THE USE OF IMMUNOCYTOCHEMICAL MARKERS FOR THE DIAGNOSIS OF MALIGNANCY IN SEROUS EFFUSIONS

by

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To my parents

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Abstract

The cytological diagnosis of malignancy in serous effusions, at the present time, depends upon the recognition by light microscopy of morphological differences between stained malignant and non-malignant cells. Not infrequently reactive mesothelial cells present morphological changes indistinguishable from those of the 'benign-looking' malignant cells thereby presenting the cytologist with differential diagnostic problems.

The distribution of the Epithelial Membrane Antigen (EMA) and the Carcinoembryonic Antigen (CEA) on cells in effusions has been examined to ascertain whether this approach is of value in discriminating between malignant and mesothelial cells. The markers were demonstrated by an indirect immunoalkaline phosphatase staining technique on smear preparations of cells from serous effusions subsequently fixed in 95% ethanol. This research was extended to include radioimmunoassay for CEA in serous effusions. A third aspect of this thesis involved an attempt to raise monoclonal antibodies to non-neoplastic mesothelial cells.

Three hundred and nine routine serous effusion specimens were stained for EMA. The immunocytochemical results were correlated with the cytology of the specimens and the clinical diagnoses. The pattern of EMA staining was classified as weak or strong. Weak staining was present

on non-neoplastic mesothelial cells as well as on malignant epithelial cells. Strong EMA staining was present on 63 of the 116 specimens reported as cytologically positive for malignancy. In addition, malignant cells in five cytologically suspicious and three negative effusions from patients with epithelial malignant disease were picked out by strong EMA staining. However, in three cases strong EMA staining was also observed in specimens from patients without evidence of malignancy indicating that this method may give rise to false-positive results.

One hundred and eighty-two of the 309 specimens investigated in the EMA study were also stained for CEA. The results indicated that CEA staining is specific for malignant epithelial cells. However, the sensitivity of detection is low as only 22 out of the 59 cytologically positive cases stained for CEA. Nevertheless, malignant cells in 1 cytologically suspicious and 3 negative specimens from patients with malignant epithelial disease were picked out by this approach.

The CEA levels in 88 effusions from patients with malignant disease and 63 fluids of benign origins were measured. The results indicated that CEA levels above 19 ng/ml were confined to effusions from patients with carcinomas.

An attempt to raise monoclonal antibodies to non-neoplastic mesothelial cells was unsuccessful. The

monoclonal antibodies obtained were not specific for these cells and also stained malignant cells.

In conclusion, the demonstration of EMA and CEA on the cells in effusions, in conjunction with the clinical data,can be of value (1) in the characterisation of cells which on morphological grounds are of indeterminate origin; (2) in picking out small numbers of malignant cells (whose identity can be confirmed by a subsequent Papanicolaou stain) from specimens reported as cytologically negative; (3) in identifying the epithelial or mesothelial origin of morphologically malignant cells. CEA assay of serous effusions can provide information of the presence of malignant epithelial disease in patients, although an elevated CEA level does not necessarily indicate the presence of malignant cells in the fluids. In this respect cytology and CEA assay represent two different approaches to the diagnosis of malignancy in patients with effusions.

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Chapter 1

Purpose of study

Within the last few years, immunocytochemistry has assumed a growing importance in histopathology (Mesa-Tejada et al, 1977). With the introduction of the hybridoma technique and the production of monoclonal antibodies (Kohler and Milstein , 1975), this functional approach to human pathology may come to have increasing importance. Immunocytochemistry has the potential to extend conventional microscopic analysis of surgical specimens by providing pathologists with the opportunity to study simultaneously a particular type of cell or tissue and the product which the cells or tissue express. This approach has been of value in aiding the conventional diagnosis and histological classification of tumours as well as in detecting micrometastasis. (Delellis et al, 1979; Sloane et al, 1980a) For example, B-cell lymphoma may be distinguished from undifferentiated carcinoma by immunocytochemical staining for immunoglobulins (Taylor, 1978a; Mason et al, 1980) The demonstration of the Epithelial Membrane Antigen in bone marrow smears has been useful for the detection of micrometastasis in patients presenting with a localised malignant lesion of the breast (Dearnaley et al, 1981) . However, to date, few of the advances made in the application of immunocytochemical techniques to histopathological diagnoses have been made in diagnostic cytology, as there has been little research in this field. Where immunocytochemical staining has been attempted in cytopathology, it has been confined to the demonstration of viral antigens in colposcopic biopsies, for

example, the identification of cervical wart virus infection in patients with dysplastic lesions of the cervix (Morin et al, 1981). The potential of tumour marker studies has not been explored.

