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Immunocytological analysis of the Tn associated antigen $83D_4$ in serous effusions from patients with cancer: comparison with Tn soluble glycoprotein

M Beuzelin-Yvraut, A Bourguignat, E Phillips, A Roseto, E Osinaga

Abstract

***Aims*—To determine whether the monoclonal antibody (MoAb) $83D_4$, previously shown to be highly specific for carcinoma cells, can be used as an immunocytological marker to discriminate between benign and malignant cells in serous effusions; and to test for a correlation between expression of the antigen reacting with MoAb $83D_4$ on effusion cells and the amount of soluble $83D_4$ antigen in effusion fluids.**

***Methods*—Thirty three pleural and 23 peritoneal effusions from 56 cancer patients with metastatic disease were tested for the presence of Tn associated $83D_4$ antigen by immunocytochemical staining, and for the presence of soluble antigen in supernatants. The patients had undergone various chemotherapy and radiation therapy protocols.**

***Results*—As a result of the various types of treatment, the cytological characteristics of the cells were often modified and the antigenic epitopes may have been altered. Positive staining for $83D_4$ MoAb was obtained in 36 (97%) of the 37 malignant effusions, eight (73%) of 11 suspect effusions, and three (38%) of the eight apparently benign effusions (free of malignant cells). In these latter cases, cytological reassessment showed a few suspect cells in two cases. $83D_4$ soluble antigen was detected in 30 of 37 malignant effusions (81%), five of 11 suspect infusions (46%), and five of eight apparently benign effusions (63%).**

***Conclusions*—Immunocytochemical staining with anti-$83D_4$ antibody is useful for differentiating reactive or atypical mesothelial cells from epithelial cells, especially in breast cancer effusions.**

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Département de Biologie, Centre René Huguenin, 35 rue Dailly, F-92211 Saint-Cloud, France
M Beuzelin-Yvraut
A Bourguignat
E Phillips

Division d'Immunocytologie Appliquée, Université de Technology de Compiègne, Compiègne, France
A Roseto

Laboratorio de Oncologia Basica y Departamento de Bioquimica, Facultad de Medicina, Montevideo, Uruguay
E Osinaga

Correspondence to:
Dr Michele Beuzelin.

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Serous effusions are common during the course of some cancers. Lung, breast, and gynaecological cancers are the malignant diseases most frequently associated with pleural or peritoneal effusions. Effusions generally occur in metastatic disease; however, they can be due to benign conditions or treatment complications. The diagnosis of malignancy in such cases is one of the most difficult problems in diagnostic cytology since carcinoma cells must be distinguished from reactive mesothelial cells.

Immunostaining can be used to detect cancer cells in fluids which appear to be benign. Monoclonal antibodies (MoAb) or panels of antibodies have been tested to detect carcinoma cells in smears or soluble tumour associated antigens in fluids. Most of them, including antikeratin antibodies, have failed to distinguish carcinoma cells from mesothelial cells.[1-13]

We have previously reported that MoAb $83D_4$ has remarkable specificity for a variety of carcinomas, especially of the breast, ovary, and uterus.[14,15] This antibody defines a heterogeneous group of acidic high molecular weight (>400 kDa) glycoproteins.[16] $83D_4$ antigen has been isolated from the MCF-7 breast cancer cell line and characterised.[17] The results of that study show the strong heterogeneity of the molecules recognised by MoAb $83D_4$ and distinguish them from known cancer associated mucins. The $83D_4$ defined epitope involves the GalNAca-*O*-Ser/Thr residues (Tn determinant)[18] which form one of the most specific carcinoma associated structures.[19,20] MoAb $83D_4$ has also been used to detect the soluble antigen in pleural and ascitic effusions.[21] High values were found in 81·5% of breast cancer associated effusions, whereas little or no soluble antigen was detectable in effusions from patients with haematological malignancies or non-malignant diseases.

The aim of this study was to determine whether MoAb $83D_4$ can be used as an immunocytological marker to discriminate between benign (particularly reactive mesothelial cells) and malignant cells in effusions from cancer patients, and to test for a correlation between expression of the antigen reacting with MoAb $83D_4$ on effusion cells and the level of soluble antigen $83D_4$ in effusion fluids.

