

Immunocytochemistry and DNA-image cytometry in diagnostic effusion cytology

I. Prevalence of markers in tumour cell positive and negative smears

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To determine the prevalence of immunocytochemical positivities for a panel of antibodies in benign and malignant cells in effusions with known follow-up in order to use these as diagnostic markers. Besides their ability to identify malignant epithelial cells their contribution to the differential diagnosis between carcinomas and mesotheliomas was investigated.

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101 tumour cell positive and 53 negative effusions were stained with 12 different antibodies. Results were scored semiquantitatively per cell type. Furthermore, DNA-image cytometry was performed.

While prevalence of Ber-EP4 positivity was 95.4% in metastatic carcinoma cells, it was 0% in those from mesotheliomas. No cell type reacted with this marker in benign effusions (0%). Ber-EP4 correctly differentiated between metastatic carcinoma and mesothelioma in 98.0%. Prevalence of DNA-aneuploidy was 95.4% in metastatic carcinomas, 57.1% in mesotheliomas and 0% in reactive effusions.

Combining immunocytochemistry (Ber-EP4 positivity) and DNA-image cytometry (aneuploidy) results in a 100% detection of metastatic carcinomas and 57.1% of mesotheliomas. Both markers furthermore allowed a correct differentiation of these entities in 98%.

1. Introduction

Sensitivity of conventional cytology for the detection of malignant cells in effusions is unsatisfactory, about 58%, specificity is about 97% (average from a literature review of 6,001 cases from 6 studies [66]). This diagnostic uncertainty manifests also in diagnostically "equivocal" effusions showing "inconclusive cells" or "cells suspicious for malignancy". Improvement of diagnostic accuracy is therefore necessary in effusion cytology. Not so much the largest possible number of metastatic cancers correctly diagnosed, but the smallest number of carcinomas incorrectly diagnosed is important [42]. Thus, sensitivity should be improved without lowering specificity.

The diagnostic value of immunocytochemistry for the identification of malignant cells in effusions has often been emphasized [7,8,37,50,62,63]. There is a great number of publications on immunohistochemical markers distinguishing between primary (mesothe-

liomas) and secondary tumours (metastatic carcinomas) of the serous membranes on paraffin embedded histologic material [24,25,32,45,49,54,55,59,61]. On the other hand there is a number of publications on the use of specific antibodies for the immunocytochemical identification of primary sites of different metastatic carcinomas in effusions, e.g., of the ovary [20,35], the breast [34] or both [5], or of bronchial carcinoma [36] in effusions. In our study of the literature we have considered only those publications in which antibodies were applied without restriction to a specific primary tumour. 41 publications [1–4,6,10,17–19,22,23,26,28–33,39–41,43,44,46–48,50–53,58,60,62–65,67–71] were found in which prevalences of markers for malignant and benign cells in effusions were recorded. The data for the ten most commonly applied antibodies were compared. 17 of these 41 studies [3,19,23,26,28,33,41,43,44,48,50,58,60,62,65,67,69] differentiate between effusions due to metastatic carcinomas and those due to mesotheliomas. Although 12 of these publications [2,10,22,23,29,39–41,46,51,64,68] have also studied cytodiagnostically equivocal effusions only two of them, Illingworth et al. [39] and Matter-Walstra and Kraft [51] have calculated sensitivity, specificity, positive or negative predictive values of markers to identify malignant cells, but without differentiating between those due to metastatic carcinomas and those due to malignant mesotheliomas. Eight further authors [6,26,31,41,48,53,62,70] have reported on “sensitivity”, “specificity”, “positive and negative predictive values”, but as they have not included equivocal effusions in their diagnostic categories, to our opinion they have incorrectly applied these definitions. Eight other authors [17,26,29,31,33,46,50,62] suggest the combined use of different immunocytochemical markers (“panels”) for diagnostic purposes.

The extreme differences in reported prevalences of marker positivities in different cell types seem, apart from influences by different patient populations, to be due to differences in methodology. This makes data difficult to compare:

Studies used different fixations: air-drying, acetone, methanol, acetone plus methanol, ethanol, Carnoy's solution, Bouin's fixative, formalin, paraformaldehyde and formol sublimate. Furthermore, the investigations differ in techniques of preparation: cell blocks (paraffin-histology), cytopins or sediment smears (cytology). Yet, both fixation and cell preparation largely influence stabilization and presentation of antigen epitopes [9]. Furthermore, a wide

variety of staining methods was applied, which accounts for differences in specificity and amplification of the reaction [9] the indirect peroxidase system (IP), peroxidase–anti-peroxidase-complex system (PAP), alkaline phosphatase–anti-alkaline-phosphatase-complex system (APAAP), (strept-)avidin–biotin-complex system ((strept-)ABC). The antibodies chosen by different authors vary strongly in their potential to detect epithelial cells and in their ability to differentiate these from benign or malignant cells of mesothelial origin: anti-AUA-1, -BCA-225, Ber-EP4, -B72.3, -calretinin, -CA1/2, -CEA, -cytokeratins, -83kD, -desmin, -EMA, -HEA 125, -HBME-1, -HMFG-2, -LCA, -Leu-M1, -MOC-31, -p53, -S-100 protein, -vimentin. The number of cases investigated differs from 25 to 227 (average 100.5). In several studies it is not obvious whether immunocytochemical results were compared with conventional cytologic diagnoses, with clinic or histologic follow-up.

