of disseminated tumor cells in bone marrow has shown a prognostic deterioration for afflicted patients, the results are still contradictory as regards lymph glands, and too few as regards peritoneal lavage and venous blood [13, 25, 26]. The goal of our prospective six year continuous study is to compare the rating of the IDT BM with established prognosis factors and to reach conclusions for the practice.

PATIENTS AND METHODS

Patients and Gathering Diagnostic Samples

We present a prospective study involving 197 patients with a breast carcinoma in any stage (195 women, 2 men) who were operated between 1993–1997 and subsequently observed up to 1999 (mean observation-time was 42 months, 72–12 months). 20.3% (n=40) of the patients were subsequently punctured. We used the UICC's 1993 TNM classification of malignant tumors for classifying the different stages [27].

The average age of the patients was 65.2 years with a disease frequency peak between 55 and 65 years. In the examined malignancy were 187 (94.9%) curative operations and 10 (5.1%) palliations on the patients carried out. In cases of local tumor, a R0 resection could be carried out in 189 (95.5%) patients and a R1 or R2 resection was carried out in 8 (4.1%) patients. The punch biopsy of the iliac crest was made on both sides using the customary method under narcosis immediately postoperative as well as with local anaesthetic for the subsequent puncture, where 5 ml of aspirate was drawn.

Immune Cytochemistry

We used a diffused immune cytochemical double marking [12, 16, 18]. In four cytocentrifugation steps we separated the cells out of the bone marrow aspirate using Ficoll-Paque density centrifugation and made a cytospin preparation. We adjusted the cell concentra-

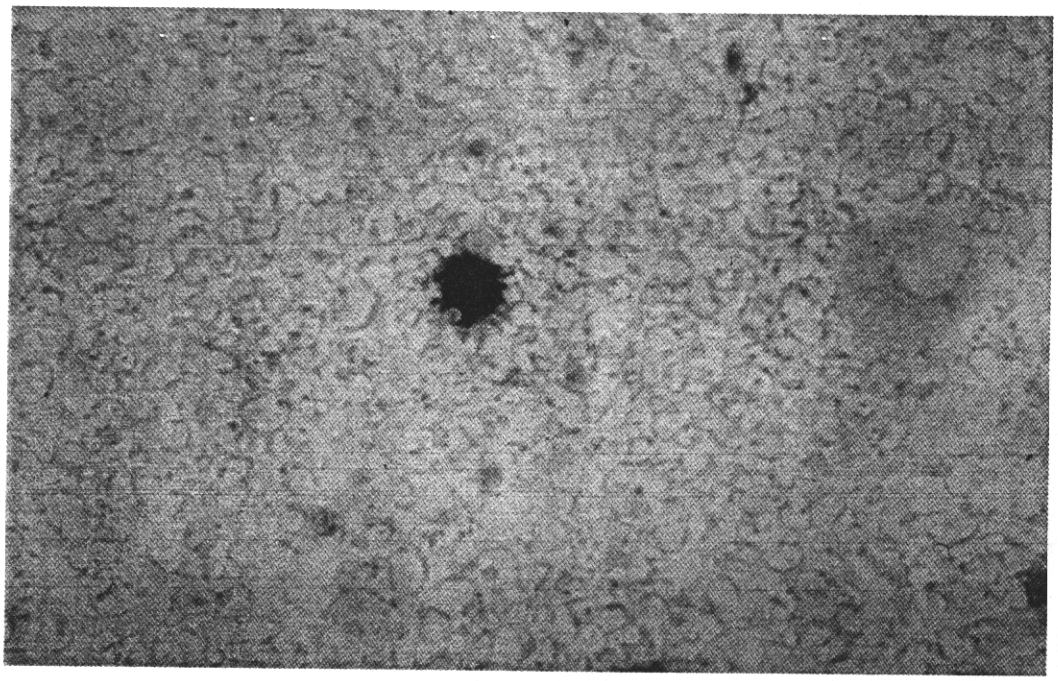

FIGURE 1 Cytokeratin-positive cells and cell complexes with the CK II antibody (see Color Plate I at the back of this issue)

tion in order to have 100,000 cells per microscope slide. Per punch biopsy of the iliac crest we equipped 5 slides so that 1 million cells per patient could be analyzed. At the beginning of the coloration we incubated the cytospin with a 10% AB serum in order to block the unspecific bonding sites. The immuno-cytological identification was achieved partially through incubation with CK 2 from Boehringer Mannheim, a monoclonal antibody against cytokeratin 18, and partially through the "Epithelial Cell Detection Kit" (EPIMET®) from the Mikromet GmbH / Baxter Deutschland GmbH company, which contains a monoclonal pancytokeratin antibody against A45-B/B3. In the first case we incubated the preparation with a bridging antibody marked with preformed alkali phosphatase antialkali phosphatase (APAAP) immune complexes. After "almost blue" coloration we obtained the cells represented in figure 1.