Methods

PATIENTS AND SAMPLES

Pleural (n = 33) and ascitic (n = 23) fluids were obtained from 56 patients with metastatic adenocarcinoma or malignant mesothelioma during normal diagnostic procedures. The diagnoses and types of effusion are shown in table 1.

Fluid samples were centrifuged at 400 *g* for 10 minutes. The supernatants were stored at −80°C until they were assayed for $83D_4$ sol-

Table 1 Origin of effusions

Primary	*Pleural*	*Peritoneal*	*Total*
Breast	28	10	38
Ovary	3	6	9
Colon	—	1	1
Bronchus	—	2	2
Liver	—	2	2
Larynx	1	—	1
Kidney	1	—	1
Mesothelioma	—	1	1
Unknown	—	1	1
Total	33	23	56

uble antigen. The cell pellets were washed in phosphate buffered saline (PBS). If the specimen was haemorrhagic, it was centrifuged on a Ficoll gradient for 20 minutes at 1000 *g*. The cells were recovered from the interface and washed in PBS.

Twelve smears were prepared from each sample by cytocentrifugation at 600 rpm for five minutes on Cytospin (Shandon).

CYTOLOGIC DIAGNOSIS

One smear was stained with May-Grunwald-Giemsa (MGG) for cytological diagnosis. When there was disagreement between the results of cytology and $83D_4$ immunostaining or the soluble antigen assay, the MGG slide was read again to confirm the original cytological diagnosis.

MONOCLONAL ANTIBODY $83D_4$

The generation and characterisation of MoAb $83D_4$ (IgM) has been reported in detail elsewhere.[15] Briefly, the antibody was precipitated from ascitic fluids by dialysis against demineralised water at 4°C.[22] The precipitated IgM was dissolved in a small volume of 0·5 M NaCl in PBS, applied to a Sephacryl S-200 gel column (2·5 × 85 cm), and eluted with PBS. The IgM was excluded from the gel and recovered in the first elution peak. Column fractions were analysed by SDS-PAGE, and the protein concentrations of purified IgM were determined by the method of Lowry *et al.*[23]

IMMUNOCYTOCHEMICAL STAINING

The Cytospin smears were allowed to dry at room temperature for at least one hour and then stored at −20°C until staining. After removal from the deep freeze, they were left at room temperature for 10 minutes, fixed in cold acetone (4°C) for 10 minutes, and rehydrated in PBS at room temperature for 30 minutes.

Ten slides were incubated with MoAb $83D_4$ at a final concentration of 45 µg/ml for 60 minutes at room temperature. This optimum concentration was determined by using a range of concentrations from 15 to 200 µg/ml on the MCF7 breast metastatic carcinoma cell line (data not shown). NS1 mouse myeloma cell line supernatant was used as negative control. After extensive washing in PBS, immunostaining was performed with an avidin-biotin-glucose oxidase immunoglobulin M complex (Vector) according to the manufacturer's instructions. This enzyme complex was preferred to peroxidase because effusion fluids contain many inflammatory cells with endogeneous peroxidase which is very difficult to inhibit completely. Glucose oxidase activity was revealed using tetranitroblue tetrazolium. The slides were counterstained with haematoxylin and mounted using Eukitt (O Kindler, Freiburg, Germany).

All immunostained smears were examined by at least two investigators. In each evaluation the percentage of positive cells was determined and the intensity of the staining reaction was noted as strong +++, moderate ++, or weak +.

DETECTION OF SOLUBLE Tn GLYCOPROTEINS

Soluble Tn glycoproteins were detected by an immuno-lectino-enzymatic assay (method CA83.4).[17] Briefly, microtitre wells (Dynatech) were coated with 100 µl/ml of MoAb $83D_4$ (10 µg/ml in 0·1 M carbonate buffer, pH 9·6) by overnight incubation at room temperature. The wells were washed with 0·1% Tween 20 in PBS, and incubated with 1% gelatine in PBS at 37°C for one hour. After three washes, the wells were incubated with 100 µl of antigen samples diluted in PBS overnight at 4°C. MCF-7 cell membrane extracts and human milk fat globules (HMFG) were used as positive and negative controls, respectively. After washes, the wells were incubated with biotinylated Lectin Vicia Villosa B4 (VVLB4) (5 µg/ml) in 0·5% gelatin, 0·1% Tween 20 in PBS (PBS/TG), at 37°C for one hour. Unbound material then was washed off and 100 µl of 1/2000 diluted avidin-peroxidase complex (Vector) in PBS/TG was added for one hour at 37°C. Peroxidase activity was revealed with a combination of ABTS (3 mg) and 30% hydrogen peroxide (5 ml) in phosphate-citrate buffer pH 5·0 (10 ml). The reaction was allowed to proceed for 30 minutes at room temperature and absorbance was read at 405 nm with an ELISA reader. The CA83.4 level in each sample is expressed in activity units. One unit of the antigen was defined arbitrarily as CA83.4 reactivity in 1 µg of MCF-7 membrane extract protein.