In this study we applied twelve different antibodies against the following epitopes: Ber-EP4 (surface and cytoplasmic glycoprotein), Lu5 and MNF 116 (cytoplasmic cytokeratins), EMA (epithelial membrane antigen, a human milk fat globulin), B72.3 (cytoplasmic tumour-associated glycoprotein), Leu-M1 (cytoplasmic oligosaccharid), CEA (cytoplasmic carcinoembryonic antigen, a heavily glycosylated protein), CA-125 (membrane highmolecular mucin), HBME-1 (cytoplasmic mesenchymal marker), vimentin and desmin (cytoplasmic intermediate filaments) and MAC 387 (cytoplasmic myeloid-histiocyte). The choice of these antibodies was made, as on the one hand the most commonly applied antibodies (e.g., anti-CEA, -EMA, -cytokeratins), on the other hand those described as the most sensitive ones to detect epithelial cells and the most specific ones to differentiate between epithelial and mesothelial cells (e.g., Ber-EP4, -Leu-M1). Only recently we routinely apply the antibody anti-calretinin, a mesothelial marker [6,27].

The diagnostic value of DNA-cytometry for the identification of malignant cells in effusions has been demonstrated by our group in two previous studies [56, 57].

The aim of the present study was to investigate the prevalence of cellular positivity for different antibodies in tumour cell negative, and positive effusions and to consider the usefulness of their combination as a panel. In parallel, the prevalence of DNA-aneuploidy in malignant and benign cells of effusions was determined in order to analyze the ability of both adjuvant methods to increase the diagnostic accuracy of effusion cytology.

Part II of this study presents the ability of the same immunocytochemical markers and DNA-cytometry to detect malignant epithelial and mesothelial cells in diagnostically equivocal effusions.

2. Materials and methods

2.1. Specimens and patient population

Subject of our study were 154 effusions of the serous cavities with cytologically tumour cell positive (101) and negative (53) diagnoses. 107 pleural, 43 peritoneal, three pericardial effusions and one coul de sac aspiration were routinely investigated between April 1996 and December 1997 in the Institute of Cytopathology. The patients were from the University Hospital of Düsseldorf as well as from hospitals of the surrounding area. Effusions were selected as containing a sufficient number of reactive or malignant mesothelial or epithelial cells for application of adjuvant methods.

2.2. Staining of specimens

Native materials were centrifuged at 300g for 5 min. Four slides were processed by pipetting a drop of the sediment, containing some residual liquid on each slide. A second slide each was used to top the first four in order to produce eight smears. Three of these slides were air-dried and stained according to May-Grünwald-Giemsa, one of these was used for DNA-image cytometry. The other slides were prepared for Papanicolaou staining and subsequent immunocytochemistry (ICC) by immediate fixation in Delaunay's fixative [21] (500 ml ethanol plus 500 ml acetone (Riedel-de-Haën, Seelze, Germany, no. 24201) plus ten drops of 1 M trichloroacetic acid (Merck, Darmstadt, Germany, no. 1.00807.0100)) for 10 min. Coverslips were removed in xylene before immunocytochemistry or DNA-cytometry were performed.

2.3. Cytological diagnosis

Specimens were evaluated according to generally accepted diagnostic criteria [7,11,15,42] by an experienced cytotechnician (K. K.) and by cytopathologists (H. M. or A. B.) as well. The following categories of cytologic diagnoses were chosen: "insufficient" for specimens without any or with exclusively autolytic or necrotic cells, "negative" for inconspicuous, reactive or inflammatory cellular changes, "doubtful" in cases

with atypical cellular activation or degeneration, "suspicious" if only sparse abnormal cells were seen or the diagnostic criteria for malignancy were only vague and "positive" for effusions containing unequivocal malignant cells [7,16].

2.4. Immunocytochemistry

The application of immunocytochemistry on cytological specimens was developed by P. D. and B. B. in the Division of Cytopathology, Institute of Pathology, University of Basel [21]. For immunocytochemistry on slides previously fixed according to Delaunay and stained according to Papanicolaou cells of interest were marked by felt-tip pen. Coverslips were then removed in xylene. If only few suspicious cells were present the slides were divided into two or three sections using a DakoPen (Dako, Glostrup, Denmark, no. S2002) so that several antibodies could be applied simultaneously on the same slide. The Avidin-Biotin-Complex method (ABC) [21] was applied for immunological reaction. The incubations were carried out in a horizontal position of the slides in a humid chamber. All other steps were carried out in an upright position of slides in cuvettes. Endogenous peroxidase activity was stopped by incubation with 1 ml H₂O₂ (30% Perhydrol, Merck, Darmstadt, Germany, no. 1.07209.0250) in 100 ml methanol (Merck, Darmstadt, Germany, no. 1.06008.250) for 30 min at room temperature (RT). After shortly rinsing the slides manually three times in phosphate buffered saline (PBS, Sigma, Deisenhofen, Germany, no. D-1408) they were then placed twice in PBS at RT for 10 min each. They were then incubated in normal (horse) serum (for mouse antibodies) (225 µl: 15 ml aqua dest.) (Vector, Camon Laboratory Service, Wiesbaden, Germany, no. S-2000) for 20 min at RT. Letting the residual liquid drip off the slide by slanting, the slides were incubated with the primary antibody (antibodies applied, as well as their specifications and dilutions are described below) for 12 h at 25°C. The slides were then rinsed twice in 0.5 M. TRIS (tris(hydroxymethyl)aminomethane), pH = 7.6 in PBS 1 : 10 for 5 min at RT each. This was followed by incubation with the link (biotinylated) antibody (LINK) [21] (Vector, Camon Laboratory Service, Wiesbaden, Germany, no. BA-2000) (30 min at RT) and once again the slides were rinsed twice in 0.5 M. TRIS in PBS 1 : 10 for 5 min at RT. The slides were then incubated with the ABC-Elite-Standard [21] (Vectastain, Camon Laboratory Service, Wiesbaden, Germany, no. PK-6100) for further 30 min at RT and