With the "Epithelial Cell Detection Kit", following cytocentrifugation the cells are incubated with fixation solutions A and B; afterwards, the prepared antibody solution is added and then the microscope slides are colored (Figure 2).

For quality control we conducted a negative control without antibodies, a positive control of the coloration, and a positive control through a series of tumor cell cultures. 51 patients without detectable carcinoma illness were examined for IDT BM by using the same method. None of the patients from this group showed IDT BM with the A45-B/B3 antibody. 9.8% (n=5) were (falsely) cytokeratin-positive with the CK II antibody.

For in vitro cell separation of vital tumor cells we drew 10 ml of blood from the veins and separated the cells using cytocentrifugation methods and Ficoll-Paque density centrifugation. We employed Human Epithelial Antigen (HEA-125) MicroBeads from Milteny Biotec to mark the cells. These are colloidal supramagnetic MACS MicroBeads conjugated with monoclonal mouse anti-human epithelial HEA-125 antibodies, isotope: Mouse IgG1. MACS separation columns (capacity 2–10 myl) were used and there were three runs in any case. The specificity of the evidence was increased by using the FcR blocking reagent from Milteny Biotec. The reagent blocks the bonding of cells expriming human Fc receptor, e.g., monocytes and macrophages.

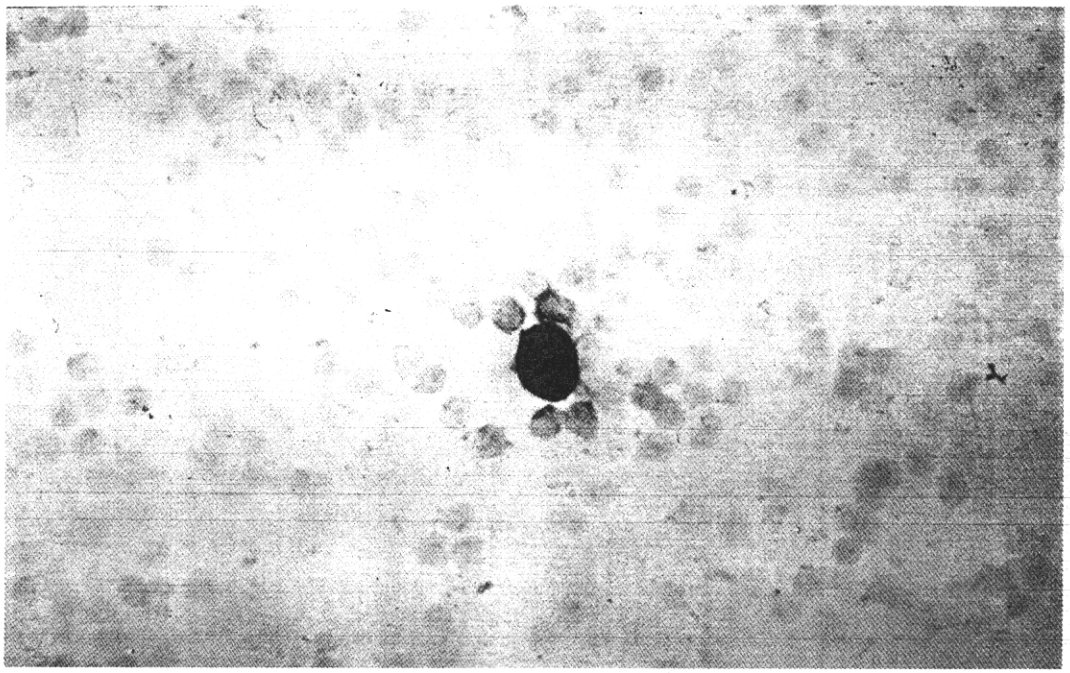

FIGURE 2 Cytokeratin-positive cells with the A45-B/B3 antibody (see Color Plate II at the back of this issue)

Statistical Analysis

The cell preparations were examined by two independent researchers through transmitted light microscopy. At 200x and 400x magnification, strong blue (CK II antibody) or strong red (A45-B/B3 antibody) colored CK positive cells could easily be identified and counted. The following statistical procedures were used for the evaluations: the Wilcoxon Test, the chisquare test, the Fischer-Exact Test, the Kaplan-Meier-Schätzer, the log rank test, and the Cox regression [1].

