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Immunocytochemical staining of cells in pleural and peritoneal effusions with a panel of monoclonal antibodies

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SUMMARY A panel of seven monoclonal antibodies was applied to smears of cell deposit from 70 pleural and peritoneal fluids, using an immunoalkaline phosphatase (IAP) procedure. The cases were chosen to show typical cytological patterns, both benign and malignant, and in this way the diagnostic value of the method could be assessed.The antibodies used were 2D1(anti-leucocyte), Ca 1, HMFG-2 (anti-milk fat globule membrane), LE61 and M73 (both anti-intermediate filament antibodies), anti-CEA, and K92 (anti-keratin).

The anti-leucocyte antibody was found useful for distinguishing lymphoma from carcinoma. Anti-CEA gave positive reactions in 80% of carcinoma cases and was not seen to react with any other cell types. Ca 1 was positive with some cells in 95% of carcinoma cases, but mesothelial cells reacted with it in two cases. A strong reaction with the anti-milk fat globule membrane antibody was very constant in carcinoma but was also seen in mesothelial cells in 30% of benign effusions. The anti-keratin reacted with malignant cells in only a small proportion of cases. The antibodies against epithelial intermediate filaments reacted equally strongly with benign mesothelial cells and carcinoma cells, but gave negative reactions with lymphoma cells.

It is concluded that a suitably chosen panel of monoclonal antibodies can be of great value in identifying neoplastic cells in serous effusions.

The identification of malignant cells in pleural, pericardial and peritoneal effusions is usually based on the subjective opinion of a person experienced in cell morphology, occasionally assisted by histochemical tests (such as the periodic acid-Schiff reaction for mucins) or by cytogenetic analysis. Although the trained observer can usually recognise malignant cells among the numerous different benign cell types which may be found in serous effusions,[1] there are a number of cases where a firm diagnosis cannot be made on morphological criteria alone. Techniques which can provide objective evidence of malignancy in such cases have therefore been long awaited.

Immunocytochemical staining procedures for demonstrating various cell and tissue antigens offer one means of increasing the accuracy of diagnosis in problem cases, and several laboratories have reported recently on the potential value of these techniques in diagnostic cytology (Table 1). In more than half of these studies, however, malignant cell identification depended on the reactions of a single antibody. Furthermore, in six of these investigations polyclonal antisera were used, which often give less reproducible results than do monoclonal antibodies and may produce non-specific reactions.

In the present study we have investigated the use of a panel of seven different monoclonal antibodies, chosen to include those reactive with epithelial and lymphoid antigens, to determine whether they would allow malignant and benign cells in serous effusions to be distinguished. The problem of background staining was overcome by the use of a recently developed immunoalkaline phosphatase (IAP) procedure.[11] Since the aim of this investigation was to assess the practical value of the method, all staining was performed on spare smears made by the routine method used in the Laboratory of Clinical Cytology, rather than on cell samples which had been subjected to special preliminary steps such as

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Table 1 *Previous studies of the application of immunochemical staining in diagnostic cytology*

Reference	*Antigens studied*	*Preparation of samples*	*Type of antibody*	*Staining technique*	*Samples studied (No of cases)*	*Comments*
Pascal and Fenoglio[2]	CEA	Paraffin embedded cell blocks of body fluids	Polyclonal	IP	Pericardial, pleural and peritoneal fluids, urine, bronchial washings (112)	Positive staining for CEA was found in all cases of adenocarcinoma. Benign cases were negative.
Singh *et al*[3]	Mesothelial cells	Methanol fixed smears of washed cells	"	IF	Pleural and peritoneal effusions.	A diagnosis of mesothelioma was confirmed in two cases and refuted in one.
Nadji[4]	Intracellular constituents—for example, immunoglobulins, enzymes, hormones & tumour markers (CEA, AFP)	95% ethanol fixed cell smears and paraffin embedded cell blocks	"	IP	Various types of routine cytologic specimens (number not given)	Several examples of the potential value of immunohistochemical staining of cytological samples were presented.
O'Brien *et al*[5]	CEA, ZGM and EMA	10% formalin fixed, paraffin embedded cell blocks	"	IP	Pleural and peritoneal effusions (62)	CEA, ZGM and EMA found respectively in 44%, 68% and 88% of malignant effusions.
To *et al*[6]	EMA	95% ethanol fixed smears of washed cells	"	IAP	Pleural and peritoneal effusions (127)	Intense staining of malignant epithelial cells. Weak staining of mesothelial cells.
To *et al*[7]	EMA	as above	"	IAP	as above (309)	54% of cytologically positive carcinoma samples stained strongly with EMA.
Woods *et al*[8]	Ca	Acetone fixed smears of unwashed cells	Monoclonal	IP	Pericardial, pleural and peritoneal fluids (50)	The antibody detected malignant cells in 91% of carcinoma samples, but did not react with mesothelial cells.
Mariani-Constantini *et al*[9]	Low molecular weight glycolipid	Fresh cells in suspension	"	IF	Pleural and peritoneal fluids from breast cancer patients (9)	Breast carcinoma cells could be identified in all cytologically positive cases. Mesothelial cells, leucocytes and red blood cells were negative.
Epenetos *et al*[10]	HMFG–2 AUA 1	Ether/ethanol fixed smears of washed cells	"	IP	Pleural and peritoneal fluids (70)	Both antibodies reacted with malignant epithelial cells but not with mesothelial cells.