once again rinsed twice in TRIS 0.5 M (Boehringer, Mannheim, Germany, no. 812838) in PBS 1 : 10 for 5 min at RT. The substrate-chromagen-reagent AEC (3-amino-9-ethylcarbazole) [21] (Sigma, Deisenhofen, Germany, no. A5754) was then applied for 40 min at RT followed by rinsing twice in sterile aqua dest. for 5 min at RT each. Counterstaining was performed with Mayers Haematoxylin (1 min RT) rinsed under tap water and coverslipped in Aquatex (water-based mounting medium, Merck, Darmstadt, Germany, no. 1.08562).

Absence of staining in other than the cell type under consideration served as negative controls. (As we realize this was not an optimal approach, we meanwhile apply isotypic Ig as negative controls.) Positive staining of other cases with the same antibody simultaneously applied served as positive control.

Specifications and applied dilutions (in PBS) of primary antibodies towards:

Ber-EP4	1 : 200, Dako, Glostrup, Denmark, no. M804; marks surface and cytoplasmic glycoprotein,	MAC 387	1 : 2000, Dako, Glostrup, Denmark, no. M0747; marks cytoplasmic myeloid-histiocyte,
B72.3	1 : 1600, BioGenex Lab, S. Remon, C., USA, no. MAU054UC; marks cytoplasmic tumour-associated glycoprotein,	MNF 116	1 : 500, Dako, Glostrup, Denmark, no. M0821; marks cytoplasmic cytokeratin,
CA125	1 : 80, Cis bio international, Sur Yvette, France, no. CA125II; marks membrane hihmolecular mucin,	Vimentin	1 : 600, Dako, Glostrup, Denmark, no. M725; marks cytoplasmic intermediate filaments.
CEA	1 : 200, Dako, Glostrup, Denmark, no. M707; marks cytoplasmic carcinoembryonic antigen, a heavily glycosilated protein,		
Desmin	1 : 500, Dako, Glostrup, Denmark, no. M0760; marks cytoplasmic intermediate filaments,		
EMA	1 : 1600, Dako, Glostrup, Denmark, no. M0613; marks epithelial membrane antigen, a human milk fat globulin,		
HBME-1	1 : 1400, Dako, Glostrup, Denmark, no. M3505; marks cytoplasmic intermediate filaments,		
Leu-M1	1 : 1000, Dako, Glostrup, Denmark, no. M0733; marks cytoplasmic oligosaccharid,		
Lu5	1 : 2500, Biochemicals AG, Augst, Switzerland, no. T-1302; marks cytoplasmic cytokeratin,		

2.5. Evaluation of immunocytochemically stained slides

Slides were evaluated independently by two experts (M. K. and H. M.). The following semiquantitative score was applied: “–” (negative) for no cellular staining detectable. “(+)” (negative) for less than 5% of the respective cell population weakly stained. “+” (positive) for 6–30% of the respective cell population stained, “++” (positive) for 31–70% of the respective cell population stained and “+++” (positive) for 71–100% of the respective cell population stained. Staining intensity was usually strong but played no role in scoring the quantity of immunocytochemical reaction. To exclude false positive results due to artificial or un-specific staining, less than 5% of weakly stained cells (“(+)”) was declared as the cut-off value for interpretation of staining results as negative. Antibody reactions were evaluated per case, without knowledge of patient follow-up in order to avoid any bias in decision making. Results were then discussed between both observers. There were no discrepancies in the interpretation of staining results as positive or negative.

Prevalence of staining was thus evaluated for each antibody in reactive mesothelial cells, macrophages and in the respective abnormal cell population in each effusion. Possible unspecific cellular staining of lymphocytes and granulocytes was registered.

2.6. Staining of specimens for DNA-cytometry

Slides prestained according to May–Grünwald–Giemsa were uncovered in xylene and Feulgen stained in a temperature-controlled staining machine with Schiff’s reagent according to the protocol described earlier [13,14,56]. In brief, after rehydration in decreasing ethanol concentrations and re-fixation in buffered 10% formalin, 5 N HCl for acid hydrolysis was applied at 27°C for one hour, followed by staining in Schiff’s reagent (Merck, Darmstadt, Germany, no. 1.09033.0500) for another hour, followed by rinsing in

SO₂-water and dehydration at increasing ethanol concentrations. The slides were then covered with Entellan (Merck, Darmstadt, Germany, no. 1.07961.0500).

2.7. Measurement

Measurements of nuclear DNA were performed as described in our previous papers [13,14,38,56]. In brief, 30 lymphocytes were measured as reference cells, meeting a coefficient of variation of $\leq 5\%$. Subsequently, if present, 300 reactive mesothelial, atypical or abnormal cells were measured per specimen interactively at random. The ZEISS CYRES workstation (Zeiss, Jena, Germany) was used for measurements. It consists of a conventional light microscope Axio-plan 2 (Zeiss, Jena, Germany) with a 40 \times objective; NA 0.75; Köhler illumination was performed to reduce the stray light and a 570 nm interference filter ± 10 nm half value width was used. A CCD black and white camera (VarioCam (Modell CCIR), PCO Computer Optics, Kehlheim, Germany) with 572 lines resolution was adapted to the microscope. The images were analyzed using a PC Pentium I processor and framegrabber (Kontron, Eching, Germany) and VGA screen, mouse and laser printer. The performance of the system meets the standards of the European Society for Analytical Cellular Pathology (ESACP) task force on standardization of diagnostic image cytometry [14, 38]. Segmentation was performed automatically on individual nuclei by grey level thresholding taking the local background into consideration. The glare error was corrected by software as previously described [13], at a rate of 2.2%.