A 5 U/ml cutoff was determined in effusions from patients with non-malignant diseases.[21] When the $83D_4$ soluble antigen level was higher than twice the cutoff (10 U/ml) it was con-

Table 2 Cytological diagnosis of effusions

Origin and cytology	*Pleural*	*Peritoneal*	*Total*
Breast cancer			
Malignant	21	3	24
Suspect	5	4	9
Benign	2	3	5
Total	28	10	38
Ovarian cancer			
Malignant	2	6	8
Suspect	—	—	0
Benign	1	—	1
Total	3	6	9
Other			
Malignant	2	3	5
Suspect	—	2	2
Benign	—	2	2
Total	2	7	9

Table 3 MoAb $83D_4$ staining of effusion cells

Cytological diagnosis	Pleural N	Pleural +	Ascitic N	Ascitic +	Total N	Total +	Total %
Breast							
Malignant	21	20	3	3	24	23	95·8
Suspect	5	4	4	4	9	8	88·9
Benign	2	0	3	3	5	3	60·0
Ovary							
Malignant	2	2	6	6	8	8	100
Benign	1	0	—	—	1	0	0
Other							
Malignant	2	2	3	3	5	5	100
Suspect	—	—	2	0	2	0	0
Benign	—	—	2	0	2	0	0

N = number of cases; + = stained with MoAb $83D_4$.

Table 4 $83D_4$ soluble antigen level in effusions

Cytological diagnosis	Pleural N	Pleural +	Pleural −	Pleural ±	Peritoneal +	Peritoneal −	Peritoneal ±
Breast							
Malignant	24	16	3	2	2	1	—
Suspect	9	3	—	2	1	1	2
Benign	5	2	—	—	2	1	—
Ovary							
Malignant	8	2	—	—	6	—	—
Benign	1	1	—	—	—	—	—
Other							
Malignant	5	2	1	—	2	—	—
Suspect	2	—	—	—	1	—	1
Benign	2	—	—	—	—	2	—

$83D_4$ soluble antigen level: + = ≥ 10 U/ml; − = <5 U/ml; ± = 5 U/ml < $83D_4$ < 10 U/ml.

sidered positive. Values between 5 U/ml and 10 U/ml were defined as borderline.

Data were analysed by means of analysis of variance.

Results

CYTOLOGICAL DIAGNOSIS *(table 2)*

Of the 38 breast carcinoma effusions, 24 (63%) were malignant, nine (24%) suspect, and five (13%) benign.

Eight (89%) of the nine ovarian carcinoma effusions were malignant and one (11%) benign. In the nine other cancers, five effusions (56%) were malignant, two (22%) suspect, and two (22%) benign.

$83D_4$ STAINING OF EFFUSION CELLS *(table 3 and fig 1)*

Intracytoplasmic and membrane staining was generally observed.

Thirty six (97%) of the 37 malignant effusions, eight (73%) of the 11 suspect effusions, and three (38%) of the eight apparently benign effusions stained positively. The three apparently benign effusions which stained with $83D_4$ MoAb were breast cancer peritoneal effusions. In other carcinomas there were no false negative reactions with the 13 malignant effusions and no false positive reactions with the three benign effusions (including a sample from a mesothelioma patient).

$83D_4$ SOLUBLE ANTIGEN LEVEL *(table 4 and fig 2)*

Thirty of the 37 malignant effusions (81%) were positive for $83D_4$ soluble antigen, two were borderline, and five were negative.

