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Immunocytochemical reaction of Ca1 and HMFG2 monoclonal antibodies with cells from serous effusions

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SUMMARY The Ca1 antibody was used in an alkaline phosphatase immunocytochemical method on cells obtained from 150 specimens of pleural and ascitic fluids. The results were compared with the routine cytology report based on the light microscopical appearances. The Ca1 antibody identified tumour cells in 51 of 57 specimens with malignant cells. The exceptions were four small cell carcinomas, one malignant lymphoma, and one adenocarcinoma. A further seven specimens reported as containing atypical cells but without conclusive evidence of malignancy were Ca1 positive. The Ca1 antibody did not give a positive reaction with benign mesothelial cells.

Similar results were obtained with the HMFG2 antibody and malignant cells, but in eight of 18 benign effusions it reacted with mesothelial cells.

An effusion may be the presenting sign of malignant disease or the first indication of recurrence. Identification of tumour cells in the fluid is an important first line investigation. In a proportion of cases the malignant cells are easily recognised, but in some fluids they are not seen or cannot be confidently identified with the use of routine staining methods. Immunocytochemical techniques increase the diagnostic accuracy.[1–6] Monoclonal antibodies have been used in panels and singly with various techniques on both histological and cytological material.[7–9] These methods require expensive reagents and considerable technical time and expertise. This paper records the reaction of Ca1 antibody with a series of effusion fluids received in two district general hospitals. The objective was to see if expenditure on this monoclonal antibody applied to specimens prepared by routine laboratory techniques improved the diagnosis based on morphological characteristics alone. A second monoclonal antibody, HMFG2 (antihuman milk fat globule membrane),[10] was introduced in a smaller series to see if it increased further the accuracy of diagnosis.

Material and methods

PATIENTS AND SPECIMENS

A total of 178 consecutive specimens of pleural and ascitic fluid received in the cytology laboratories of two district general hospitals were stained with the Ca1 antibody using the immunoalkaline phosphatase technique. From the results, 11 specimens were discounted because they contained only blood. Histological confirmation of the diagnosis was sought from the patient's records when the series was complete.

The specimens from nine patients were excluded from the report because no other evidence could be found for a firm diagnosis of either benign or malignant disease. A further eight patients had had malignant disease at some time but had no tumour cells in their effusions. They were also excluded from the final analysis because morphological confirmation of the Ca1 specificity was essential for this study. This left 150 specimens, which were assessed in three groups according to the report based on morphology alone.

1 Positive: this group consisted of 57 effusions with positive identification of the presence of malignant cells.
2 Negative: this group consisted of 84 effusions which showed no morphological evidence of malignancy.
3 Atypical: this group consisted of nine effusions which showed cellular atypia but had no conclusive evidence of malignancy.

The HMFG2 monoclonal antibody was applied to

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36 of the 150 specimens using the immunoalkaline phosphatase technique. Eighteen of these effusions were from the positive group and 18 from the negative group.

The effusion fluids were collected into 3·8% sterile citrate anticoagulant. They were spun at 2000 rpm for 10 min and smears were made from the cell deposit for the routine Papanicolaou, May-Grunwald Giemsa, and mucin stains. Four additional rapidly air dried, unfixed smears were wrapped in aluminium foil and stored at −20°C for subsequent staining with the monoclonal antibodies.

REAGENTS

Antibodies

1 Ca1 (given by Wellcome Diagnostics)
2 HMFG2 (given by Dr J Taylor-Papadimitriou. The Imperial Cancer Research Fund, Lincoln's Inn Fields, London)
3 Rabbit antimouse immunoglobulin (from Nordic Immunological Laboratories Ltd)
4 Normal human serum
5 Alkaline phosphatase—antialkaline phosphatase complex (APAAP) (given by Dr DY Mason, Nuffield Department of Pathology, University of Oxford, John Radcliffe Hospital, Oxford).

Buffers

1 0·5 M Tris buffer, pH 7·6, was used as a diluent for the Ca1 antibody, the HMFG2 antibody, the rabbit antimouse antiserum, the human serum, and alkaline phosphatase. It was also used for washes between incubation steps.
2 0·1 M Tris buffer, pH 8·2, was used in the substrate.

Enzyme

Alkaline phosphatase from calf intestine (obtained from Sigma Chemical Co Ltd) was used.

Substrate

In a glass tube 2 mg of napthol AS-MX phosphate was dissolved in 0·2 ml of dimethyl formamide. To this 9·8 ml of Tris buffer, pH 8·2, and levamisole of 1 mmol/l were added. Immediately before staining 10 mg of fast red TR was dissolved in the solution, which was then filtered on to the slides. All these reagents were obtained from the Sigma Chemical Co Ltd.

IMMUNOALKALINE PHOSPHATASE STAINING TECHNIQUE

1 Fix in acetone at room temperature for 10 min.
2 Incubate with monoclonal antibody for 45 min. The dilutions used were Ca1, 1/2; HMFG2, 1/20.
3 Incubate with rabbit antimouse (1/20) containing normal human serum (1/20) for 45 min.
4 Incubate with the APAAP complex for 30 min.
5 Incubate with alkaline phosphatase (5 mg/ml) for 30 min.
6 Develop the reaction with the prepared substrate for 15 min.
7 Counterstain with haematoxylin and mount in Apathy's medium (aqueous mountant).