The data were diagnostically interpreted as described in a previous paper [56]. DNA-aneuploidy was assumed, if the DNA-index of an abnormal stemline was $< 0.925 > 1.075$ or $< 1.80 > 2.20$ or $< 3.60 > 4.40$, a coefficient of variation (CV) of the first stemline was $\geq 10\%$ and/or cells $> 9c$ occurred ($9c$ exceeding events ($9c$ EE)).

2.8. Validation of cytologic diagnoses

According to patient follow-up (periods of 6–18 months) the investigated effusions were classified as either containing malignant cells or not. Patient histories were only accepted for evaluation if retrospectively presenting sufficient evidence for the presence or absence of tumour cells in effusions. These revealed either histologic follow-up of a tumour of the serous membranes itself ($43/154 = 27.9\%$) or of

the respective primary tumour (with clinical evidence of a malignant tumour) ($57/154 = 37.0\%$) or clinical evidence for the malignant or benign nature of the effusion, applying advanced diagnostic techniques (i.e., radiology, computed tomography, laparoscopy, $54/154 = 35.1\%$) was found. Patients presenting tumour cell positive effusions revealed the following primary tumours: carcinomas of the breast (17), the ovary (22), the endometrium (1), the lip (1), the thyroid (1), the lung (19), the esophagus (1), the stomach (2), the colon sigmoideum (2), the pancreas (1), the kidney (1), the urothelium (1), as well as carcinomas of unknown primary (CUP) (18). Furthermore, malignant mesotheliomas (without further histologic classification) (14) as well as a non-Hodgkin-lymphoma (1) occurred. Although cytologically mimicking cells of a carcinomatosis, the effusion due to lymphoma was excluded from the evaluation as immunocytochemical markers were selected to solely distinguish between epithelial and mesothelial cells and not cells of lymphoma. According to histologic follow-up, among the tumour cell positive effusions, there were two cytodiagnostically seemingly “false positive” cases both due to chronic unspecific pleuritis. Both cases undoubtedly revealed tumour cells even on second-look inspection. This was confirmed by immunocytochemistry and DNA-cytometry. Patients presenting tumour cell negative effusions showed the following basic diseases: pneumonia/pleuritis (17), congestive heart failure (21), renal insufficiency (1), cirrhosis of the liver (6), endometriosis (2), ovarian cyst (1), gastritis (1), pancreatitis (1), insufficiency of rectum anastomosis (1) and cholecystitis (1). Furthermore, there was one cytologically “false negative” pleural effusion due to a carcinoma of the lung, which was detected as tumour cell positive by immunocytochemistry and by DNA-cytometry. It was re-evaluated as tumour cell positive on cytologic second-look inspection.

A tumour cell positive effusion was considered as truly positive if it was confirmed by medical report with one of the above mentioned techniques. In two cases with positive cytological diagnosis but without further clinical, radiological or histopathological evidence of a malignant tumour re-evaluation of the slides (by two expert cytopathologists: H. M. and A. B.) led to a definitively positive morphological diagnosis, confirmed by immunocytochemistry and DNA-cytometry.

To evaluate the specificity of the method, the reports on patients with any non-positive diagnosis in effusion cytology were checked for further evidence of a malignant tumour. If there were no discrepancies between

the cytologic diagnosis and the medical report, the diagnosis was considered as truly negative. A cytologic diagnosis was also considered as truly negative if clinical evidence of a non-tumourous origin of the effusion was given, also despite the fact that a patient might have had a malignant tumour as well. It was classified as false negative ($n = 1$) if a malignant tumour was diagnosed clinically, radiologically or histopathologically during the patients follow-up period and if no non-tumourous origin of the effusion was found. By re-evaluation of slides tumour cells were found on second-look inspection.

After comparing results of cytology, immunocytochemistry and DNA-cytometry with histologic follow-up diagnoses seven cases were re-evaluated. In these cytologically correctly positive diagnosed cases histologic follow-up showed discrepancies with combined cytological-, immunocytochemical- and DNA-cytometric diagnoses (these three methods were in accordance) concerning the histogenetic tumour typing. In these cases histologic specimens were re-evaluated and the primary diagnosis doubted. Five patients with the histologic diagnosis "suspicious for mesothelioma" were re-evaluated as carcinomas, as cytology, as well as both adjuvant methods clearly demonstrated the epithelial origin of the cells and histology was also consistent with this finding. One histologic diagnosis of carcinoma was re-evaluated as mesothelioma, as cytology, as well as both adjuvant methods clearly demonstrated the mesothelial origin of the cells and histology was consistent with this finding as

well. Specimens of one further patient with history of a chronic lymphatic leukemia were re-evaluated and diagnosed to contain cells of an adenocarcinoma as all three methods: cytology, immunocytochemistry and DNA-cytometry were in accordance, so that it must be concluded that the patient has an occult adenocarcinoma additionally to his leukemia.

