Use of antisera to epithelial membrane antigen for the cytodiagnosis of malignancy in serous effusions

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SUMMARY A new human antigen, designated epithelial membrane antigen (EMA), has recently been described on surface membranes of a wide variety of normal epithelium but not on connective tissue cells. The antigen is only weakly expressed on normal or reactive mesothelium. Increased expression of the antigen has been observed in most neoplasms of epithelial origin and in malignant mesothelioma.

We have investigated the possibility of using this difference in the expression of the antigen to distinguish between mesothelial cells and malignant cells in cytological smears of serous effusions. This distinction cannot always be made on morphological grounds alone and problems of differential diagnosis are encountered in about 15% of all specimens of serous effusions sent for cytological examination.

Using antisera to EMA we have applied an indirect immunoalkaline phosphatase technique to alcohol-fixed smears prepared from serous effusions and have found that intense staining of the antigen is confined to effusions from patients in whom there is either clinical or cytological evidence of malignancy. The technique proved to be especially useful in cytologically equivocal cases, where there were problems of differential diagnosis.

The cytodiagnosis of malignancy in serous effusions at present depends upon the recognition of morphological differences between stained malignant and non-malignant cells in the light microscope. When the malignant cells are small and characterised by isonucleosis, they are often morphologically indistinguishable from reactive mesothelial cells thereby presenting the pathologist with problems of differential diagnosis. Such diagnostic problems are not rare and, in our experience, may be encountered in as many as 15% of all specimens of serous effusions sent for cytological examination. A number of attempts to improve the diagnostic accuracy have been made using histochemical,¹ electron microscopic² and cytogenetic techniques,^{3,4} but none has been entirely successful and the problem of differential diagnosis remains unresolved.

In 1979, a new antigen was reported to be present on human epithelial cells and was designated Epithelial Membrane Antigen (EMA).⁵ The antigen

was demonstrated in a wide variety of normal and neoplastic epithelial cells in formalin-fixed, paraffin-embedded sections of human tissues. The antigen appeared to be localised to epithelium and was not expressed by haemopoietic, lymphoid, osseous or other connective tissues. Studies of conventional histological sections of the mesothelium have shown only weak antigen expression.^{6,7} On the other hand, carcinoma cells in locally invasive tumours or metastases and malignant mesothelioma were often found to stain strongly for EMA, the antigen frequently being localised in the cytoplasm as well as on cell membranes.⁶ We have carried out this study to see if this qualitative difference in the expression of the Epithelial Membrane Antigen between carcinoma cells and mesothelial cells can be used for diagnostic purposes.

We have applied an indirect immunoalkaline phosphatase technique to alcohol-fixed smears prepared from 127 effusions of pleural or peritoneal origin. The results of our investigation of the distri-

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Table 1 Clinical diagnoses of the 100 patients

Malignant disease	No of patients		Non-malignant disease	No of patients
Carcinoma of breast	22		Cardiac failure	8
Carcinoma of ovary	14		Pulmonary tuberculosis	4
Carcinoma of gastrointestinal tract*	10		Pneumonia	4
Carcinoma of bronchus†	7		Pulmonary embolus	2
Other epithelial malignancies of known site‡	6		Hepatic cirrhosis	6
Epithelial malignancies: primary site unknown	5		Renal failure	2
Mesothelioma	2		Unknown aetiology	4
Lymphoma	4			
Total	70			30

*Stomach 2; oesophagus 1; colorectal 7.

†Squamous 5; adenocarcinoma 2.

‡Fallopian tube 1; cervix 1; pancreas 1; bladder 1; kidney 1; pyiform fossa 1.

bution of EMA on the cells in serous effusions and our evaluation of its role in complementing the conventional morphological diagnosis of malignancy are presented in this paper.

Material and methods

All adequate specimens of pleural or peritoneal fluid sent routinely to the Cytology laboratory at St Mary's Hospital, London W2 between 1 January and 30 September 1980, were included in this study. Specimens were also received from the Royal Marsden Hospital, Sutton, Surrey and St Bartholomew's Hospital, West Smithfield, London. A total of 127 effusions from 100 patients were investigated. Eighty-six of the effusions were pleural in origin and 41 were ascitic fluids. Of the 100 patients, 70 were diagnosed as having malignant disease while the remaining 30 patients were considered on clinical grounds to have no evidence of malignancy. The clinical diagnosis of all the patients are shown in Table 1. The majority of the patients with proven malignancy had primary tumours of epithelial origin.