IP = immunoperoxidase.
IF = immunofluorescence.
IAP = immunoalkaline phosphatase.
CEA = carcinoembryonic antigen.
ZGM = zinc glycinate marker.
EMA = epithelial membrane antigen.
HMFG = human milk fat globule.

washing, removal of red cells, or embedding of cell deposit in paraffin blocks.

Material and methods

SAMPLES

A total of 70 specimens of pleural and peritoneal fluid which had been sent to the Cytology Laboratory at the Churchill Hospital, Oxford formed the basis of this study. These samples were not a random or consecutive series, but were either unequivocally malignant effusions or else good examples of typical benign effusions (usually containing numerous mesothelial cells). Details of the type of effusion and the clinical diagnoses are shown in Table 2.

Samples were collected into EDTA (4 mg/ml final conc.) as anticoagulant. The fluid was centrifuged at 3000 rev/min for 5 min and the cell deposit smeared on slides to produce rapid drying. Where possible at least 10 slides were made. Two air dried smears were stained by a standard Giemsa method, two were fixed immediately for a minimum of 30 min in 95% alcohol prior to Papanicolaou staining, whilst the remaining air dried smears were stored unfixed wrapped in aluminium foil at −20°C for subsequent staining by the IAP technique. Cytological diagnosis was based on examination of the Giemsa and Papanicolaou stained smears.

REAGENTS

Antibodies

Details of the monoclonal antibodies used in this

Table 2 *Diagnosis and site of effusion*

Malignant disease			*Non-malignant disease*		
	Pleural	*Peritoneal*		*Pleural*	*Peritoneal*
Ca breast	8	2	Post-pneumonic	1	0
Ca ovary	1	8	Cardiac surgery	1	0
Ca endometrium	2	1	Cardiac failure	5	1
Ca prostate	1	0	Cardiac & renal failure	1	0
Ca bronchus	5	0	Renal failure	0	1
Ca bronchus (oat cell)	2	0	Hepatic and renal failure	1	0
Anaplastic carcinoma, probably of lung	1	0	Empyema	1	0
Ca pancreas	0	1	Liver cirrhosis	0	2
Ca colon	1	3	Constrictive pericarditis	0	1
			Benign ovarian cyst	0	2
			Nephrotic syndrome	0	1
Primary unknown	1	2	Pulmonary infarct	1	0
*Mesothelioma	1	0	Venous obstruction	0	1
			Rheumatoid arthritis	1	0
Lymphoma	6	2	Cause unknown	1	0
Total	29	19		13	9

*This was a subsequent specimen from the same case illustrated by Woods *et al.*[8]

Table 3 *Details of the monoclonal antibodies used for the immunocytochemical analysis of serous effusions*

Antibody	*References*	*Antigen*	*Comments*
2D1	Beverley[12]	Leucocyte common antigen	Leucocyte common antigen is confined to cells of haemopoietic and lymphoid origin. Its use for the immunohistochemical diagnosis of undifferentiated tumours has been described previously.[13,14]
Ca 1	Ashall *et al*[15]	Cancer-associated glycoprotein	This antigen appears to be preferentially expressed on malignant cells.[16] Its use for the immunocytochemical detection of malignant cells in serous effusions has been described previously.[8]
HMFG–2 (14.A.3)	Taylor-Papadimitriou *et al*[17]	Human milk fat globule membrane	This antibody is of similar specificity to the polyclonal antiserum (anti-EMA) used previously for the immunocytochemical analysis of serous effusions[6] (see Table 1). In tissue sections it reacts with all normal glandular epithelia, with the exception of prostate and liver.
LE 61	Lane[18]	Simple epithelium antigen on cytokeratin filaments	This antibody reacts with cells in simple epithelia and has been used for the immunohistochemical diagnosis of epithelial tumours.[14]
M 73	Cordell J (unpublished)	Intermediate filaments	This antibody was raised against Mallory bodies. Its immunohistochemical reactivity pattern is essentially identical to that of other antibodies raised against Mallory bodies[19] and to antibody LE 61 (see above).
11.285.14	Corvalan JRF, Woodhouse CS (unpublished)	Carcinoembryonic antigen	This antibody reacts with gastro-intestinal epithelium, non-keratinising squamous epithelium and many epithelial tumours.[14]
K 92	Pulford K (unpublished)	Keratin associated component	This antibody reacts with cutaneous epithelium and a minority of mammary duct epithelial cells. Non-keratinising squamous epithelium and other types of glandular epithelia are negative with this antibody.