The study in hand is of a descriptive character; that is, the results should rather be seen as a description. The reasons for this lie in the multitude of tests carried out on one and the same set of data. Thus, the problem of multiple testing arises, which can be circumvented by adjusting the levels of significance. Each test delivers a significant finding only when the P value is less than 0.05 per number of tests. Therefore, with ten tests, each individual test is only significant if $P<0.005$. The total probability of error then remains at 5%. Only major significant effects can be proven in this manner; significant tests should be seen as expected tendencies, not as statistical proof. Interesting phenomena can thus be filtered out.

RESULTS

1. Description of the Data Material

15.9% (20/126) of the patients were cytokeratin positive with the A45-B/B3 antibody. 83.2% (164/197) of the patients were cytokeratin positive with the CK II antibody. A comparison between both sides of the iliac crest did not show any noticeable differences as regards evidencing cytokeratin-positiveness. In cases of positiveness with the A45-B/B3-antibody, 1 to 16 cytokeratin-positive cells were detected; with the CK II antibody the respective rate was 1 to 3000 cytokeratin-positive cells out of 10^6 mononuclear cells.

2. Simple Statistics: Median Comparisons and Correlation Analysis

There was evidence of a correlation between CK II antibody positiveness and proven metastases in the lymph glands (P<0.001) and diagnosed distant metastasis (Fischer's Exact Test, P:0.015). A correlation between tumor size and grading was not detected.

3. Univariate Analysis of Survival Time

In this form of analysis the parameters for the multivariate Cox analysis were preselected. With the A45-B/B3 antibody there was a correlation between cytokeratin-positiveness and survival time (P value: 0.004). No correlation could be proven with the CK II antibody. An examination of the conventional prognosis factors showed a significant correlation between tumor size and survival time and the time until progression of the tumor disease (P value <0.001). A correlation could also be established between lymph node infestation (pN stage) and the survival time (P value 0.0032) and the time until progression (P value<0.001). The correlation between M stage and the survival time and the time until the tumor disease yielded the same significance level of <0.001.

A significance of operations for local relapses of breast carcinoma could not be established for the survival times (P value 0.3037). However, the data suggested a significance as regards the time until advancement of the breast carcinoma (P value 0.0013). In 9 cases a second carcinoma appeared on the same organ system; in 4 cases it appeared on a different system; and in one case a second carcinoma appeared on both the same and a different organ system. A palliative or curative approach of treatment, though indicated by the stage of the tumor, had a significant influence on the survival time of the patients (P value<0.0003) and, still more distinctly, on the time until the progression of the carcinoma disease of the patients (P value<0.001). Younger patients (separated at the median of the group) showed worse survival times (P value: 0.0663).

4. Multivariate Analysis of Survival Time (Cox Regression)

4.1 Survival Time

Only the detection of isolated disseminated tumor cells through the A45-B/B3 antibody (P value 0.0049) represented an independent prognostic factor in this research. The other factors examined did not have this prognostic relevance. The risk of an earlier death at least doubled with detection of cytokeratin-positive cells in bone marrow with the A45-B/B3 antibody (confidence interval: 2.1 to 62.9) in comparison with cytokeratin-negative patients.

4.2 Time until Advancement of the Carcinoma Illness

The presence of a local relapse (P value<0.001) and the detection of isolated disseminated tumor cells by the A45-B/B3 antibody (P value<0.001) represented an independent prognostic factor in our investigation for the time until advancement of the tumor. No other factors examined had this prognostic relevance. The risk of experiencing a tumor relapse increased with an actual local relapse of the breast carcinoma by a factor of 1.9 to 25.5 (confidence interval) as compared with patients with a first diagnosis of breast cancer, and, in the case of detecting disseminated tumor cells in bone marrow by the A45-B/B3 antibody, by a factor of 4.5 to 48.7 (confidence interval) as compared with cytokeratin-negative patients. This holds true on condition that the second symptom remains constant in the respective case. Patients with a local relapse and detection of disseminated tumor cells have, on average, approximately a 100x greater risk.