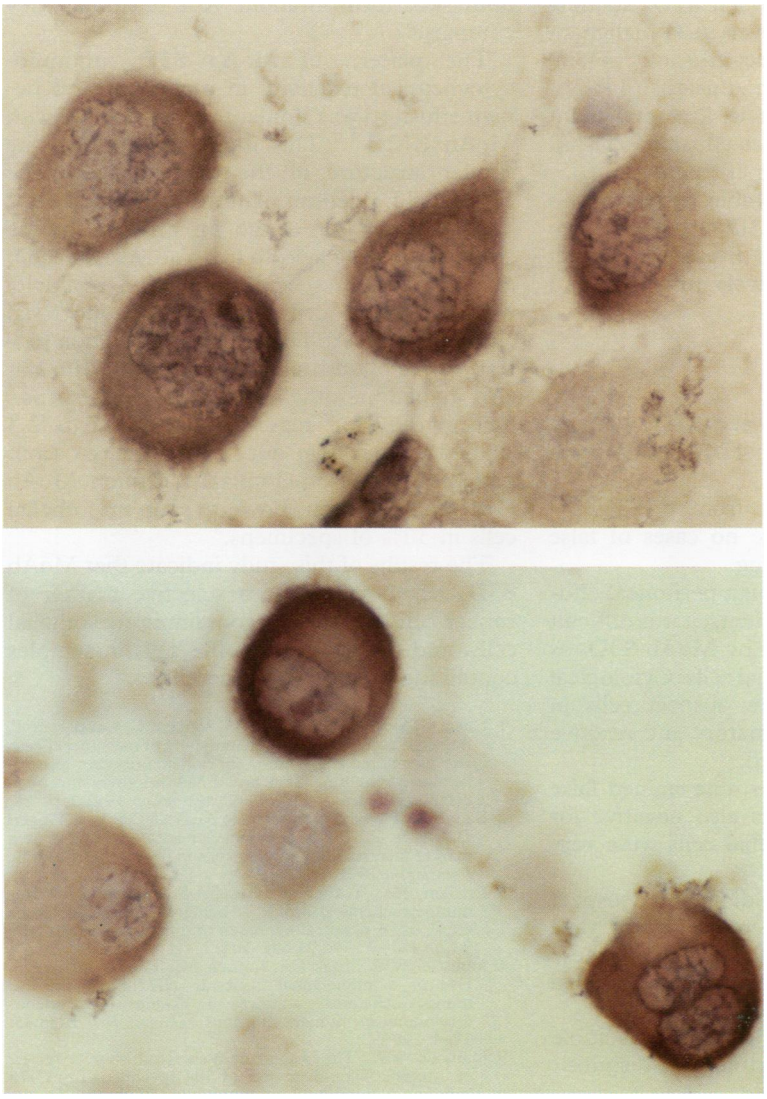

Figure 1 Immunoglucosidase staining with $83D_4$ MoAb of cell smear from pleural and peritoneal effusions of breast carcinoma.

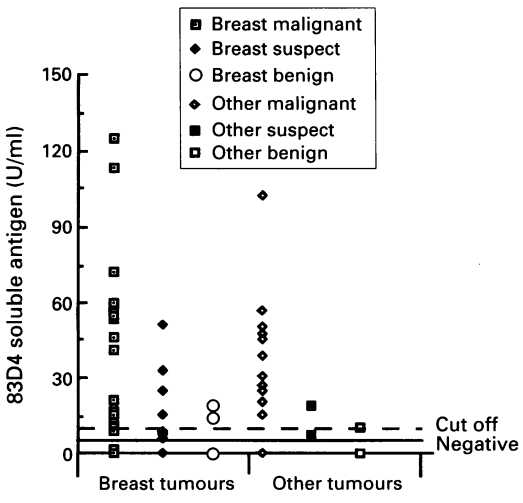

Figure 2 Level of $83D_4$ soluble antigen: relationship with conventional cytology.

Table 5 Concordance between 83D$_4$ staining and 83D$_4$ soluble antigen levels in effusions

Cytological diagnosis	N	Concordance n	Concordance %	No concordance n	No concordance %	Doubtful n	Doubtful %
Breast							
Malignant	24	19	79·2	3	12·5	2	8·3
Suspect	9	4	44·4	1	11·2	4	44·4
Benign	5	2	40·0	3	60·0	—	—
Ovary							
Malignant	8	8	100	—	—	—	—
Benign	1	—	—	1	100	—	—
Other							
Malignant	5	4	80·0	1	20·0	—	—
Suspect	2	—	—	1	50·0	1	50·0
Benign	2	2	100	—	—	—	—

N = total number of cases; n = number of cases in subgroup.