Plate Ia *Peritoneal fluid from a case of serous cystadenocarcinoma of the ovary. The cell shown has a dense covering of microvilli at the distal pole. May-Grünwald-Giemsa × 450.*

Plate Ib *Same sample as (a), reacted with Ca 1. The most intense staining is in the area with microvilli, where cell membrane is most abundant. IAP × 450.*

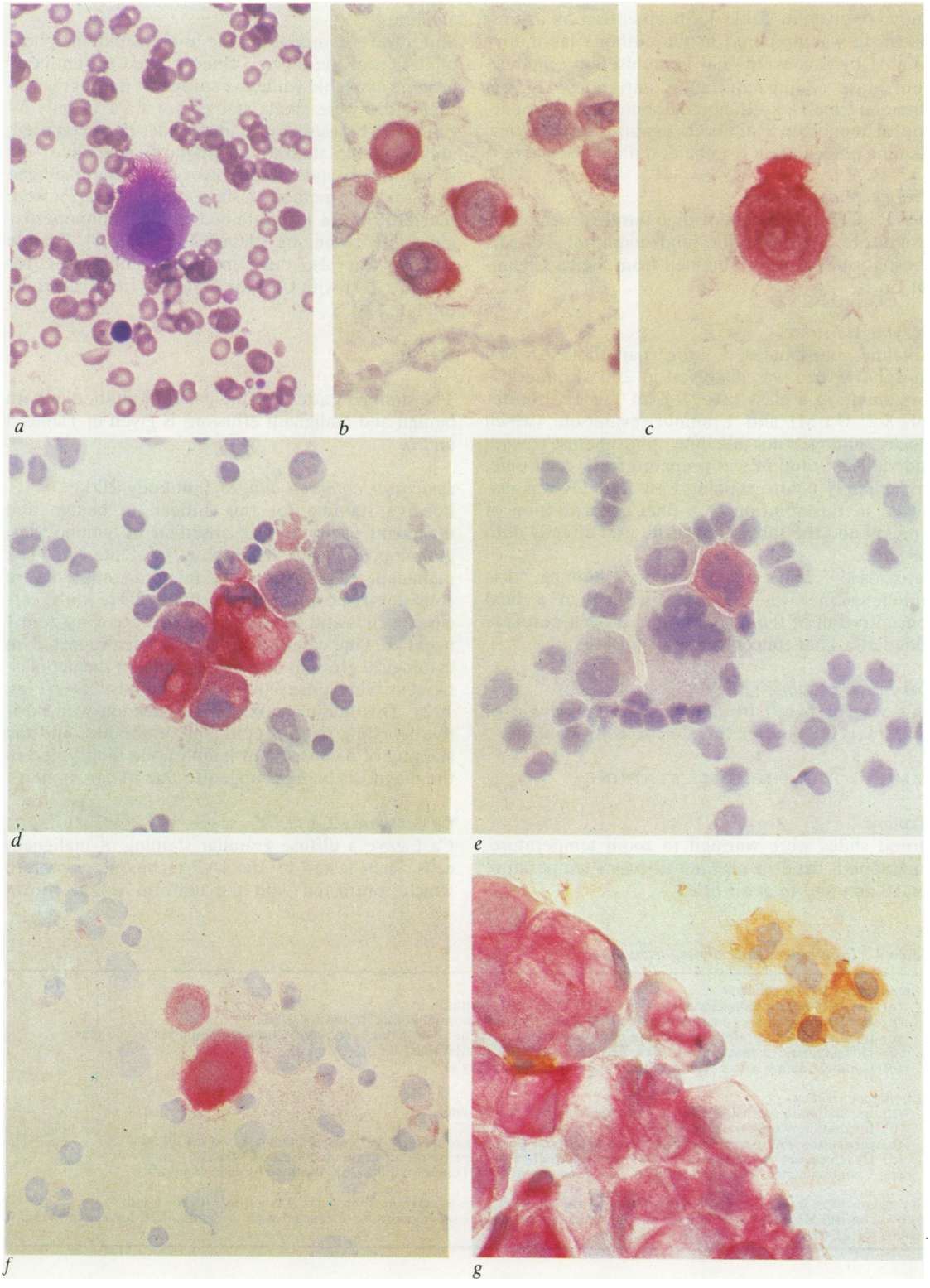
Plate Ic *Same case as (a) and (b). This cell has been reacted with HMFG–2, and shows strong positive staining, particularly of microvilli. IAP × 450.*

Plate Id *Pleural fluid from a case of carcinoma of the breast. A group of malignant cells is shown, reacted with anti-CEA. Several cells show strong positive staining. IAP × 450.*

Plate Ie *Same case as (d). A group of carcinoma cells reacted with Ca 1. Only one shows significant positive staining. IAP × 450.*

Plate If *Pleural fluid from a woman with cardiac and renal failure. Two mesothelial cells are shown, reacting positively with HMFG–2. (Most mesothelial cells stain weakly or not at all with this antibody). IAP × 450.*

Plate Ig *Peritoneal fluid from a case of carcinoma of ovary, illustrating double immunochemical staining. Clusters of carcinoma cells are shown stained pink by the IAP method for epithelial intermediate filaments (antibody LE61), while macrophages and lymphocytes, reacting with anti-leucocyte (antibody 2D1), are stained yellow-brown by immunoperoxidase. × 450.*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study are given in Table 3. Sheep antiserum against mouse Ig was prepared in the author's laboratory (DYM) by a conventional immunisation schedule. Peroxidase conjugated rabbit anti-mouse Ig was obtained from Dako Immunoglobulins. The production of monoclonal antibody against calf intestinal alkaline phosphatase is to be described elsewhere.[11]

Histochemical reagents
Fast Red TR, diaminobenzidine tetrahydrochloride, levamisole hydrochloride and naphthol AS-MX phosphoric acid were obtained from Sigma Chemical Co.