5. Additional Investigations

5.1 Follow-up Puncture of Patients Examined at the Time of Operation for Isolated Disseminated Tumor Cells (IDT BM)

A control puncture was carried out during the follow-up examination period using the same method on 40 (20.3%) of the 197 patients examined at the time of operation. Of the 40 follow-up puncture patients, 51.4% (n=13) were CK II antibody negative and

42.2% (n=27) were positive. With the A45-B/B3 antibody, disseminated tumor cells were not detectable in 53.3% (n=32) of the patients; in 28.6% (n=8) of the patients they were detected. Table I represents the correlation with the clinical development. Convincing statistical calculations cannot be made because of the limited number of cases.

A quantitative analysis of the follow-up punctures showed 7 cases of tumor advancement in A45-B/B3 patients; however, no patients remained tumor-free. Cytokeratin positiveness at the time of the initial puncture and at the time of the follow-up punctures is represented in detail in Table II.

A comparison between the initial and multiple follow-up punctures with respect to quantitative cytokeratin positiveness shows in the majority of cases a correlation with the clinical development and is represented in detail in Table III.

5.2 In vitro cell separation of disseminated tumor cells and vital tumor cell cultures

Disseminated tumor cells from the venous blood of 9 patients with metastasis colorectal carcinomas were given vital markings of the monoclonal HEA-125 antibody and isolated and accumulated by magnetic cell sorting (MACS). After seven days a growth of tumor cells was ascertainable, as shown in figure 3, which later stagnated.

TABLE I Correlation between follow-up punctures and clinical development

	CK II antibody		*A45-B/B3 antibody*	
	cytokeratin negative	*cytokeratin positive*	*cytokeratin negative*	*cytokeratin positive*
tumor-free	12	15	28	0
tumor advancement	1	12	4	8

TABLE II Quantitative comparison between initial puncture and follow-up puncture with respect to IDT BM

	Increase of CK+ cells		*Decrease of CK+ cells*		*Constant of the CK+ cells*	
	CK II	*A45-B/B3*	*CK II*	*A45-B/B3*	*CK II*	*A45-B/B3*
tumor-free (n=27)	4	0	19	3	4	24
Tumor Advancement (n=13)	8	7	5	2	0	4

TABLE III Quantitative comparison between multiple punctures with respect to IDT BM

Identification	*Result*	*1st Puncture*		*2nd Puncture*		*3rd Puncture*	
		CK	*A45-B/B3*	*CK*	*A45-B/B3*	*CK*	*A45-B/B3*
hc080741	free	0	0	0	0	0	0
tc240160	free	154	0	0	0	0	0
gu040655	met. (dead)	170	1	1120	7	1515	6
ba230426	met.	1210	1	780	2	1100	1
ei060447	met. (dead)	3080	5	1210	12	1780	7
jw190438	met.	1670	16	540	6	470	0

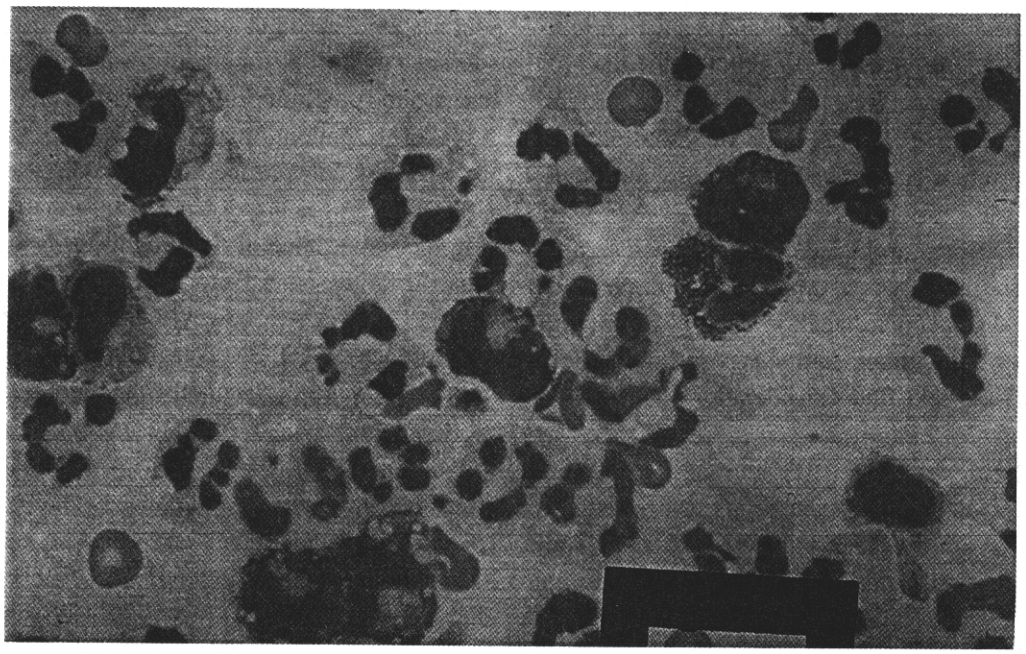

FIGURE 3 Carcinoma cell cultures from venous blood after 7 days (see Color Plate III at the back of this issue)

