

## Papers

## Flow cytometric immunophenotyping of serous effusions and peritoneal washings: comparison with immunocytochemistry and morphological findings

Bjørn Risberg, Ben Davidson, Hiep P Dong, Jahn M Nesland, Aasmund Berner

### Abstract

**Aim**—To evaluate immunophenotyping by means of flow cytometry as a complementary method for the detection of malignant cells in serous effusions and peritoneal washings.

**Material and methods**—Frozen samples of 49 fresh serous effusions and peritoneal washings were analysed by flow cytometry, using monoclonal antibodies against CD45, Ber-EP4, and N-cadherin. Results were compared with smear and cell block morphology, as well as immunocytochemistry on paraffin wax embedded cell blocks.

**Results**—Seventeen specimens were cytologically diagnosed as malignant, whereas 25 were interpreted as benign. The remaining seven specimens were diagnosed as indeterminate or suspicious for malignancy. Ber-EP4 positive cells were detected in 16 of the 17 cytologically malignant effusions, as well as in five of seven suspicious cases and five of 25 specimens with benign cytology. In the latter group, three specimens showed atypical or malignant cell groups that were missed in routine morphological evaluation. In two additional samples, obtained from patients with benign and borderline ovarian tumours, Ber-EP4 positive cells showed benign or mildly atypical features, and were interpreted as exfoliated benign or borderline malignant epithelial cells of tubal origin, or as endosalpingiosis. All five Ber-EP4 positive indeterminate specimens showed atypical or malignant cells on re-evaluation, and were Ber-EP4 positive in four of five cases using immunohistochemistry in cell block sections. Large numbers of CD45 positive and relatively few N-cadherin positive cells were detected in most specimens with the use of flow cytometry, when compared with morphological evaluation.

**Conclusions**—Flow cytometry is a rapid and highly effective method for the evaluation of effusions and peritoneal washings. The detection of Ber-EP4 positive cells using flow cytometry is strongly indicative

of the presence of carcinoma cells in effusions and peritoneal washings. Although false positives are relatively infrequent, all specimens should be carefully evaluated morphologically to prevent the diagnosis of benign epithelial clusters as malignant.

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The differential diagnosis between reactive mesothelial cells and metastatic adenocarcinoma is often extremely difficult in serous effusion specimens and peritoneal washings.

Immunocytochemistry, mainly on sections from paraffin wax embedded cell blocks, appears to be of use in increasing the diagnostic accuracy in many of these cases,<sup>1 2</sup> and is often used in routine practice for this purpose. However, the role of flow cytometry, a method that is widely used for the immunophenotyping of haematological specimens, in the analysis of serous effusions has not been fully investigated. To date, flow cytometry has mainly been used to detect aneuploid cell populations,<sup>3–8</sup> sometimes in combination with immunophenotyping of admixed lymphoid cells.<sup>9–11</sup> The number of natural killer (NK) cells has been used to predict the presence of malignant cells in serous effusions.<sup>12</sup> However, limited data are available regarding flow cytometric immunophenotyping of epithelial cells in effusions.<sup>13 14</sup>

Ber-EP4 is a monoclonal antibody directed against the protein moiety of two glycopeptides on human epithelial cells. First discovered in the breast carcinoma cell line MCF-7 by Latza and co-workers in 1989,<sup>15</sup> its role in the diagnosis of epithelial malignancies has been the subject of extensive research, which has been reviewed recently.<sup>16</sup> Although results of these studies vary greatly, several studies have shown its high specificity and sensitivity for distinguishing between epithelial and mesothelial cells.<sup>2 17–19</sup>

Cadherins are tissue specific integral membrane glycoproteins with a central role in cell–cell adhesion. They are located at the cell–cell adherens junction, forming homophilic con-

Department of Pathology, Division of Cytology, The Norwegian Radium Hospital, The University of Oslo, Montebello N-0310 Oslo, Norway  
B Risberg  
B Davidson  
H P Dong  
J M Nesland  
A Berner

Correspondence to:  
Dr Risberg  
email: brisberg@ulrik.uio.no

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Table 1 Clinical diagnosis and sample site

Diagnosis	Peritoneal effusion	Pleural effusion	Pericardial effusion	Peritoneal washing	Total
Breast carcinoma	0	15	0	0	15
Benign ovarian cyst	1	1	0	4	6
Ovarian borderline tumour	1	0	0	1	2
Ovarian carcinoma	2	2	0	6	10
Endometrial carcinoma	1	0	0	3	4
Tubal carcinoma	1	0	0	0	1
Cervix carcinoma	0	1	0	0	1
Colon carcinoma	1	1	0	0	2
Lung carcinoma	0	0	1	0	1
Adenocarcinoma NOS	0	1	0	0	1
Non-epithelial malignancy	1	2	0	0	3
Benign tumour NOS	1	2	0	0	3
Total	9	25	1	14	49

NOS, not otherwise specified.