Enzyme substrates
Alkaline phosphatase: 2 mg naphthol AS-MX phosphoric acid was dissolved in 200 μl dimethylformamide in a glass tube; 9·8 ml Tris HCl buffer (pH 8·2, 0·1 M) and 1 mmol/l levamisole (which blocks endogenous alkaline phosphatase) were added. This solution was prepared fresh each time. Immediately before staining Fast Red TR was dissolved in this solution at a final concentration of 1 mg/ml and the substrate was filtered directly onto the slide.
Peroxidase: Immediately before staining diaminobenzidine was dissolved in TBS at a final concentration of 0·6 mg/ml and hydrogen peroxide added at a final concentration of 0·01%.

Tris-buffered saline (TBS)
This was prepared by adding a tenth volume of 0·5 M Tris HCl buffer (pH 7·6) to 0·15 M saline.

IMMUNOCYTOCHEMICAL STAINING

Fixation
Stored slides were warmed to room temperature, unwrapped, fixed in acetone at room temperature for 10 min and then air dried.

Staining
Slides were stained using the immunoalkaline phosphatase procedure (see Table 4). The Ca 1 antibody became available while the study was in progress, so that slides were single-stained for Ca 1 in only 54 cases. In 16 cases all unstained slides had been used, and a double staining technique was applied; the slides already stained for leucocyte common antigen by the IAP method (using antibody 2D1) were reacted with Ca 1 and stained by the immunoperoxidase (IP) technique (detailed in Table 4). Double staining was also done in a few other cases, for instance 2D1 with Ca 1, or 2D1 with LE61 as shown in Plate I(g).

Results

The staining pattern of the panel of antibodies with benign and malignant effusions is given in Tables 5 and 6.

Leucocyte common antigen (antibody 2D1)[12–14]
Positive staining for this antigen in benign and malignant effusions was observed in lymphocytes, granulocytes and macrophages (Plate Ig). The malignant cells from all lymphoma samples were consistently positive with antibody 2D1, whilst carcinoma cells and mesothelial cells were consistently negative. One case had previously been reported on cytological grounds as a lymphoma, but the neoplastic cells were unreactive with antibody 2D1 (Figs. 1, 2). This finding, taken in conjunction with positive labelling by anti-epithelial antibodies, and the staining of a subsequent lymph node biopsy, led to the diagnosis being changed to one of carcinoma.

Ca (antibody Ca 1)[15 16]
Ca 1 gave a diffuse granular staining of malignant cells when tested by the IAP technique, a pattern which contrasted with the uniform yellow brown

Table 4 *Immunoenzyme staining techniques*

1 *Immunoalkaline phosphatase*
(a) Incubation with monoclonal antibody (see Table 3) for 60 min.
(b) Incubation with sheep antimouse Ig (1/20) containing 5% normal human serum for 30 min.
(c) Incubation with preformed alkaline phosphatase-anti alkaline phosphatase (APAAP) immune complexes for 60 min.
(d) Development of reaction with Fast Red TR substrate (see Methods) for 10–15 min.
(e) Counterstaining with haematoxylin and mounting in Apathy's.

2 *Immunoperoxidase*
(a) Incubation in methanol +0·5% hydrogen peroxide for 30 mins.
(b) Incubation with monoclonal antibody for 60 min.
(c) Incubation with peroxidase conjugated antimouse Ig (1/50) containing 5% normal human serum for 30 min.
(d) Development of reaction with diaminobenzidine/hydrogen peroxide substrate (see Methods) for 5–10 min.
(e) Counterstaining with haematoxylin, and mounting in DPX (single staining), or Apathy's (double staining).

All reagents were diluted in TBS unless otherwise stated. After each incubation, slides were washed in TBS for 5 min.
Positive controls were included in each run, in which appropriate tissue sections were reacted with the antibodies, for example breast sections for LE61.