DISCUSSION

Alongside the newer methods for detecting the "minimal residual disease", such as the RT PCR [29, 30] is the immuno-cytochemical detection of isolated tumor cells in bone marrow (IDT BM) by using monoclonal antibodies is currently considered the standard method [13, 15]. The antibodies we used against CK II and A45-B/B3 have been generally tested and are acknowledged for detection purposes [3, 14, 22].

15.9% of the patients with the A45-B/B3 antibody and 83.2% of the patients with the CK II antibody were detected as cytokeratin positive. In the cases of positiveness, 4 or 362 cytokeratin-positive cells out of 10^6 mononuclear cells could be identified on an average with with the A45-B/B3 antibody and with the CK II antibody, respectively. Compared with reference figures, the detection rates of the A45-B/B3 antibody are at the lower margin of the average; those of the CK II antibody lie over the specified detection rates of 4–45% [2, 4, 11, 14, 17]. On an average, such cells were found in 35% of the patients covered by a meta-analysis of 20 studies of all types of carcinoma [6].

In our opinion, the differences in the detection rates can be attributed to methodological aspects. In peroxidase-rich tissue, such as bone marrow, the CK II antibody does not succeed with the immuno-peroxidase reaction in sufficiently reducing the unspecific background color; and the co-reaction of the bone marrow cells containing peroxidase make a clear identification of the antibody-positive cells difficult. Conjugates where the alkali phosphatase is directly bonded to the antigen-building fab-fragment of the primary antibody, such as the A45-B/B3 antibody ("Epithelial Cell Detection Kit"), raise the specificity and simplify the implementation of the methods [13]. Moreover, a direct comparison of the studies is not possible since polyclonal (EMA) antibodies (11), antimuzin antibodies (2E11) [2], and other antibodies have been used in addition to the CK II antibody (4, 16, 21). Method standardization is needed in order to simplify this problem in the future.

As regards the A45-/B/3 antibody, our analysis showed a significant correlation between cytokeratin-positiveness and the pN-stage and a clear correlation to the M-stage. No such correlation was found for the CK II antibody with respect to the TNM system. Most authors [5, 6, 20, 21, 24] saw a correlation with the different tumor stages as did we in previous studies [23].

The conventional prognosis factors show a significant relationship between tumor size, lymph node infestation, distant metastazation and the time until progression of the tumor, and survival times. These are known facts and find consideration in the TNM classification [26, 27].

Patients with the A45-B/B3 antibody who were analyzed showed a significant relationship between cytokeratin-positiveness and survival time. In accordance with this fact Funke et al. [6] found a positive correlation between tumor cell detection and a reduction of the illness-free interval in in 14 of 20 studies of their meta-analysis of 2494 patients from 1980 to 1997 by the univariate analysis.

In the multivariate survival time analysis (Cox regression) [1], merely the detection of isolated disseminated tumor cells by the A45-B/B3 antibody represented an independent prognostic factor. The risk of an early death at least doubled with the detection of IDT BM. A local relapse situation at the time of the operation and the detection of IDT BM with the A45-B/B3 antibody represented independent prognostic factors for the time until tumor advancement. The risk of experiencing an early local or systemic advancement of the tumor was at least 3x greater with an actual local relapse and at least 4x greater with the detection of IDT BM. Interestingly, the symptoms of the TNM system did not supply any additional information as prognostic factors when including IDT BM in the multivariate analysis. This means that other tumor-biological effects independent from the conventional prognostic factors could just as well be measured through the detection of IDT BM and find expression in this prognostic relevance. In comparison with bibliographical references, only 5 of the 11 studies of the meta-analysis made by Funke et al. [6] showed positive bone marrow evidence as an independent prognostic factor for a shorter disease-free interval. Moreover, 5 out of 12 studies provided univariate evidence, but only 2 studies provided multivariate evidence that the positive bone marrow condition is an independent factor for the overall survival of the patient. In a Mantel-Haenszel analysis the relative risk for a reduction of the disease-free interval was 1.34 for breast cancer. Information concerning the importance of the prognostic factor IDT BM apart from the other factors is otherwise not normally given [2, 11, 24]; however, it is indicative of the importance of IDT BM detection.