3. Results

Prevalences of cellular staining for different antibodies in benign and malignant effusions (totally as well as separately for carcinomas and mesotheliomas) are given in Figs 1–4.

Ber-EP4, anti-Leu-M1, -CEA and -EMA were the immunocytochemical markers with the most distinct results in the detection of malignant cells and the differential diagnosis between benign and malignant mesothelial cells and cells of metastatic carcinomas (Table 1). While staining for Ber-EP4 in tumour cells had a prevalence of 82.2% in malignant effusions, no cell type reacted with this marker in benign effusions (0%). Within malignant effusions prevalence was 95.4% in metastatic carcinomas and 0% in mesotheliomas. Ber-EP4 stained adenocarcinomas, as well as squamous cell- and small cell carcinomas. The four Ber-EP4 negative malignant effusions were due to ovarian ($n = 2$), thyroid ($n = 1$) and urothelial ($n = 1$) cancers, this number of cases being too small to detect any correlation between different types of pri-

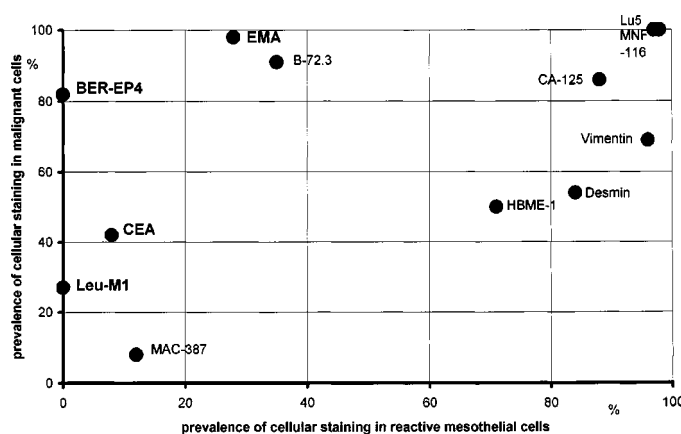


Fig. 1. Prevalence of cellular staining by twelve different immunocytochemical markers in malignant cells in 101 tumour cell positive effusions as opposed to reactive mesothelial cells in 53 tumour cell negative effusions.

Note: Ber-EP4 marks a surface and cytoplasmic glycoprotein, Lu5 and MNF 116 mark cytoplasmic cytokeratins, EMA (epithelial membrane antigen) marks a human milk fat globulin, B72.3 a cytoplasmic tumour-associated glycoprotein, Leu-M1 a cytoplasmic oligosaccharid, CEA (carcinoembryonic antigen) a heavily glycosilated cytoplasmic protein, CA-125 a membrane highmolecular mucin, HBME-1 cytoplasmic mesenchym, vimentin and desmin mark cytoplasmic intermediate filaments, and MAC 387 marks cytoplasmic myeloid-histiocyte structures.

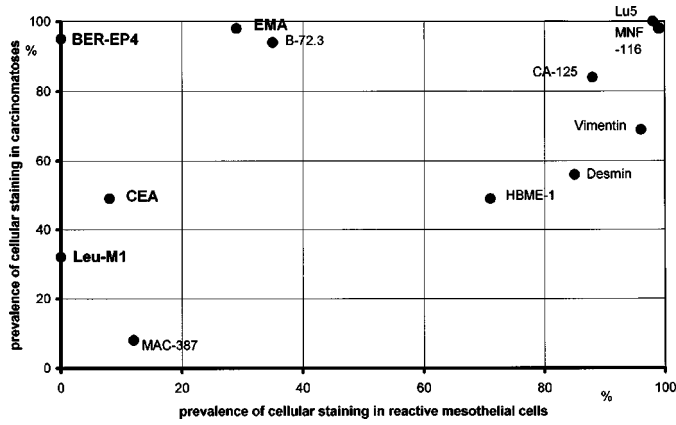


Fig. 2. Prevalence of cellular staining by twelve different immunocytochemical markers in carcinomatoses in 87 tumour cell positive effusions as opposed to reactive mesothelial cells in 53 tumour cell negative effusions.
Note: (see Fig. 1).

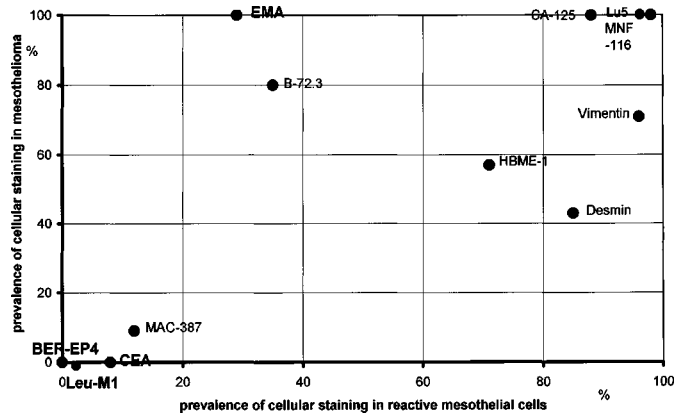


Fig. 3. Prevalence of cellular staining by twelve different immunocytochemical markers in mesotheliomas in 14 tumour cell positive effusions as opposed to reactive mesothelial cells in 53 tumour cell negative.
Note: (see Fig. 1).