Table 5 *Reaction of panel of monoclonal antibodies with malignant cells*

Type or origin	*No of cases*	*2D1*	*Ca 1*	*HMFG-2*	*LE61*	*M73*	*CEA*	*K92*
Ca breast	8	−	+	+	+	+	+	−
	2	−	+	+	+	+	NT	−
Ca ovary	3	−	+	+	+	+	+	−
	2	−	+	+	+	+	+	+
	2	−	+	+	+	+	−	−
	1	−	−	+	+	+	−	−
	1	NT	+	+	+	NT	+	NT
Ca endometrium	2	−	+	+	+	+	−	−
	1	−	+	+	+	+	−	NT
Ca prostate	1	−	+	+	+	+	+	−
Ca bronchus	4	−	+	+	+	+	+	−
	1	−	+	+	+	+	+	+
Ca bronchus (oat cell)	1	NT	+	+	+	+	+	−
	1	−	+	+	+	+	NT	NT
Anaplastic carcinoma probably of lung	1	−	−	+	−	NT	−	NT
Ca pancreas	1	NT	+	+	+	+	+	−
Ca colon	2	NT	+	+	+	+	+	−
	2	−	+	+	+	+	+	−
Primary unknown	2	−	+	+	+	+	+	−
	1	−	+	+	+	+	+	+
Mesothelioma	1	−	+	+	+	+	+*	+
Lymphoma	7	+	−	−	−	NT	NT	NT
	1	+	−	−	−	NT	−	−

NT = not tested.
+ = positive.
− = negative.
* very weak.

Table 6 *Reaction of panel of antibodies with "active" mesothelial cells in benign effusions*

	Positive	*Negative*
2D1	0	22
Ca 1	2	20
HMFG-2	7 strong 7 weak	8
LE61	22	0
M73	22	0
CEA	0	22
K92	0	22

colour obtained by the IP method. The stain was often weak compared with that obtained with other antibodies. Table 5 shows that of the 40 effusions which were cytologically positive for carcinoma cells, 38 contained cells reactive with Ca 1. One Ca 1 negative case was the anaplastic tumour referred to above, while the other sample was from a patient with carcinoma of the ovary in which only small numbers of carcinoma cells were present in the smear. There was marked variation in the intensity of staining for Ca 1 within any tumour cell population. In three of five cases of cancer of the bronchus Ca 1 stained a very small proportion of morphologically malignant cells strongly while the rest were very weakly stained or negative. This heterogeneity of staining in a smear was also seen in two of 10 cases of carcinoma of the breast (Plate Ie), two of nine cases of carcinoma of the ovary, one of three cases of carcinoma of endometrium, and one of four cases of carcinoma of the colon. Adenocarcinoma cells carrying localised tufts of microvilli showed much stronger staining in these areas; a good example from carcinoma of the ovary is shown in Plate Ia,b. The one case of mesothelioma tested gave a positive reaction with Ca 1. None of the lymphoma cases tested was positive with Ca 1.

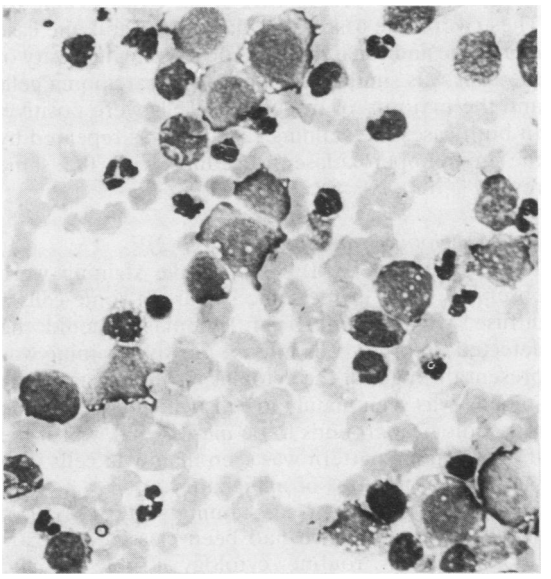

Fig. 1 *Numerous anaplastic malignant cells in pleural fluid of a woman aged 27yr. They were wrongly interpreted as lymphoma cells. May-Grünwald Giemsa × 460.*

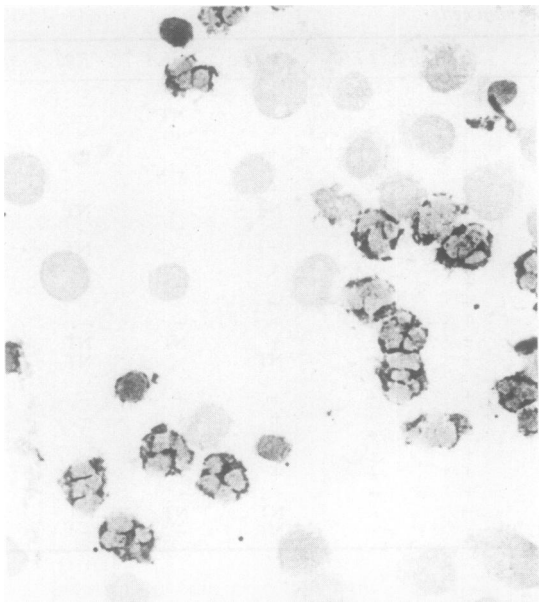

Fig. 2 *Same case as Fig. 1. The smear has been incubated with the monoclonal antibody 2D1, which reacts with leucocytes and lymphoma cells. Only the neutrophils are stained, the tumour cells remaining negative. The tumour was subsequently identified as an anaplastic carcinoma. IAP* × *460.*

In the benign effusions Ca 1 positive mesothelial cells were observed in two of 22 cases (Table 6). These were one case of cardiac failure and one case of cardiac and renal failure. The staining intensity of the cells was similar to that seen in carcinoma cells and the majority of mesothelial cells were positive. In both cases the staining reaction was repeated by the immunoperoxidase procedure with the same result.