The follow-up puncture of the cytokeratin-positive patients with the A45-B/B3 antibody revealed an advancement of the tumor in 8 of them; none remained tumor-free. 28 of the cytokeratin-negative patients were tumor-free and 4 had an advancement. The correlation of this trend was still less clear with the CK II antibody. Cytokeratin-positiveness was by no means an indicator for an approaching advancement, which was mainly due to the randomly selected one-time follow-up puncture during the first to third postoperative years. A quantitative analysis of the follow-up puncture and the multiple follow-up punctures showed predominantly a decrease of IDT BM with freeness from tumor and an increase in tumor advancement. Multiple follow-up punctures at time intervals have been suggested also by other authors as a possibility of therapy monitoring [15, 21]; investigations made by others, but also earlier own studies [7–10] show a correlation with clinical developments. At any rate is optimization and standardization of detection methods a decisive requirement for effective monitoring.

In the metastasis stage we isolated the tumor cells with the HEA125 antibody with the help of magnetic cell sorting (MACS method) and cultivated the vital cells. Cell growth stagnated after approximately one week. Data on cultures of tumor cells isolated from venous blood or bone marrow are rather rare; the aforementioned fact was also observed by other authors [15]. The possibility of the vital cell culture indicates a proliferative potential for the IDT BM. The atrophy of the tumor cells in the culture after some time could perhaps be traced back to the still not

fully developed metastatic potential of these cells and the lack of endogenic factors for tumor development [19]. An additional therapeutic option using a cytostatic resistogramm from venous blood is desirable though not feasible because of the facts mentioned above.

CONCLUSION

Our comparison of the CK II and A45-B/B3 antibodies showed better correlation of the latter antibody with other literary references and clinical developments. Therefore, we should give preference to the "Epithelial Cell Detection Kit" in which the alkali phosphatase is directly bonded to the antigen-bonding fab fragment of the primary antibody, since it enhances specificity and simplifies the use of the methods. A generally accepted standardization of the methods is desirable. Due to the importance of the independent prognostic factor of IDT BM, the method should be established at institutions of higher learning for ascertaining the pathological stages. With accumulating experience a decision should be taken on whether patients who would not receive adjuvant additional therapy because of the stage of their tumor, would nevertheless be advised to undergo an adjuvant therapy because of the sole detection of disseminated tumor cells (ID-BM).