Table 2 The antibodies used for flow cytometry

Antibody	Source	Lot number	Clone
FITC-Ber-EP4	Dako (Glostrup, Denmark)	026 (301)	Ber-EP4
PE-CD45	Dako (Glostrup, Denmark)	117 (301)	T29/33
FITC-CD14	Dako (Glostrup, Denmark)	016 (30)	Tük4
N-Cadherin	Becton-Dickinson (San-Jose, California, USA)	80956	NCAD2

FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Table 3 The association between cytological diagnosis and Ber-EP4 immunophenotyping using flow cytometry

Cytological diagnosis	The fraction of Ber-EP4 positive cells in the studied specimens				Total
	0%	>0.01 to <1%	≥1 to <5%	≥5%	
Benign	20 (80%)	2 (8%)	2 (8%)	1 (4%)	25 (100%)
Malignant	1 (6%)	7 (41%)	4 (24%)	5 (29%)	17 (100%)
Indeterminate	2 (29%)	5 (71%)	0	0	7 (100%)
Total	23 (47%)	14 (29%)	6 (12%)	6 (12%)	49 (100%)

tacts with neighbouring cells.<sup>20-22</sup> N-Cadherin is a 135 kDa protein found in nerve and muscle cells,<sup>23-25</sup> as well as in pleural mesothelial cells.<sup>26</sup> N-Cadherin expression was shown to be highly specific for cells of mesothelial origin, and its combined use with an antibody against the epithelial cadherin, E-cadherin, has been shown to aid in the differentiation of malignant mesotheliomas from adenocarcinomas.<sup>27-29</sup> However, the value of N-cadherin in the latter setting has been questioned recently in a large study of formalin fixed, paraffin wax embedded cell blocks from serous effusion specimens.<sup>30</sup>

The object of our study was to investigate the potential for flow cytometric immunophenotyping using a limited antibody panel, directed against Ber-EP4, N-cadherin, and the haematopoietic marker CD45, in the evaluation of 49 serous effusion and peritoneal washing specimens.

### Materials and methods

The studied material comprised 49 fresh non-fixed peritoneal washing and effusion specimens that were submitted to the division of cytology, department of pathology, the Norwegian Radium Hospital, during the period of January 1998 to July 1999. Table 1 lists the clinical diagnoses and specimen sites. Upon receipt, all specimens underwent a standard treatment and evaluation procedure, as described previously.<sup>2</sup> Morphological evaluation of cytological smears and cell block haematoxylin-eosin stained sections was carried out as reported previously.<sup>2</sup>

### FLOW CYTOMETRY

Flow cytometry was undertaken using the FACSCalibur flow cytometer (Becton-Dickinson, San Jose, California, USA). The frozen material was carefully thawed and 10 ml RPMI with 10% calf serum was added. After centrifugation at 290 ×g for 10 minutes, the supernatant was decanted. The cells were fixed in fixative A (Fix and Perm; Caltag Laboratories, Burlingame, California, USA) for 15 minutes at room temperature, washed in 5 ml of phosphate buffered saline (PBS), centrifuged for eight minutes at 200 ×g, and subsequently divided into different tubes for direct and indirect staining. The mean number of cells analysed (gated cells) was 6300. Table 2 details the antibodies used. The combinations used were phycoerythrin (PE)-CD45/fluorescein isothiocyanate (FITC)-Ber-EP4, N-cadherin + indirect PE/FITC-Ber-EP4, and, in a few cases, PE-CD45/FITC-CD14 also.

### DIRECT STAINING PROCEDURE

Aliquots of 50 µl (1/10) PE and FITC conjugated monoclonal antibodies were added to the wells. The cells were then incubated in the dark at room temperature for 25 minutes.

A few drops of PBS were then added and the cells centrifuged for eight minutes at 200 ×g. The supernatant was decanted, and 500 µl FACSFlow was added, followed by filtration of the samples through a 70 µm nylon filter. The samples were then put on ice until analysis.

### INDIRECT STAINING PROCEDURE (N-CADHERIN)

Non-specific staining was blocked by incubation with 100 µl mouse myeloma IgG1 protein (1/50) for 25 minutes at room temperature. Cells were subsequently washed with a few drops of PBS, and 50 µl primary non-conjugated antibody (1/20) was added, followed by incubation for 25 minutes at room temperature. Cells were then washed again with a few drops of PBS and centrifuged for eight minutes at 200 ×g.