Milk fat globule (antibody HMFG-2)[17]

Two patterns of HMFG-2 positive staining were observed, often in the same smear. In some cells a diffuse weak staining of cytoplasm could be detected; in other cells intense positive staining was present throughout the cytoplasm. The diffuse weak staining was seen mainly in cells which appeared on morphological grounds to be mesothelial, whilst the strong staining pattern was seen mainly in cells with the classical features of malignancy.

HMFG-2 gave a strong staining pattern in 39 of the 40 effusions which had been classified as carcinomatous on routine cytology (Table 5, and Plate Ic). The one exception was an effusion containing clearly malignant cells (of unknown origin) which stained only weakly for HMFG-2. In the case of malignant mesothelioma the cells gave a strong reaction (Fig. 3).

In contrast to the frequency with which HMFG-2 stained carcinoma cells, this antibody gave consistently negative reactions with each of the eight samples containing lymphoma cells.

In 14 of 22 benign effusions, HMFG-2 positive mesothelial cells were observed (Table 6). The majority of the mesothelial cells in these samples were positive and stained with a diffuse weak pattern. In seven of them (six pleural and one peritoneal) a few mesothelial cells gave strong staining (Plate If).

"Simple epithelium cytokeratin" antigen (antibody LE61)[18]

Antibody LE61 gave a positive reaction with malignant cells in all of the effusions from clinically and cytologically proven cases of carcinoma or mesothelioma (Table 5, and Plate Ig), with the exception of the anaplastic carcinoma referred to already. The commonest staining pattern was an intense positive labelling throughout the cytoplasm (Fig. 4). LE61 also reacted strongly with many mesothelial cells in benign and malignant effusions (Figs. 4, 5) as might be expected since similar cytokeratins have been demonstrated in these cells.[20] LE61 did not react with malignant cells from any of the lymphoma cases studied.

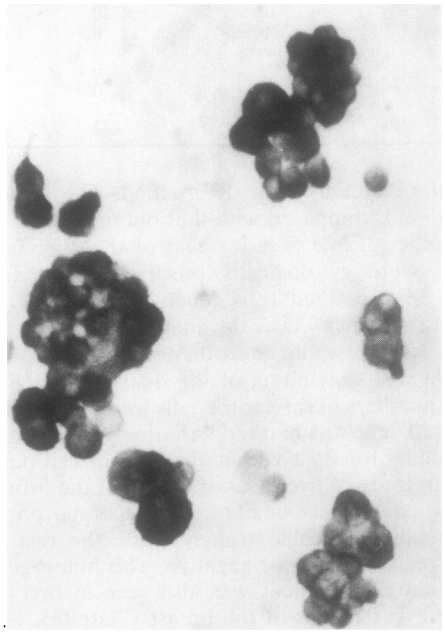

Fig. 3 *Clusters of malignant cells in pleural fluid deposit from a case of mesothelioma, reacted with HMFG-2. IAP* × *240.*

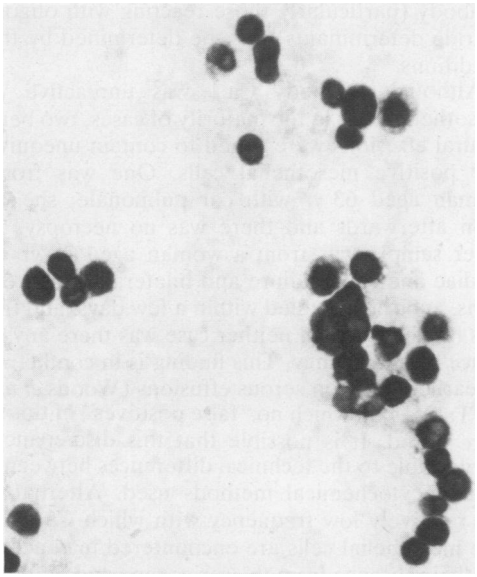

Fig. 4 *Pleural fluid from a case of endometrial carcinoma, showing tumour cells and mesothelial cells equally strongly stained using the antibody LE61 against cytoplasmic filaments (cytokeratin). IAP × 150.*

Intermediate filaments (antibody M73)
The staining reactions for the determinant recognised by antibody M73 were similar to LE61 (Tables 5, 6). M73 gave a positive reaction with carcinoma cells, mesothelioma and mesothelial cells, but showed no labelling of lymphoid cells. In some cells a diffuse or granular staining of the cytoplasm was observed but in the majority of cells intense positive staining was seen throughout the cytoplasm. The type of staining did not show any obvious correlation with the site of the primary tumour.

Carcino-embryonic antigen (anti-CEA)
Positive cells were detected in 30 of 36 effusions from clinically and cytologically proven cases of carcinoma (Table 5), all of them showing strong staining throughout the cytoplasm (Plate Id). Anti-CEA gave consistently negative reactions with all benign effusions and with lymphoma cells.