The smears used in this study were prepared from individual effusions by the following technique. A minimum of 50 ml of a serous fluid was centrifuged at 300 *g* for 5 min. The sediment was then washed with 10 ml of normal saline, recentrifuged and washed again. An attempt to remove the red blood cells and to concentrate mesothelial and malignant cells was made by treating the sediment in one of two ways. The sediment was drawn up into six capillary tubes, sealed at one end with Cristaseal (Gelman-Hawksley Ltd, England) and centrifuged at 700 *g* for 10 min; the cells collected in the buffy layer were used for making the smears. Alternatively, the sediment was layered onto a mixture of Ficoll and

sodium metrizoate (Lymphoprep, density 1.077, Nyegaard & Co, Oslo) and centrifuged at 400 *g* for 20 min, at which time the red cells had formed a pellet leaving the majority of tumour and mesothelial cells together with lymphocytes at the interface. These were aspirated and washed in phosphate-buffered saline (PBS) and subsequently used to make smears. At least six smears were made using either of these techniques. Air-dried smears were stained by the standard Giemsa method. The others were fixed immediately in 95% alcohol for a minimum of 30 min and subsequently stained by the Papanicolaou, periodic acid Schiff-diasase and alcian blue methods. Two alcohol-fixed smears were stained by an indirect immunoalkaline phosphatase technique using rabbit antiserum to EMA. All smears were examined in a Leitz Dialux microscope at a magnification of $\times 100$. The cytological assessment of the smears was based on the morphological studies of the Papanicolaou and Giemsa-stained cells. Mucin staining was used to confirm the presence of adenocarcinoma cells. The results of the EMA staining were compared with those of the morphological studies and the mucin staining.

IMMUNOCYTOCHEMICAL STAIN

Antibody to EMA was prepared as previously described⁵ and alkaline phosphatase conjugated to the second antibody by the method of Avrameas and Ternynck.⁸

Ethanol-fixed smears were immersed in 20% acetic acid for 10 min to block endogenous alkaline phosphatase activity and then washed in PBS. Staining was performed using rabbit anti-EMA at a suitable dilution for 90 min followed by sheep anti-rabbit alkaline phosphatase conjugated second antibody for 90 min. Smears were carefully washed between these steps with (i) 0.5% bovine serum albumin in PBS, (ii) PBS to which a small amount of detergent (Tween 80) had been added, and (iii) PBS. An additional final wash with distilled water was included after incubation with the conjugated antibody. Dilutions of both first and second antibodies were made in 1/20 non-immune goat serum, and were determined by staining tissue sections of breast carcinomas, smears of carcinoma cell lines and smears of benign mesothelial cells. Alkaline phosphatase was visualised using Naphthol AS-BI and Brentamine fast red TR as substrates, after which the smears were washed in distilled water and counter-stained with Mayer's haemalum for 30 min. To confirm antibody specificity, human breast carcinoma sections and smears of malignant cell lines were incubated with antiserum which had been absorbed with a preparation of EMA;^{6,7} staining activity was completely abolished.

Fig. 1 Weak staining of tumour cells in a smear from a patient with primary carcinoma of breast showing a "rim" pattern of EMA positive staining. Alkaline phosphatase stain $\times 1000$.

Fig. 2 Weak staining of tumour cells in a smear from a patient with primary carcinoma of breast showing a "diffuse" pattern of EMA staining. Alkaline phosphatase stain $\times 400$.

Fig. 3 Strong staining of tumour cells in a smear from a patient with primary gastric carcinoma showing a "dense central" pattern of EMA positive staining. Alkaline phosphatase stain $\times 1000$.

Fig. 4 Selective staining in a smear from a patient with primary squamous carcinoma of the bronchus. Carcinoma cells are strongly EMA positive, mesothelial cells are negative. Alkaline phosphatase stain $\times 400$.

Results

A firm diagnosis of malignancy was made on the morphological appearances of the exfoliated cells in Papanicolaou and Giemsa smears in 62 of the 127 (49%) serous fluids investigated. Fifteen effusions (12%) were observed to contain cells showing changes suggestive of malignancy and a suspicious report was given. In the remaining 50 (39%) effusions a negative report was given as no evidence of malignancy was found. Mucin secretion was demonstrated in the malignant cells in 34 of the 62 cytologically positive effusions (55%) by the PAS-diacetate and alcian blue staining techniques. EMA was demonstrated in exfoliated cells in alkaline phosphatase-stained smears prepared from 67 cytologically positive and suspicious effusions and nine cytologically negative specimens.