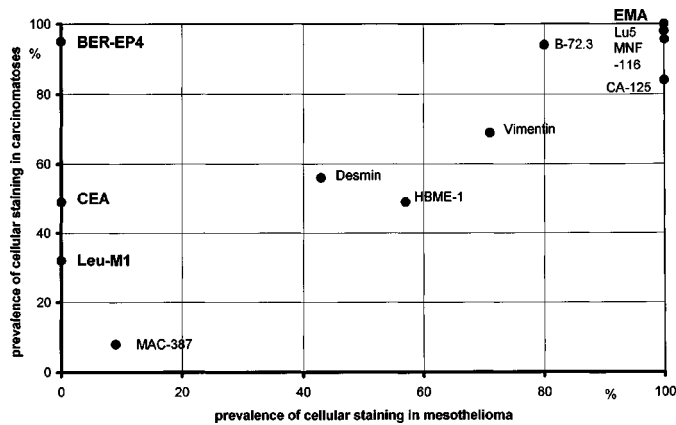


Fig. 4. Prevalence of cellular staining by twelve different immunocytochemical markers in carcinomatoses in 87 tumour cell positive effusions as opposed to in mesotheliomas in 14 tumour cell positive effusions.
Note: (see Fig. 1).

Table 1
Prevalence of cellular staining by four antibodies in effusions

Follow-up diagnoses	Ber-EP4	Leu-M1	EMA	CEA
Without tumour cells <i>n</i> = 53	0% (0/53)	0% (0/53)	28.8% (15/52)	7.7% (4/52)
Carcinomatosis <i>n</i> = 87	95.4% (83/87)	32.2% (28/87)	97.7% (85/87)	49.4% (42/85)
Mesothelioma <i>n</i> = 14	0% (0/14)	0% (0/14)	100% (14/14)	0% (0/14)
Total with tumour cells <i>n</i> = 101	82.2% (83/101)	27.7% (28/101)	98.0% (99/101)	42.4% (42/99)

mary tumours and absent Ber-EP4 staining. Anti-Leu-M1, -EMA and -CEA revealed prevalences of 27.2, 98.0 and 42.6% in malignant cells in effusions (32.2, 97.7 and 49.4% in metastatic carcinomas and 0, 100 and 0% in mesotheliomas). Prevalences of staining of reactive mesothelial cells in benign effusions was 0% for anti-Leu-M1, 28.8% for -EMA and, 7.6% for -CEA, respectively. There was no special pattern of staining of antibodies to be detected that could be considered as distinctive for a certain cell type. Especially EMA did not consistently show a thick membrane pattern in mesotheliomas (as sometimes mentioned in the literature [7]), but instead sometimes also showed it in reactive mesothelial cells or carcinomas. Ber-EP4 as well seldomly stained in a thick membrane pattern without specificity for a certain cell type. Of the four above mentioned antibodies anti-Leu-M1 was the only marker unspecifically staining granulocytes. No staining of lymphocytes or macrophages was seen.

In effusions containing malignant cells immunocytochemistry was able to correctly differentiate between metastatic carcinomas and mesotheliomas in 98.0% (99/101) by applying Ber-EP4 and/or anti-Leu-M1 which were both exclusively present in cells of metastatic carcinomas and/or -CEA which was in malignant effusions only present in metastatic carcinomas.

Table 1 demonstrates in detail the results of immunocytochemistry in the differential diagnosis of benign and malignant mesothelial and metastatic carcinoma cells. While anti-Ber-EP4 showed positive cellular staining in 95.4% of malignant effusions due to metastatic carcinomas, it never showed positive staining of any cell type in benign effusions (0%) or mesotheliomas (0%). Positive cellular staining (+, ++, +++) by anti-Leu-M1 was exclusively seen in effusions due to metastatic carcinomas (in 32.2%). Anti-EMA showed positive cellular staining in all three differential diagnoses mentioned above, but

nearly 100% positivity of all abnormal cells is only seen in malignant effusions. Cellular staining for anti-CEA was observed in benign effusions (7.6%) and those due to carcinomas (49.9%), but not in effusions due to mesotheliomas (0%).

The other immunocytochemical markers were not as helpful distinguishing benign or malignant mesothelial cells or those from carcinomas. In spite of optimized protocols they either showed constant positive (anti-Lu5 and anti-MNF 116) or negative (anti-MAC 387) staining of both reactive mesothelial cells in benign as well as abnormal cells in malignant effusions due to metastatic carcinoma as well as to mesothelioma and therefore did not contribute to the differential diagnosis. Or they revealed strong differences in scoring (from negative to “+++” positive) (anti-CA125, anti-desmin and anti-vimentin). Furthermore, technical difficulties were encountered such as improper fixation or overstaining and therefore evaluation was almost impossible (anti-B72.3, anti-HBME-1) leading to high interobserver variability. Some of these antibodies showed staining of other cell types in effusions, making interpretation difficult: anti-Leu-M1 (strongly), MAC387 (strongly) and -CEA (weakly) stained neutrophil granulocytes and monocytes; MAC387 of course stained macrophages as well.

Combinations of staining results of the four most helpful immunocytochemical markers: Ber-EP4, anti-Leu-M1, -EMA and -CEA were considered (Table 1). Their ability to detect malignant cells in effusions, without loss of specificity of 100%, was regarded. Ber-EP4 as the most sensitive and specific marker was able to detect abnormal cells in 82.2% (83/101) of the malignant effusions. Of the 18 cases not staining positive for Ber-EP4, 14 were effusions due to mesothelioma, four to carcinomas. Anti-Leu-M1 as a marker for the detection of malignant cells found only one additional carcinoma.