Keratin (antibody K92)
The main pattern of staining observed with K92 was a weak stain throughout the cytoplasm. K92 gave a positive reaction with cells from two cases of carcinoma of the ovary (although not all malignant cells were positive), one case of carcinoma of unknown origin and one case of carcinoma of the bronchus. A weak diffuse staining reaction was observed in cells from the patient with mesothelioma (Table 5). K92 did not react with cells from benign effusions or with the one case of lymphoma tested.

Discussion

The purpose of this study was to compare the immunocytochemical reactivity patterns of a number of monoclonal antibodies with cells in malignant and benign effusions and to assess their potential value in routine diagnostic cytology. An earlier study of normal and neoplastic human tissues[14] has clearly established the usefulness of a number of these antibodies in routine histopathology, particularly in elucidating the true nature of anaplastic neoplasms.

The reactions of the different cell types found in effusions may be summarised as follows:
Leucocytes, including granulocytes and lymphoid cells, were labelled by 2D1 (anti-leucocyte common antibody) but by none of the other monoclonal

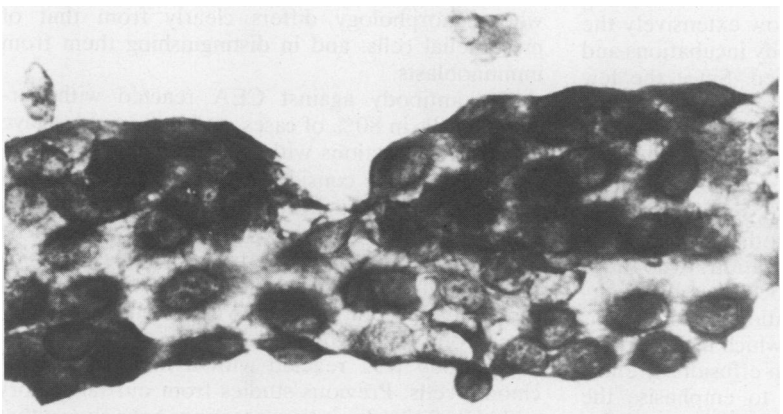

Fig. 5 *A sheet of detached mesothelial cells, from peritoneal fluid obtained at laparotomy, reacted with LE61. Cell attachments are clearly seen as "bridges" IAP × 560.*

reagents. In several subsequent cases a strong non-specific positive IAP reaction has been seen with Ca 1, HMFG-2 and anti-CEA in occasional lymphoplasmacytoid cells and plasma cells. The possibility that it is due to intracellular heterophile antibodies is being investigated.

Lymphoma cells, in all cases tested, also reacted only with 2D1, providing one means of objectively distinguishing this type of neoplasm from carcinoma in cytologically equivocal cases. A negative reaction of neoplastic cells for 2D1 may also be informative, as illustrated by the case described above, in which an initial cytological diagnosis of lymphoma was revised to one of carcinoma.

Macrophages also reacted with 2D1 only: this applied both to the smaller types and to those of large size, which are common in effusions and which are morphologically indistinguishable from "degenerating" mesothelial cells.

Mesothelial cells, at least those classified as "active" mesothelial cells,[1] gave negative reactions with 2D1, CEA and K92. They stained strongly with LE61 and M73. With HMFG–2 they usually stained weakly or not at all, but a few strongly stained cells were present in seven cases. Using a polyclonal antiserum of related but broader specificity, To *et al*[6] also detected weak staining of mesothelial cells. This is in contrast to Epenetos *et al*[10] who used HMFG–2 and found that it did not react with normal mesothelial cells. These different observations may be due to differences in the techniques used in the fixation and staining of the smears. The antibody HMFG–2 is directed to an oligosaccharide determinant on a large molecular weight component of the human milk fat globule. When expressed by cells, this determinant may be carried on more than one type of molecule which may have different affinities for the antibody.[21] Whether low affinity binding sites are detected as positive depends on the staining techniques and particularly on how extensively the slides are washed between antibody incubations and what fixation procedures are used. Since the few mesotheliomas so far tested strongly express the HMFG–2 determinant, it is not unlikely that other abnormal mesothelial cells, such as the reactive ones found in benign serous effusions, could also express HMFG–2 sites. However, these have not been detected by Epenetos *et al*[10] and other investigators (A Griffiths, personal communication) who have used more extensive washing procedures than ourselves as well as a different fixation method. This suggests that any HMFG–2 sites which may be present on mesothelial cells in benign effusions are low affinity sites. Our results serve to emphasise the need to use well defined conditions in immune staining procedures, since the spectrum of reactivity of an antibody (particularly those reacting with oligosaccharide determinants) may be determined by these conditions.