Three patterns of EMA-positive staining were observed. In some cells a "rim" of positive staining was present around the edge of the cells (Fig. 1). In other cells, a "diffuse" staining of the cytoplasm could be detected (Fig. 2). The third pattern of staining was characterised by an intense positive staining throughout the cytoplasm (Fig. 3). Selective EMA staining with this "dense central" pattern is shown in Fig. 4. The "rim" and "diffuse" staining is described in the Tables as "weak" and was seen on cells which appeared to be either mesothelial or malignant on morphological grounds. The type of cells showing dense central (or "strong") staining could not always be ascertained as the morphological features of the cells were partially obscured by the stain; but some had clearly visible malignant features. The results of the EMA staining in individual specimens were analysed according to these patterns. Not infrequently more than one type of staining was observed in a smear. Whenever the strong dense central staining was present it was recorded as the pattern of staining for that particular specimen.

The pattern of EMA staining in the 77 effusions from 66 patients with malignant disease of epithelial

or mesothelial origin is shown in Table 2. Of the 57 smears which contained cells classified as malignant on cytological grounds only one, from a patient with rectal carcinoma, did not express EMA. The staining pattern of malignant cells did not appear to vary appreciably with the site of primary tumours. In the cytologically negative group five smears contained only leucocytes and, as expected, these were all negative for EMA.

Two effusions were obtained from patients with cytological evidence of mesothelioma. Histological confirmation of the diagnosis had been obtained in one case. The abnormal mesothelial cells in one specimen stained strongly for EMA, weaker staining was present in the other case.

Six effusions were obtained from four patients with lymphoma—a tumour which does not normally express EMA.⁶ Three patients had non-Hodgkin's type tumour and one had Hodgkin's disease. Malignant lymphoma cells were noted in Giemsa-stained smears from five of the six specimens. However, all the specimens were negative for EMA.

The results of EMA staining for the 44 effusions from 30 patients without clinical suspicion of malignant disease are shown in Table 3. No malignant cells (as defined morphologically) were seen in smears from any of these specimens but six were reported as containing atypical cells showing features suggestive of malignancy. Of the 38 specimens which were cytologically negative, 31 were also negative for EMA. However, the other seven contained a few mesothelial cells which exhibited weak EMA staining. Of the six specimens which were classified as suspicious on cytological grounds, three showed some EMA staining, two weakly and the third strongly. The clinical diagnoses in the patients with weak EMA staining were cardiac failure and pulmonary embolus. However, the patient with strong EMA staining was an elderly woman with pneumonia. A definitive cytological diagnosis of malignancy could not be made in this case even after review of the smears, and there is still some doubt as to the clinical diagnosis of the underlying disease in this patient.

Table 2 Results of the EMA staining and cytological findings of 77 effusions from 66 patients with proven epithelial or mesothelial malignant disease

Clinical diagnosis	Cytological diagnosis	Effusion with EMA stain classified as		
		Strong	Weak	Absent
Malignant	Malignant	37 (25)	19 (9)	1
	Suspicious	3	5	1
	Non-malignant	1*	1	9

Figures in parentheses refer to number of specimens showing mucin secretion.

* Small number of probable carcinoma cells (see text).

Table 3 Results of the EMA staining and the cytological diagnosis of 44 effusions from 30 patients without proven malignant disease

Clinical diagnosis	Cytological diagnosis	Effusion with EMA stain classified as		
		Strong	Weak	Absent
Non-malignant	Malignant	0	0	0
	Suspicious	1*	2	3
	Non-malignant	0	7	31

* Elderly woman with pneumonia, neoplastic involvement could not be ruled out (see text).

The remaining three cytologically suspicious effusions showed no EMA staining, and were from patients with cardiac failure.

Discussion

The purpose of our study was to determine the distribution of the epithelial membrane antigen (EMA) in the cells of serous effusions and to establish whether demonstration of the antigen in cytological preparations of effusions was of diagnostic value. Earlier work using tissue sections^{6,7} had suggested that whereas the majority of carcinomas stain strongly for EMA, particularly when present as single cell infiltrates, normal or reactive mesothelium is either negative or stains weakly and inconsistently.