Table 2
Prevalence of DNA-aneuploidy in tumour cell positive and negative effusions

DNA-ploidy-status	Follow-up diagnosis			Total with tumour cells <i>n</i> = 101
	Without tumour cells <i>n</i> = 52 (100%)	Carcinomatoses <i>n</i> = 87 (100%)	Mesothelioma <i>n</i> = 14 (100%)	
Non-aneuploid	52 (100.0%)	4 (4.6%)	6 (42.9%)	10 (9.9%)
Aneuploid	0 (0.0%)	83 (95.4%)	8 (57.1%)	91 (90.1%)

Table 3
Prevalence of cellular staining by Ber-EP4 and DNA-aneuploidy in effusions

Follow-up diagnoses	ICC: BER-EP4	DNA-ICM: aneuploidy	ICC and/or DNA-ICM
Without tumour cells <i>n</i> = 53	0% (0/53)	0% (0/53)	0% (0/53)
Carcinomatosis <i>n</i> = 87	95.4% (83/87)	95.4% (83/87)	100% (87/87)
Mesothelioma <i>n</i> = 14	0% (0/14)	57.1% (8/14)	57.1% (8/14)
Total with tumour cells <i>n</i> = 101	82.2% (83/101)	90.1% (91/101)	94.1% (95/101)

Regarding Figs 1–4 it can be concluded that it is possible to choose panel combinations resulting in an increase of sensitivity yet associated with a loss of specificity.

None of the DNA-histograms of mesothelial cells in non-malignant, inflammatory or reactive effusions revealed any of the above mentioned criteria of DNA-aneuploidy and were therefore all interpreted as DNA-euploid (Table 2). This corresponds to a prevalence of 100% of the marker DNA-euploidy in reactive mesothelial cells.

The DNA-histograms of effusions containing malignant cells presented one, two or three aspects of DNA-aneuploidy mentioned above. On this basis 83/87 of the effusions due to metastatic carcinomas and 8/14 due to mesotheliomas were DNA-aneuploid (Table 2). This corresponds to a prevalence of DNA-aneuploidy in malignant cells in effusions of 95.4% for metastatic carcinomatoses and 57.1% for mesotheliomas. The detection rate of DNA-aneuploidy depends on the application of one to three different algorithms. Nuclear DNA-values $> 9c$ represented the most frequent aspect of DNA-aneuploidy (86.1%). Additionally accepting an abnormal stemline, present in 79.2%, as an aspect of DNA-aneuploidy, prevalence increased to 90.1%. Applying a CV $\geq 10\%$ as another aspect of DNA-aneuploidy, present in 21.8% of cases, did not result in a further increase of detection rate.

While 64.3% (9/14) of mesotheliomas showed their greatest DNA-stemline within the range of 1.80c and

2.20c, only 9.2% (8/87) of carcinomatoses showed their greatest stemline in this region.

Combining immunocytochemistry solely applying Ber-EP4 as marker with DNA-cytometry leads to an increase in detection of cells of carcinomatoses from 95.4% only applying the former and 95.4% the latter to 100% applying both methods (Table 3). For malignant mesothelioma cells the detection rate in effusions is 0% solely applying immunocytochemistry and 57.1% DNA-cytometry (Table 3).

4. Discussion

We have investigated 101 tumour cell positive and 53 -negative effusions concerning immunocytochemical staining for 12 antibodies as well as the marker DNA-aneuploidy by DNA-image cytometry.

Staining for Ber-EP4 had a prevalence in tumour cells of 82.2% (95.4% in metastatic carcinomas and 0% in mesotheliomas). Since our analysis achieved a specificity of 100% of negative staining for the immunocytochemical marker Ber-EP4 in benign effusions, false positive diagnosis due to unspecific cellular staining in reactive effusions are not to be expected. We propose that staining of cells for Ber-EP4 in effusions may be used as a highly specific and sufficiently sensitive marker for neoplastic epithelial cells. This marker can furthermore differentiate between cells of metastatic carcinoma and mesothelioma in 98.0%.

The application of immunocytochemistry as a panel obviously reflects insufficient sensitivity and specificity of single markers which were so far applied. Our study on prevalences of 12 different antibodies in cells of benign and malignant effusions demonstrates that the sole use of Ber-EP4 results in an excellent detection rate of malignant epithelial cells. We therefore do not recommend the use of a panel of antibodies for the detection of malignant cells in effusions. For the differential diagnosis between malignant cells due to carcinomas and mesotheliomas we suggest the additional use of anti-Leu-M1 and/or -CEA which were only positive in cells of metastatic carcinomatosis. Recently, we additionally apply anti-calretinin to differentiate between mesothelial and epithelial cells in effusions. It seems that anti-calretinin is a useful additional marker for the differentiation between carcinomas and mesotheliomas as it is a highly sensitive and rather specific marker for reactive and malignant mesothelial cells. The reduction of an immunocytochemical panel to 1–2 antibodies helps to reduce laboratory and personnel costs without loss of sensitivity and specificity. We do not recommend the application of an immunocytochemical panel using different coefficients attributed to various antibodies for the differentiation between metastatic carcinomatosis and mesothelioma [24,25].

Altogether, immunocytochemistry as an adjuvant method seems to be a valuable tool for the detection of malignant cells in effusions and in the differential diagnosis between metastatic carcinoma and mesothelioma. Thus, this method should be able to increase diagnostic accuracy of conventional effusion cytology.