Although antibody Ca 1 was unreactive with mesothelial cells in the majority of cases, two benign pleural effusions were found to contain unequivocally positive mesothelial cells. One was from a woman aged 63 yr with cor pulmonale; she died soon afterwards and there was no necropsy. The other sample was from a woman aged 78 yr with cardiac and renal failure and bilateral pleural effusions, and she also died within a few days, and there was no necropsy. In neither case was there any evidence of malignancy. This finding is in conflict with an earlier study on serous effusions (Woods *et al*[8]—see Table 1) in which no "false positives" of this sort were noted. It is possible that this discrepancy is attributable to the technical differences between the immunocytochemical methods used. Alternatively the relatively low frequency with which Ca 1 positive mesothelial cells are encountered may account for their absence from the series reported by Woods *et al*.[8] Whatever the explanation of this phenomenon its existence should be borne in mind when using Ca 1 antibody for the detection of malignant cells in cytological samples. In this context it may be noted that the finding of Ca 1 antigen in normal cells is not without precedent, since in immunohistological studies of human tissue sections this antibody has been shown to react with certain epithelial cell types.[16]

Carcinoma cells consistently gave strong positive reactions with LE61 and M73, but since mesothelial cells commonly react in the same way, this has no discriminatory value. They also gave strongly positive staining with HMFG–2, stronger than mesothelial cells generally show, so that this provides some confirmatory evidence; it would be valuable, for instance, in searching for small numbers of oat-cells, whose morphology differs clearly from that of mesothelial cells, and in distinguishing them from immunoblasts.

The antibody against CEA reacted with carcinoma cells in 80% of cases, and in this series gave no positive reactions with other cell types. Consequently it can be considered of high discriminatory value. Ca 1 also gave a positive reaction in nearly every case. However this was often weak, and the occurrence of two benign effusion samples in which mesothelial cells were positively stained (see above) should lead to caution in drawing diagnostic conclusions from this antibody alone.

Antibody K92 reacted with a minority of carcinoma cells. Previous studies from our laboratory with this antibody on tissue sections have shown that antibody K92 is reactive with keratinising squamous

epithelium (and tumours) but also with a few cells in adenocarcinoma samples.[14] Keratinised carcinoma cells are very rare in effusions, and adenocarcinoma cells are very common. Consequently a positive reaction is of no help in determining cell type.

Finally, staining with 2D1 was consistently negative, and this is of value in distinguishing carcinoma from lymphoma, as illustrated in the case discussed above.

Mesothelioma was represented in this series by only one case, so that no general conclusions should be drawn. Besides the reaction with Ca 1 (described by Woods *et al*)[8] there was a very strong reaction with HMFG–2. If this finding is confirmed in other cases, a useful method would be available for distinguishing mesothelioma from benign mesothelial proliferation.

Conclusions

Previous studies on the application of immunochemical staining techniques to cytological samples have usually been based on the detection of a single antigen (see Table 1). This approach has obvious limitations which we have attempted to overcome by using a panel of seven monoclonal antibodies directed against different antigens.

Three of the antibodies used in this study (anti-CEA, Ca 1 and HMFG–2) may form a suitable panel for the diagnosis of effusions. Of these only one (anti-CEA) was specific for carcinoma cells in the present series. However the non-specific reactions of the other two antibodies were restricted to labelling of mesothelial cells (rarely in the case of Ca 1 and more frequently with HMFG–2). Hence judicious assessment of the labelling reactions of the three antibodies taken together may prove of value in the detection of carcinoma cells which would otherwise pass unnoticed, and for confirmation of a suspected diagnosis of malignancy. It is to be hoped that other monoclonal antibodies of considerably greater specificity will become available in the future.

It is important both for cytological diagnosis and for immunoenzymatic staining, that cell smears are optimally processed. The technique used in the present study (that is, the staining of air-dried smears of centrifuged cell pellets) is used in many laboratories for the routine preparation of Romanowsky-stained cytological samples. Provided smears are air-dried rapidly, morphology is very clearly defined. Using a sensitive IAP method with monoclonal antibodies we found little or no background staining and there was no need to wash the cells or to remove red cells, as was done in other studies.[6,7,10] The alkaline phosphatase reaction product produces a vivid red colour which facilitates the identification of positively labelled cells even when the enzyme reaction is weak. This enzyme label also offers the advantage that endogenous alkaline phosphatase is present in lower amounts (and is more easily inhibited) in serous effusion cells than is endogenous peroxidase. Our experience with alkaline phosphatase as an antibody label in cytological studies is in keeping with the preference of To *et al*[6] for this enzyme.

A further application of immunocytochemical staining is the detection of two antigens in the same smear. We have already shown that smears can be stained for a lymphoid antigen and an epithelial associated antigen (Plate Ig). Double staining with light chain antibodies and with T and B cell markers may be useful in the diagnosis of lymphoma cases to indicate a neoplastic origin, and we are currently looking at other lymphoid cell markers in these cases. Additional examples of the way in which double enzymatic staining may be used for the analysis of peripheral blood cells has recently been reported from one of our laboratories by Moir *et al*.[22]

Even when using an antibody with a high specificity, it is unwise to place complete reliance on a single immunocytochemical test. The increased information obtained when further appropriate antibodies are used is much more than simply additive, since the different results confirm and control each other. Thus the use of multiple standard monoclonal antibodies can already provide compelling evidence for the presence of cells from the common kinds of cancer, and in future will increasingly help to determine the type of the primary tumour. The present study indicates the value of this approach in diagnosis of serous effusions, and the techniques described may be adapted to complement the traditional morphological assessment in many other types of cytological material.

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