Aliquots of 50 µl secondary conjugated antibody (1/10 goat antimouse, PE conjugated) were added, and the cells were incubated in the dark at room temperature for 25 minutes. Cells were then washed twice in PBS, followed by the addition of 50 µl of primary (1/10 dilution) FITC conjugated mouse antihuman antibody. After mixing, cells were incubated in the dark at room temperature for 25 minutes. A few drops of PBS were then added, followed by centrifugation for eight minutes at 200 ×g. The supernatant was decanted and 500 µl FACS-Flow added, followed by filtration of the samples through a 70 µm nylon filter. The samples were then put on ice until analysis.

### CONTROLS

A mixture of a Ber-EP4-positive mammary carcinoma cell line (T47-D) and human mononuclear leucocytes was analysed in each run. In pilot studies, the analysis of several control cell mixtures (with different breast carcinoma cell lines) showed that dot plots were optimal when cells were fixed in fixative A (Fix and Perm). Non-specific blocking with mouse

Table 4 Discrepant cases with an initial benign diagnosis

Clinical diagnosis	Age	Sample	Cytological diagnosis	FCM Ber-EP4 (% positive cells)	Diagnosis after re-evaluation
Benign ovarian cyst	68	Peritoneal washing	Benign	0.4	Benign
Endometrial carcinoma	51	Peritoneal washing	Benign	2.0	Benign
Ovarian carcinoma	27	Peritoneal washing	Benign	0.4	Atypical
Ovarian borderline tumour	45	Peritoneal washing	Benign	10	Atypical
Ovarian carcinoma	53	Peritoneal effusion	Benign	3	Malignant

FCM, flow cytometry.

myeloma protein (IgG1) was beneficial when indirect conjugation was used. Without its use, "false" double positive cells were observed with the use of directly conjugated antibodies.

## EVALUATION OF FLOW CYTOMETRY PHENOTYPING

This was undertaken in a standardised way. A simple gating procedure was used to exclude cell debris, by including only cells with higher forward scatter and side scatter than the values for lymphocytes in the analyses. Lymphocytes were easily detected in the scatter plot through the use of control values. Quadrant cursors were set by using isotypic negative controls. Quadrant setting was undertaken so that in negative controls 99% of the cells were localised to the left lower quadrant. Cell populations were interpreted as immunoreactive for CD45, Ber-EP4, and N-cadherin only when unequivocal separation from the negative controls (lymphocytes in the case of Ber-EP4, car-

cinoma cell lines in the case of CD45, and both cell types in the case of N-cadherin) could be demonstrated. The proportion of immunoreactive cells was calculated.

## IMMUNOCYTOCHEMISTRY

A comparative immunohistochemical analysis of Ber-EP4 staining on formalin fixed, paraffin wax embedded cell block sections (29 specimens) was undertaken as described previously.<sup>2</sup>

## Results

Seventeen specimens were cytologically diagnosed as malignant, whereas 25 were interpreted as benign. The remaining seven specimens were diagnosed as indeterminate or suspicious for malignancy.

Table 3 details the flow cytometry results regarding the presence of Ber-EP4-positive cells in the 49 studied specimens, as well as the relation between flow cytometry results and the cytological diagnoses. Altogether, Ber-EP4-positive cells were detected in 16 of the 17 cytologically malignant effusions, as well as in five of seven suspicious cases and five of 25 specimens with benign cytology. In re-evaluation of the latter group, three specimens showed isolated, often degenerated, atypical or clearly malignant cell groups and psammoma bodies that were missed in routine morphological evaluation. In two additional samples, obtained from patients with benign and borderline ovarian tumours, Ber-EP4 positive cells showed benign or mildly atypical features,

Table 5 Discrepant cases with an initial indeterminate diagnosis

Clinical diagnosis	Age	Sample	Cytological diagnosis	Ber-EP4 (% positive cells)		Diagnosis after re-evaluation
				FCM	IHC	
Ovarian carcinoma	54	Pleural effusion	Indeterminate	10.0	NA	Malignant
Colon carcinoma	40	Peritoneal effusion	Indeterminate	0.02	1.0	Malignant
Ovarian carcinoma	57	Pleural effusion	Indeterminate	0.10	0.1	Atypical
Breast carcinoma	53	Pleural effusion	Indeterminate	0.10	1.0	Malignant
Ovarian carcinoma	72	Peritoneal effusion	Indeterminate	0.70	1.0	Malignant
Breast carcinoma	63	Pleural effusion	Indeterminate	0	0	Benign
Benign effusion NOS	66	Peritoneal effusion	Indeterminate	0	0	Atypical

NA, not available owing to insufficient material.