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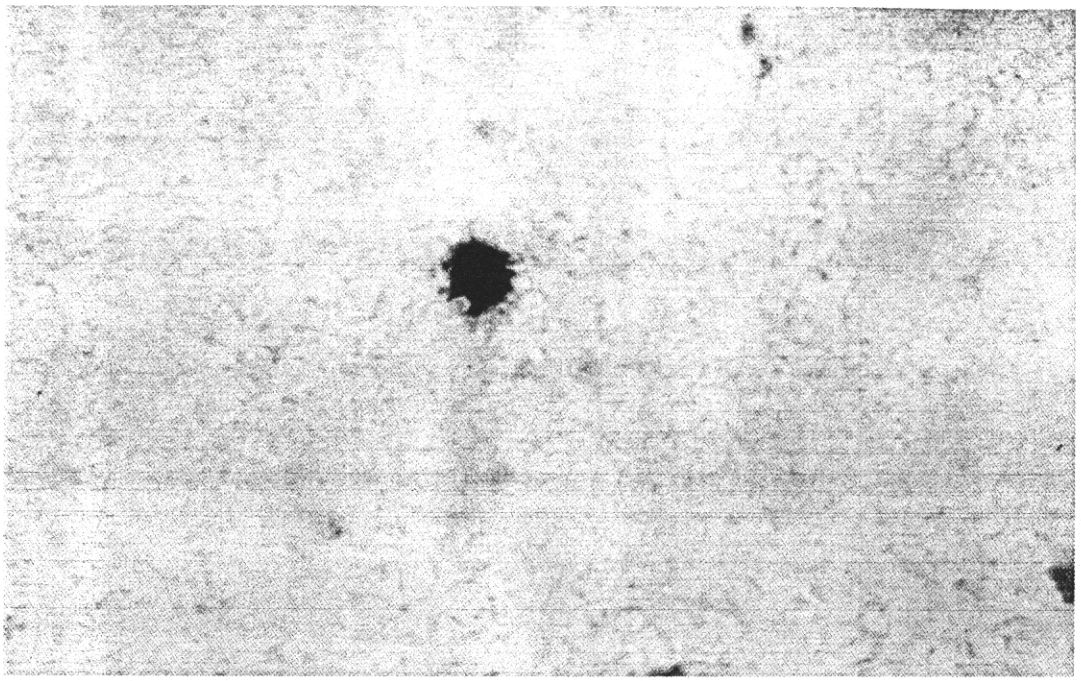
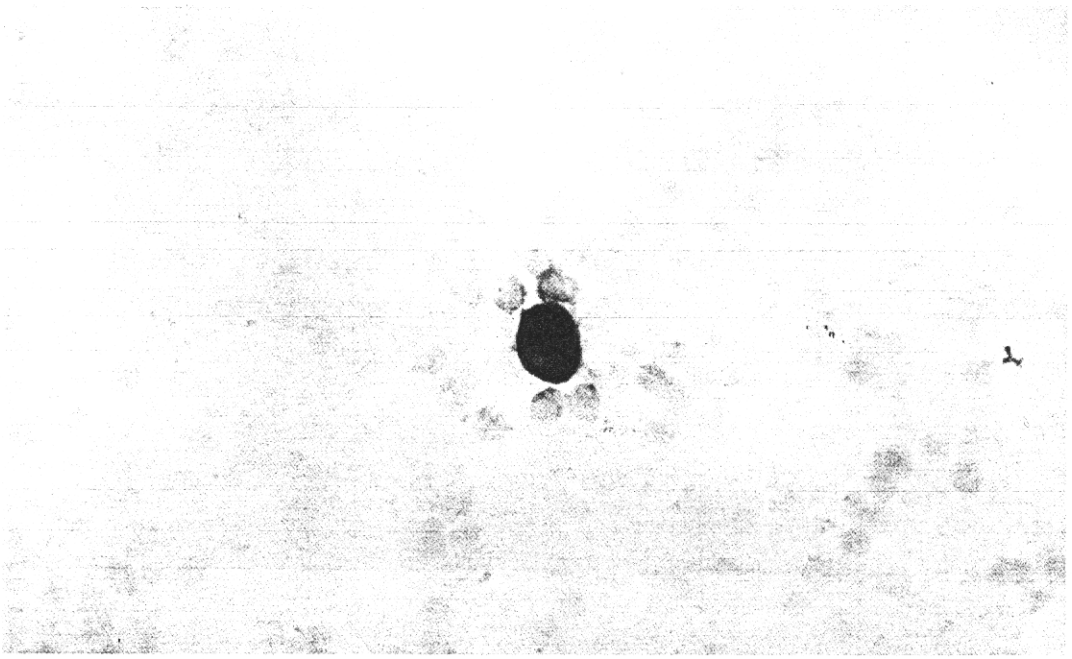
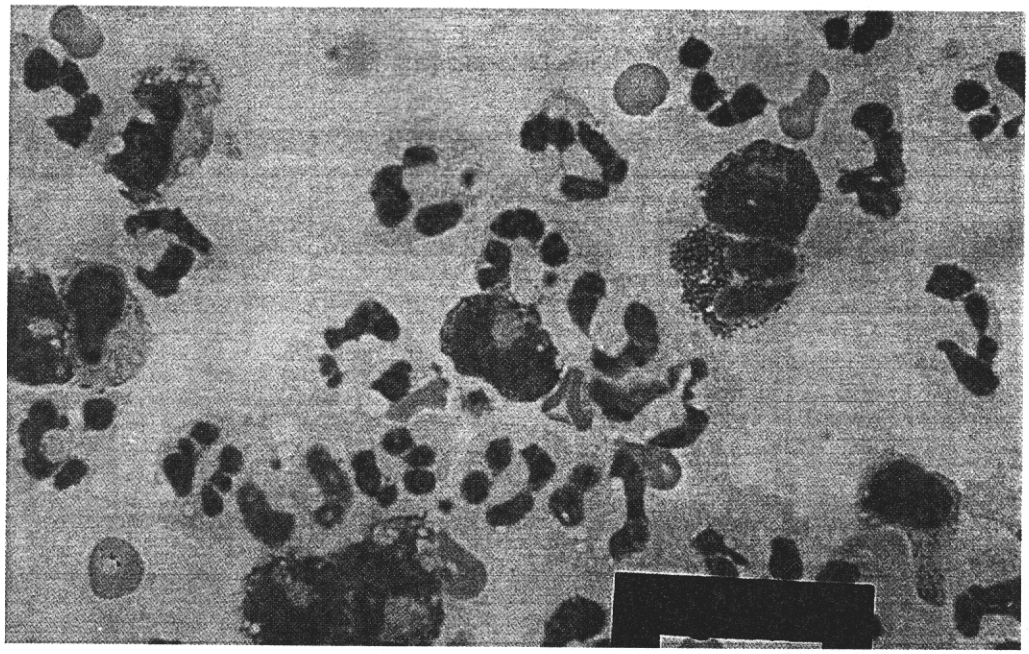