Of the 11 suspect fluids, five (45%) were positive, five were borderline, and one was negative.

Of the eight apparently benign fluids, five (63%) were positive (four breast and one ovarian cancer), one was borderline, and two were negative.

RELATIONSHIP BETWEEN MoAb 83D$_4$ STAINING AND LEVEL OF 83D$_4$ SOLUBLE ANTIGEN *(table 5)*

There was agreement between 83D$_4$ staining and 83D$_4$ soluble antigen levels in 31 malignant effusions (84%), four suspect effusions (44%), and four apparently benign effusions (44%).

There was disagreement in four malignant effusions (9%), two suspect effusions (18%), and four apparently benign effusions (44%).

In seven cases, 83D$_4$ soluble antigen levels were borderline (5 U/ml<83D$_4$<10 U/ml): two were malignant (8%), and five were suspect (45%). Five stained positively for 83D$_4$ and two were negative.

Discussion

There was agreement between 83D$_4$ staining and cytological diagnosis in 41 of the 45 cases (91%).

In the benign effusions from non-breast-cancer patients, there were no cases of false positive 83D$_4$ MoAb staining.

In three cytologically benign peritoneal effusions from breast cancer patients, it was difficult to identify the cells stained by MoAb 83D$_4$ as carcinoma cells or mesothelial cells. Cytological reassessment showed a few suspect cells in two cases and only inflammatory and reactive mesothelial cells in the third.

In breast cancer effusions, one stained false negative for 83D$_4$ and was also negative for soluble antigen, but epithelial cells were very rare in this sample.

Among the 11 effusions defined as suspect in the cytological analysis, reassessment gave the same result in every case. In general, these samples contained many reactive mesothelial cells, which stained positively in one case.

The degree of agreement between the soluble 83D$_4$ antigen assay and cytological diagnosis was 73·3% (33/45 cases).

Five effusions defined as malignant in the cytological analysis (four breast and one colon cancer), which were negative for 83D$_4$ soluble antigen, contained a few epithelial cells, which stained weakly in four cases. Five effusions defined as benign in the cytological analysis (four breast and one ovarian carcinoma) were positive for 83D$_4$ soluble antigen: two were classified as suspect after cytological reassessment, and two were previously defined as malignant were tested after chemotherapy. In the last case (ovarian carcinoma), the 83D$_4$ soluble antigen level was 10 U/ml (cutoff level).

Staining for 83D$_4$ seems to improve the detection of malignant cells in effusions defined as benign or suspect in cytological analysis. These results were confirmed by the clinical outcome.

The carcinoma associated epitope defined by MoAb 83D$_4$ involves the innermost GalNac residue of carbohydrate chains directly *O* linked to Ser or Thr residues (Tn antigen).[18] This epitope is one of the most remarkable examples of tumour associated antigens resulting from incomplete synthesis.[24,25] Incomplete glycosylation is a frequent alteration in cancer, resulting in the exposure of precursor structures that are masked in normal cells.[19] Tn antigen is not expressed in most mucins of normal human tissues. In contrast, it is expressed in unmasked form in about 90% of human carcinomas.[20]

The pattern of MoAb 83D$_4$ immunocytochemical reactivity in malignant and benign effusions was similar to that obtained with MoAb B72.3.[4-6] This is in keeping with the similar structure of the two epitopes. The MoAb B72.3 determinant involves sialyl-Tn,[26] a chain elongation of the Tn structure by the addition of sialic acid to GalNac. MoAb 83D$_4$ displays a more specific pattern of reactivity with carcinoma cells than other monoclonal antibodies. MoAb DF3 showed a reaction pattern to neoplasms similar to that of 83D$_4$ but also stained mesothelial cells in all specimens.[6] One investigator[2] reported that MoAb B72.3 stained cells in two out of 10 effusions from patients with mesothelomia. In addition, MoAb HMFG-2 has been shown to stain mesothelial cells in 30% of specimens.[8]

The results of this study indicate that MoAb 83D$_4$ has potential as a selective marker of cancer cells in samples containing mesothelial cells, and of adenocarcinoma cells in samples containing other malignant cells.

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