FCM, flow cytometry; IHC, immunohistochemistry; NOS, not otherwise specified.

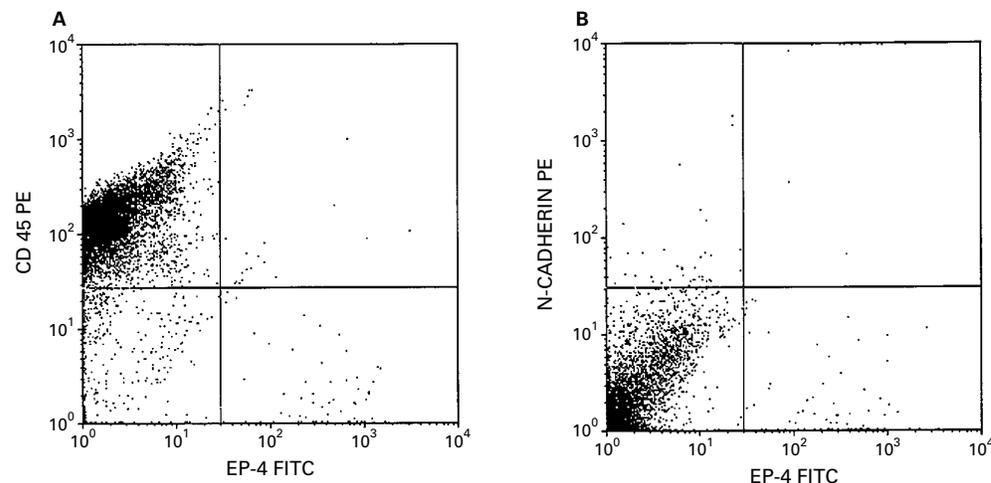


Figure 1 Flow cytometry results of a metastatic carcinoma of unknown origin, showing unequivocal Ber-EP4 positive cells, as well as large numbers of CD45 positive cells and a few N-cadherin positive cells.

and were thus interpreted as exfoliated benign or borderline malignant epithelial cells of tubal origin, or as endosalpingiosis. This finding was confirmed with the use of immunohistochemistry in one case. All five Ber-EP4 positive indeterminate specimens showed atypical or malignant cells on re-evaluation, Ber-EP4 positive in four of five cases using immunocytochemistry in cell block sections. The single specimen in the malignant specimen group that contained Ber-EP4 negative malignant cells originated from a patient with a previously diagnosed malignant melanoma. Tables 4 and 5 show detailed findings for discrepant cases in the benign and indeterminate diagnostic groups, respectively.

Large numbers of CD45 positive and relatively few N-cadherin positive cells were detected in most specimens with the use of flow cytometry, when compared with the morphological evaluation.

The mean percentage of N-cadherin positive cells was 1.1% (range, 0–14%; SD, 2.2%), and the mean percentage of CD45 positive cells was 81.4% (range, 18–99%; SD, 25.6%). The number of CD14 positive cells was much greater than that of N-cadherin positive cells in all specimens in which the former was included in the panel. Ber-EP4 and N-cadherin double positive cells were not detected in any of the specimens studied (fig 1).

A comparison between flow cytometry and immunohistochemistry regarding the presence of Ber-EP4 positive cells was possible in 29 cases. Within this group, 16 specimens were positive using both methods, 12 were negative using both, and one case was flow cytometry positive and immunohistochemistry negative. In the latter case, representative cells were probably not present in cell block sections.

### Discussion

Our study evaluated the potential of flow cytometry in the diagnosis of serous effusion and peritoneal washing specimens using a limited panel of four monoclonal antibodies.

Immunophenotyping using flow cytometry is a sensitive and rapid method of detecting cellular surface antigens in cytological material. It facilitates the evaluation of cell populations using simultaneous double or triple staining analyses, thus making it possible to characterise in a more precise manner various cell types. However, the use of flow cytometry for epithelial cell immunophenotyping has been reported in only two studies. Czerniak and co-workers demonstrated the sensitivity of flow cytometry in the detection of malignant cells using the monoclonal antibody Ca1. Fourteen of 17 malignant specimens had a more intense fluorescence when compared with controls.<sup>13</sup> Tamai and co-workers compared immunohistochemistry and flow cytometry results in nine cases of gastric carcinoma, using a monoclonal antibody against carcinoembryonic antigen (CEA). Comparable results were obtained in eight of nine specimens (four immunoreactive and four negative). However, quantitative evaluation of the immunoreactive cell populations was available