CONTROLS

1 Each test case was controlled by omitting the monoclonal antibody from one smear during staining.
2 Each batch of slides was controlled by including known positive and known negative smears with the test smears.

Results

Ca1 ANTIBODY

Positive

The Ca1 antibody stained the cytoplasm of malignant cells a diffuse granular red with the immunoalkaline phosphatase technique. There was variation in the intensity of the staining in any given tumour cell population and the cells were not evenly positive. This was particularly noticeable in the balls of tumour cells, where there was a greater intensity of staining around the edges. The malignant cells did not all react with the Ca1. The number of malignant cells stained in each effusion did not appear to be related to the type of tumour. In effusions containing few malignant cells, the cells which reacted were generally on the edges of the smears. These cells are in the thin part of the smear, where they dry more rapidly when the smear is made than the cells in the thicker part towards the centre.

Of the 57 malignant fluids stained with the Ca1 antibody, 51 gave a distinct positive reaction (Table 1). The six malignant fluids which did not react consisted of four cases of small cell carcinoma of lung, one case of centrocytic/centroblastic lymphoma, and one case of breast carcinoma.

Negative

In 77 of the 84 benign effusions, Ca1 did not react with any cells. In the remaining seven cases the mesothelial cells stained with a pale pink blush. This reaction was quite different in appearance from the positive granular red staining of malignant cells with Ca1 antibody. These seven cases appeared early in the series when technical experience was limited and the problem did not recur.

Atypical

In these nine cases which had shown morphological

Table 1 *Reaction of Ca1 monoclonal antibody with malignant effusions*

Effusions	*Site of primary tumour*	*Reaction with Ca1*	
		Positive	*Negative*
Pleural	Breast	10	1
	Bronchus	1	0
	Lung		
	Adenocarcinoma	4	0
	Adenosquamous carcinoma	1	0
	Large cell carcinoma	1	0
	Small cell carcinoma	0	4
	Lymphoma—centrocytic/centroblastic	0	1
	Ovary	4	0
	Pancreas	2	0
	Prostate	1	0
	Stomach	1	0
	Primary unknown—adenocarcinoma	3	0
Ascitic	Endometrium	1	0
	Gall bladder	1	0
	Ovary	13	0
	Primary unknown—adenocarcinoma	8	0
Total		51	6

Table 2 *Reaction of Ca1 monoclonal antibody with atypical effusions*

Effusions	*Clinical diagnosis/histological confirmation*	*Reaction with Ca1*	
		Positive	*Negative*
Pleural	Carcinoma bronchus—adenocarcinoma	1	0
	Carcinoma breast—adenocarcinoma	1	0
	Primary unknown—adenocarcinoma	2	0
	Pneumonia	0	1
Ascitic	Carcinoma breast—adenocarcinoma	2	0
	Carcinoma stomach—adenocarcinoma	1	0
	Postoperative effusion	0	1
Total		7	2

atypia but no conclusive evidence of malignancy the Ca1 antibody stained cells a granular red colour in seven. These cells showed morphological appearances consistent with malignancy (Table 2). In one case the cells which reacted were in a cluster, but in the other six they were individual cells which would be more difficult to identify with the routine Papanicolaou or May-Grunwald Giemsa methods

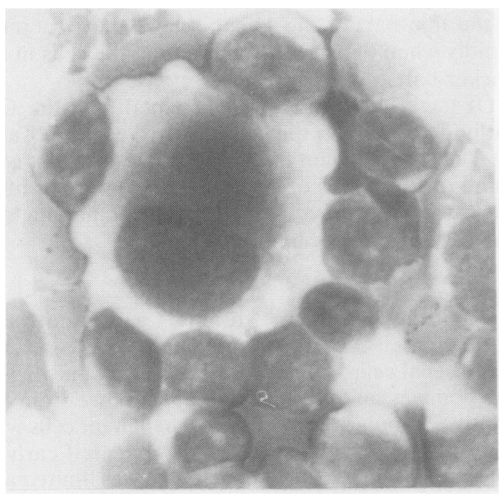

Fig. 1 *Conventional May-Grunwald Giemsa smear showing a single atypical cell with a background of lymphocytes from a pleural effusion. Magnification ×160, enlarged ×5.*

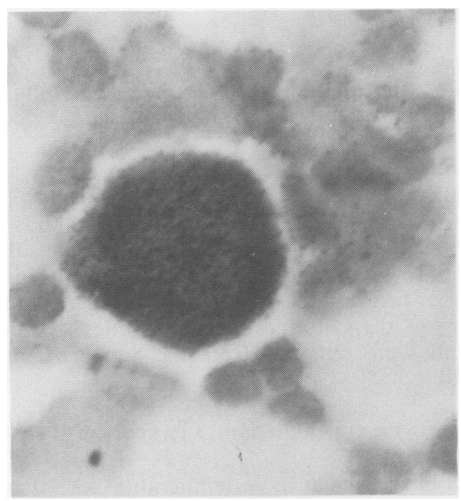

Fig. 2 *Ca1 granular positive staining confirming that the atypical cell is malignant. Magnification ×160, enlarged ×5.*