Reasons for differences in prevalences of, e.g., Ber-EP4 positive staining (32–100%) in the literature as well as compared to our study, are the following: while we applied Delaunay fixation on smears of effusion sediments followed by staining according to the Avidin–Biotin–Complex method other authors have used many different types of fixations on various types of cell preparations also applying different staining methods. All of the factors influence immunocytochemical staining results. We suggest to apply immunocytochemical methods only according to standardized protocols optimized especially for effusion specimens. Merely applying a protocol established only for histologic material without optimal fixation and tested antibody concentrations for cytologic specimens will lead to unsatisfactory results. Different study designs (i.e., patient populations, selection of effusions for cytologic evaluation) may also lead to different results.

A further important aspect concerning the interpretation of immunocytochemical staining in effusions is the “cut-off” level for acceptance of cellular staining as positive. Even if an antibody is absolutely specific in detecting a certain cell type, there remains a risk of unspecific staining of a few cells, e.g., due to cellular degeneration or necrosis or to serum precipitates. Interpretation of staining results may also be due to equivocal morphological typing of cells. Therefore, a “cut-off” level of >5% staining of the respective population of cells is recommended to accept a staining result as positive, otherwise false positive results may occur.

Qualifications for the exact judgement of the efficacy of an antibody to detect malignant cells in effusions are: cytology-adapted techniques of immunocytochemistry [21], the interpretation of staining results by a semiquantitative scoring scheme in different cell types and the calculation of prevalences of staining per cell type and antibody. These are the prerequisites to then compare the efficacy of antibodies in a panel. With these data we now may conclude that a panel of immunocytochemical markers is not necessary.

Furthermore, in our study prevalence of immunocytochemical markers is compared with that of DNA-aneuploidy in tumour cell positive and negative effusions.

The diagnostic value of DNA-cytometry for the identification of malignant cells in effusions has been demonstrated by our group in two previous studies [56, 57]. We proposed the combined use of three different algorithms for the identification of DNA-aneuploidy: position of any DNA-stemline, CV of the first stemline $\geq 10\%$ and occurrence of cells $> 9c$.

Considering the diagnostic interpretation of DNA-cytometric results, we recommend strict consideration of the standards of the European Society for Analytical Cellular Pathology (ESACP) for diagnostic DNA-image cytometry [14,38], otherwise, false positive diagnoses may occur.

In this study our measurements identified DNA-aneuploidy in 90.1% of specimens from malignant effusions (95.4% in metastatic carcinomas and 57.1% in mesotheliomas). Since our analysis achieved a specificity of 100% for the marker DNA-euploidy to confirm absence of malignant cells in effusions, false positive diagnoses in reactive effusions are not to be expected. We therefore believe that DNA-image cytometry is also able to increase diagnostic accuracy of effusion cytology. In effusions containing malignant cells the location of the greatest stemline by DNA-cytometry is able to contribute to the differential di-

agnosis between metastatic carcinomas and mesotheliomas [56]. While 64.3% of mesotheliomas showed their greatest stemline within the range of 1.80c and 2.20c, only 9.2% of carcinomas did. In our previous study [56] none of the carcinomas showed their greatest stemline at this location.

Apart from our study three other authors have suggested the combined use of immunocytochemical markers and DNA-cytometry. While Croonen et al. [19], and Joseph et al. [41], suggest the use of DNA-flow cytometry as opposed to DNA-image cytometry, which is principally a good method for the detection of aneuploid DNA-stemlines, it should be considered that rare events (9c exceeding events) are usually not detected by DNA-flow cytometry. Matter-Walstra and Kraft [51] introduced the combined use of staining for Ber-EP4 and DNA-image cytometry. Principally, this is a good methodological procedure, but the authors used $5c\ ER \geq 10\%$ as the only criterion for DNA-aneuploidy. Yet, this is not an acceptable marker in cells undergoing euploid polyploidization, like mesothelial cells, as they normally reveal cells $>5c\ EE$ [12,57]. Therefore, cells with DNA-content between 4c and 8c must regularly be expected.

Problems arise concerning the interpretation of the follow-up of patients with cytologically tumour cell positive and -negative effusions. At present there is no general agreement as to the "golden standard" for evaluation of the diagnostic accuracy of immunocytochemical or DNA-cytometric diagnoses. This aspect is handled controversially in the literature. Some studies report on histological, others only on clinical evidence of the basic disease. Most authors merely compare immunocytochemical or DNA-cytometric results with routine cytologic diagnoses. In our study the immunocytochemical finding of malignant epithelial cells or the cytometric finding of DNA-aneuploidy was classified as correctly positive if patient follow-up revealed histologic or clinical evidence for a malignant nature of the effusion. They were classified as correctly negative if the patient follow-up revealed histologic or clinical evidence for a benign nature of the effusion. We felt it necessary to re-evaluate seven cases concerning their histogenetic classification of malignant cells. As adjuvant methods indicated the origin of the specimens as epithelial or mesothelial, histology had to be re-evaluated.

Part II of this study demonstrates the application of these adjuvant methods in diagnostically equivocal effusions.

5. Conclusion

We assume that with cytology-adapted techniques of immunocytochemistry, standardized interpretation of staining results as well as with improved precision of DNA-cytometry and application of different algorithms for histogram analysis both methods may help to unequivocally detect a high percentage of tumour cells in cytologically equivocal effusions. A combination of both methods will hopefully result in an increased sensitivity for the detection of malignant cells and thus decrease the rate of cytologically definitely doubtful effusions. In tumour cell positive effusions they may furthermore help to differentiate between metastatic carcinomas and mesotheliomas.

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