only with the use of flow cytometry.<sup>14</sup> Our results are in agreement with the latter report. Flow cytometry showed a high concordance with immunohistochemistry: 28 of the 29 cases that had enough material for both analyses showed similar results (16 specimens with Ber-EP4 positive and 12 with Ber-EP4 negative cells). In addition, the evaluation of the fraction of immunoreactive cells was by far easier using flow cytometry. In small specimens, flow cytometry facilitated the evaluation of larger numbers of cells than cell block sections. Thus, flow cytometry appears to aid not only in the detection of malignant cells, but also in the evaluation of other cell types. Mesothelial cells and histiocytes are often difficult to differentiate from each other in cytological smears and cell blocks because of their similar size and overlapping morphological characteristics. In our experience, histiocytes are often interpreted as mesothelial cells, leading to erroneous evaluation of the extent of mesothelial proliferation. Because extreme mesothelial proliferation often raises the suspicion of malignant mesothelioma, an accurate evaluation of reactive cell populations is of more than academic value. Flow cytometry results, using anti-CD14 for the monocyte/macrophage lineage and anti-N-cadherin for the identification of mesothelial cells, showed far higher numbers of histiocytes than mesothelial cells in most of the specimens studied.

The use of Ber-EP4 as an epithelial marker is now an acceptable approach to the study of effusions, as well as tissue sections. Moreover, it appears to be of benefit for the detection of circulating tumour cells in the peripheral blood of patients diagnosed with epithelial malignancies.<sup>31</sup> We have previously reported the high sensitivity and specificity of Ber-EP4 in the detection of epithelial cells in serous effusions.<sup>2</sup> Our present report supports our previous findings, because Ber-EP4 immunophenotyping is both highly sensitive and highly specific, the latter confirmed by the lack of double positive cells using flow cytometry. However, there is a risk of false positive results because of the occasional presence of benign epithelium from the fallopian tube or endosalpingiosis, both of which are often Ber-EP4 positive. Therefore, the presence of Ber-EP4 positive cells should alert the cytologist to the possible presence of malignant epithelial cells, but is not a sine qua non to the diagnosis of malignancy.

The ability of N-cadherin to aid in the distinction between cells of mesothelial origin and adenocarcinoma cells has been demonstrated in three studies by Peralta-Soler *et al.*<sup>27–29</sup> Studying a total of 63 tumours, as well as two malignant mesothelioma cell lines, the authors concluded that the combined use of E-cadherin and N-cadherin could distinguish between the two cell types, and is associated with both high sensitivity and specificity. However, the anti-N-cadherin antibody used in the latter studies, which has recently become commercially available, differs from the one used by us. The antibody used in our study was evaluated recently in a study of 77 paraffin wax embedded

cytoblocks.<sup>30</sup> The authors report low specificity of the antibody, manifested by frequent staining of adenocarcinomas (48%), as well as low sensitivity, demonstrated by the staining of only 35% of the specimens containing benign mesothelial cells. Moreover, the staining pattern was mostly cytoplasmic and/or nuclear because membranous staining was detected in only two malignant mesotheliomas and one adenocarcinoma. The authors conclude that N-cadherin is useless in distinguishing mesothelial cells from adenocarcinoma cells. We analysed 10 cases of malignant mesothelioma in tissue sections, as well as 30 cell block sections using the same antibody. Although membranous staining was detected in a few malignant mesotheliomas, this pattern was detected in only one of 30 cytological specimens containing benign mesothelial cells. Granular cytoplasmic staining was often detected in carcinoma cells (B Davidson *et al*, 1999, unpublished observations). Because cadherins are adhesion molecules, located on cell membranes, we interpreted the presence of cytoplasmic or nuclear N-cadherin immunoreactivity, in the absence of membranous staining, as non-specific. These results are to be expected, because the applied anti-N-cadherin antibody is not recommended for use on paraffin wax embedded material. Therefore, we recommend its use in flow cytometric immunophenotyping in addition to the use of calretinin, another mesothelial marker, on paraffin wax embedded cell blocks.

In conclusion, 49 serous effusions and peritoneal washings were studied using flow cytometry immunophenotyping. Although immunophenotyping by flow cytometry is useful in the routine investigation of haematological malignancies, most serous effusions do not necessitate the use of ancillary studies, because the diagnosis is often accurately rendered based on morphological findings. However, occasional cases might benefit from the use of complementary methods, which can aid in making an accurate, specific, and rapid diagnosis in an otherwise challenging case. Flow cytometry appears to be one of the tools that could be used to resolve these selected cases.

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