Table 3 *Reactions of HMFG2 monoclonal antibody with malignant effusions*

Effusions	*Site of primary tumour*	*Reaction with HMFG2*	
		Positive	*Negative*
Pleural	Breast	4	0
	Bronchus	2	0
	Lymphoma—centrocytic/centroblastic	0	1
	Lung—adeno squamous carcinoma	1	0
	Small cell carcinoma	0	1
	Pancreas	1	0
	Ovary	1	0
	Primary unknown—adenocarcinoma	2	0
Ascitic	Gall bladder	1	0
	Pancreas	1	0
	Primary unknown—adenocarcinoma	3	0
Total		16	2

(Figs. 1 and 2). The two negative cases showed no reaction with the Ca1 antibody and review of the cases confirmed benign causes of the effusion.

HMFG2

Positive

Of the 18 malignant effusions stained with HMFG2, 16 showed strong positivity in a granular diffuse pattern with the immunoalkaline phosphatase technique (Table 3). The reaction was distinct and appeared to be similar to that given by the Ca1 antibody. It was generally stronger than the Ca1 reaction and was less focal, with most of the malignant cells shown. The two negative cases were a small cell carcinoma and a follicular lymphoma.

Negative

No reaction was shown with the HMFG2 antibody in 10 of the 18 benign effusions. In the remaining eight effusions the mesothelial cells were stained pink. This reaction was not as strong as a positive reaction but it was more intense than the pale pink blush that occurred in seven benign effusions with the Ca1 antibody.

Atypical

No smears from the atypical effusions were available for use with the HMFG2 antibody.

Discussion

This study was designed to see whether the use of monoclonal antibodies could increase the accuracy of the diagnosis of malignancy in effusions in a routine hospital cytology laboratory. Traditionally, this diagnosis relies on the experience of individuals using smears stained by the Papanicolaou and May-Grunwald Giemsa techniques supported by a histochemical method for mucins. A negative report issued on this evidence means that malignant cells were not recognised. Spriggs and Boddington estimated that 38% of malignant fluids are given a negative report.[11] In this study the Ca1 antibody was a useful aid to the diagnosis of malignancy in effusions; it did not give any false positive results. In specimens which showed morphological characteristics of malignancy it failed to react with one malignant lymphoma, four small cell anaplastic carcinomas, and one specimen containing only a few tumour cells. Its value was most evident when specimens appeared to contain a few suspicious cells but there was no conclusive evidence of malignancy. Nine effusions in the series fell into this category, and in seven cells were shown by Ca1 antibody which were then recognised as having malignant characteristics. All the patients with a positive Ca1 reaction had other evidence of malignant disease.

It has been noted that Ca1 does not react throughout the tumour tissue in histological sections.[7] This focal staining is also seen in the cell preparations from effusions.[2-4] Bramwell *et al*[1 12] suggested this might be due to lower levels or masking of the Ca antigen in the tumour cells which did not react or to a variation in the structure of the antigen which impaired binding of the Ca1 antibody. This will account for a number of false negative tests, particularly where the specimens contain small numbers of tumour cells. The quality of the smear preparation may also influence the result as we found that in some preparations the tumour cells near the edges of the smear were positive whereas those in the central slightly thicker areas were not stained. With good technique in making cell smears, routine rapidly air dried preparations are satisfactory for Ca1 antibody demonstration and cell washing is not essential.[2 6]

Studies with Ca1 have shown that the Ca antigen is not confined to malignant tumours as was first reported.[13-16] This series has confirmed that in the clinically important field of the cytodiagnosis of malignancy in effusions the Ca1 makes a reliable and useful contribution. It increases the proportion

in which a correct diagnosis can be made. A negative reaction does not exclude malignancy because it can be expected with certain tumours. Several workers have obtained negative reactions with malignant lymphoma.[2 4 8] Paradinas *et al* found that less than 1% of small cell carcinoma cells were positive in histological sections.[16] This may be accounted for by the almost complete absence of cytoplasm in small cell carcinoma. The unequivocal positive staining of mesothelial cells has been experienced by some workers,[2] but in this series the pink blush staining of mesothelial cells in some benign effusions was recognisably different from the red granular positive staining of malignant cells. The HMFG2 antibody gave equally good positive results with the 36 specimens tested, but it did not add any information to that obtained with the Ca1 antibody. It may be of value, however, to confirm the epithelial origin of a tumour as malignant lymphomas are consistently negative.[2 7] HMFG2 gave more staining of mesothelial cells as previously reported.[2] This is a particular disadvantage because the distinction between mesothelial cells and malignant cells is critical in the cytodiagnosis of effusions. The Ca1 reaction is therefore preferred. The use of other monoclonal antibodies, in particular against leucocytes, would provide further diagnostic information in a few cases. If, however, for economical and technical reasons resources are limited, the use of one monoclonal antibody, Ca1, can be recommended to improve the cytodiagnosis of effusions.

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