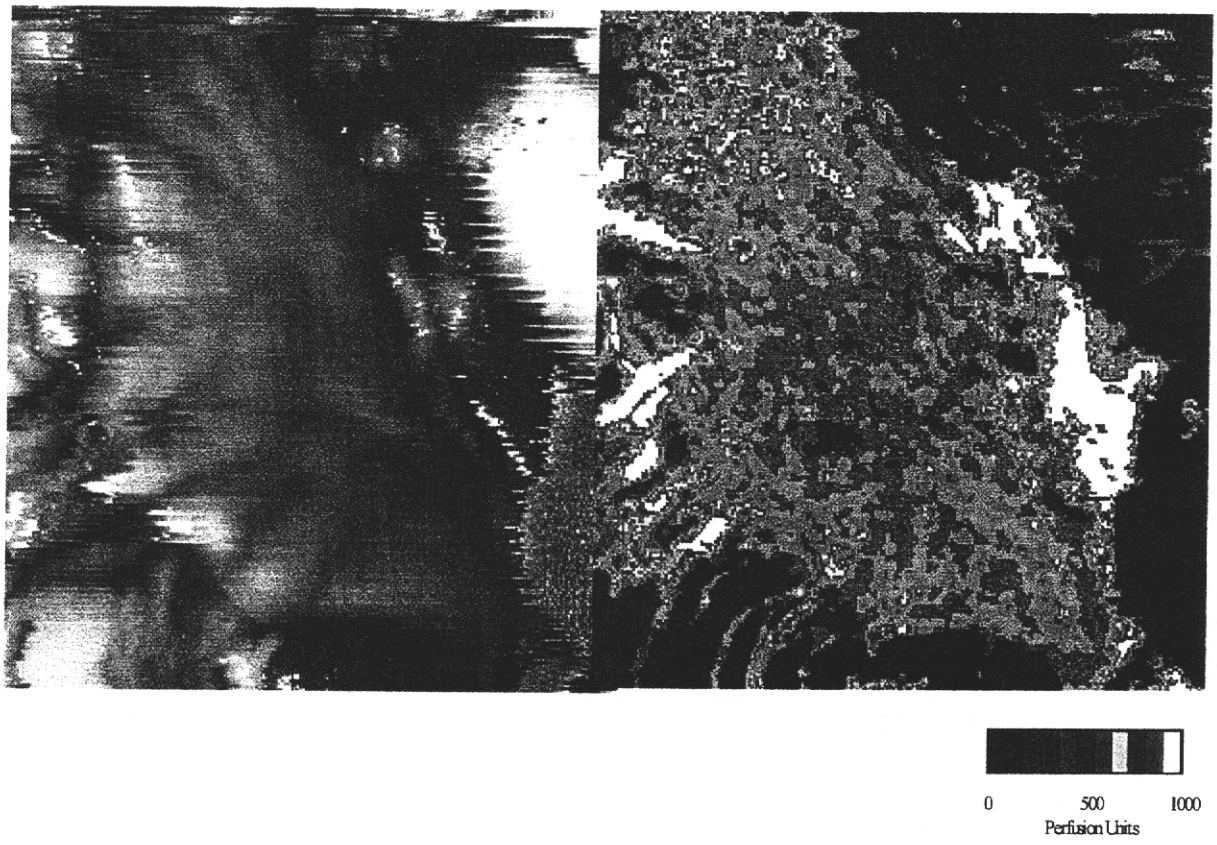
Color Plate I (See page 195, figure 1) Cytokeratin-positive cells and cell complexes with the CK II antibody



Color Plate II (See page 196, figure 2) Cytokeratin-positive cells with the A45-B/B3 antibody



Color Plate III (See page 199, figure 3) Carcinoma cell cultures from venous blood after 7 days



Color Plate IV (See page 205, figure 1) Stomach prior to mobilisation, with greater curve to left and fundus at bottom of picture. The two images were made simultaneously by the laser Doppler scanner. In the colour perfusion map blood flow is colour coded. The 1cm2 areas used to compare single point and scanning laser Doppler measurements of perfusion were made in the gastric body in the centre of the picture