The results of our investigation showed that 66 of the 77 effusions (86%) from patients with proven epithelial or mesothelial malignancy were found to express the antigen. Of these 66 EMA positive effusions, 25 showed the rim/diffuse type of staining. This weak EMA staining was also observed in nine of the effusions from patients without malignant disease. Since this pattern of staining was present on both carcinoma cells and some mesothelial cells, it cannot be used as a criterion for malignancy. However, the strong dense central staining was found to occur only in effusions where there was either a clinical or a cytological suggestion of malignancy. Forty-two effusions in this study fell into this category. Forty-one of these were from patients with proven malignant disease, and the remaining one was from an 81-year-old woman who presented with pneumonia. Cytological studies showed that this effusion contained very suspicious cells, and a diagnosis of malignancy cannot be ruled out.

Positive EMA staining with the strong dense central pattern may prove to be especially valuable when the cytological diagnosis is uncertain. In this study three of the nine effusions from patients with proven malignant disease, which on cytological evidence were classified as suspicious, showed this strong EMA staining. If subsequent studies confirm that a strong EMA stain is specific for malignancy, not only would this method establish the diagnosis in many cytologically equivocal cases, but would also correct errors of false-negative reporting. For instance, one of the 12 cytologically negative effusions from patients with proven malignant disease contained a small number of cells exhibiting the dense central pattern of staining, and it is possible that a few malignant cells were present in this specimen which were not identified on morphological grounds. Lack of EMA staining in cytologically suspicious smears may be used to indicate

the absence of carcinoma cells, and this occurred in four of 15 such smears in this study. As strong EMA staining occurred in four of the remaining 11 cases, diagnosis was aided in eight out of these 15 (53%) cytologically difficult specimens.

Correct preparation of the smears used for both the cytological diagnosis and for the EMA staining is of paramount importance. Diagnostic difficulty is more often brought about by inadequate preparation rather than morphological uncertainty. The true value of an additional diagnostic technique can only be assessed when the morphological diagnosis on satisfactory preparations is uncertain. The method of preparation of the smears used in this work produces smears where the morphology of the cells is clearly defined and the background clear. Removal of the red blood cells further adds to the quality of the preparation. The process of washing the cells in normal saline is important for immunocytochemical staining. Smears prepared from effusions with a high protein content may, if not washed, have a dirty, heavy background staining which makes microscopic analysis unreliable.

Standardisation of the EMA technique is also of prime importance in obtaining reliable results. Variations in the dilutions of the antisera, or in the incubation time with various reagents used in the technique, will give varied intensity of staining on preparations of the same specimen. Thus staining done at different times with varied protocols clearly cannot be interpreted using the same criteria. The staining procedure for this work was carried out according to a strict protocol of 10-minute treatment with 20% acetic acid, fixed concentrations and 90-minute incubation respectively with the primary and secondary antisera, and a further 60-minute incubation with the substrate.

The antigen does not survive prolonged storage in ethanol-fixed smears at room temperature. If it is necessary to keep smears for more than two weeks they should be stored in a freezer.

The intensity and distribution of the different patterns of staining may be a reflection of both the location and the quantity of the antigen on the surface or in the cytoplasm of the cells. Our present data suggest that the EMA staining with the strong dense central pattern as the sole criterion for malignancy is both specific and sensitive in detecting malignant epithelial cells in serous effusions and may be especially valuable in complementing the morphological diagnosis in equivocal cases. It is important to understand that none of the cell markers currently available is tumour-specific and therefore some staining of the mesothelium may be anticipated. We have shown that grading the strength of staining is informative, provided careful standardisation of

antibody titres and conditions is maintained. Our results apparently differ from those of O'Brien *et al.*,⁹ who investigated the usefulness of three epithelial antigens (carcinoembryonic antigen-CEA, zinc glycinat marker-ZGM, and EMA) in the diagnosis of malignant cells in sections of cell blocks of serous effusions. This is a relatively insensitive method of diagnosing malignancy, and the lack of specificity of EMA in this study was probably because no such grading system was utilised.

There are many other problems of cytological diagnosis in both gynaecological and non-gynaecological practice, for which immunocytochemical staining for epithelial surface antigens may prove useful. We are in the process of investigating the diagnostic potentials of CEA and ZGM in conjunction with EMA on cells in effusions and will be reporting our observations shortly. The methodology for immunocytochemical staining on different types of cytological specimens needs to be developed. Application of EMA staining to the detection of micrometastases in bone marrow has already been investigated.^{7,11} The application of stains for epithelial antigens to cell suspensions may accelerate the development of automated screening techniques in future, possibly if used in conjunction with a fluorescent second antibody and a suitable flow cytometer.¹⁰ EMA staining may have a wide application in diagnostic cytology and further research in this field is indicated.

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