One long standing problem in diagnostic cytology which has not been investigated by immunocytochemical techniques, and may be resolved by tumour marker studies, is one that involves the diagnosis of malignancy in serous effusions. The discrimination between reactive mesothelial cells and small malignant cells in smears of effusions is difficult by virtue of their morphological similarities. Cytologists tend to err on the side of caution when faced with this problem and report the specimens as negative. Furthermore, the primary site of the malignant cells in effusions can rarely be identified. The distinction between epithelial and non-epithelial origin of anaplastic tumours also can be difficult. Lastly, 'benign-looking' malignant cells may be missed or not readily identified in routine screening leading to false-negative reports.

This study has applied immunocytochemical techniques to the cytodiagnosis of malignancy in serous effusions with the specific aim of determining whether immunocytochemical staining for the tumour markers, EMA and CEA, could contribute to the resolution of these problems. Furthermore, because of the conflicting reports concerning the usefulness of CEA measurement in effusions, the author proposed to include

this method of investigation in order to ascertain its diagnostic value. Finally, an attempt was made to raise monoclonal antibodies to non-neoplastic mesothelial cells by the hybridoma technique. The aim of this exercise was to obtain monoclonal antibodies that could be used to stain specifically non-neoplastic mesothelial cells by immunocytochemical methods.

Chapter 2

The diagnosis of malignancy in serous effusions

2.1 The cytological approach

2.2 The histochemical approach

2.3 The electron microscopic approach

2.4 The cytogenetic approach

2.5 The histological approach

2.6 The biochemical approach

2.1 The cytological approach

The pleural, the peritoneal and the pericardial cavities are lined by a single layer of mesothelial cells. In healthy individuals, these cavities are flattened and only a minute amount of fluid is present for the purpose of lubrication. The accumulation of body fluid in these cavities constitutes an effusion, which invariably indicates a pathological process. The blood cells, the cells of the reticulo-endothelial system, the mesothelial cells and, when present, the malignant cells, are the cellular constituents found in serous effusions. The cell content of an effusion can be visualised under the light microscope by examination of either the cytological smear or the cell block prepared from the cell sediment after centrifugation. While the cell block technique is often preferred by histologists, cytological smears are mostly preferred by cytologists because when the smear preparations are of good quality, they are much easier to interpret and take less time to prepare (Canti, personal communication; Koss, 1979).

The prime object of cytological examination of serous effusions is to determine the presence of malignant cells. Whilst a negative diagnosis is non-contributory, an accurate positive diagnosis of malignancy has a practical clinical value, in that it is a cost-effective method of determining future management of the patient. At the present time, however, the cytological diagnosis of malignancy in serous

effusions is based almost exclusively upon the morphological examination of the stained, detached cells under the light microscope, from which only limited information can be obtained and for which the interpretation is subjective. Numerous reports have been published on the accuracy of cytological investigation as a method of diagnosing malignancy in serous effusions. Most of these reports are concerned with the frequency with which false cytological diagnosis is given. A review of literature was given in the publication of Kesavan Kutty et al (1981). This situation is appropriately summarised by Spriggs and Boddington (1968), who stated: "there is no particular object in recording here the statistics of correct and incorrect (cytological) diagnoses published from laboratories all over the world and down the years. These do not show a steady improvement with advances in knowledge". Reasons for this are threefold:

First, a substantial number of diagnostically difficult cases are the result of poor laboratory preparatory techniques which render the specimens unsuitable for reliable morphological assessment under the light microscope. Failure to appreciate that proper preparation of the specimen is of paramount importance for accurate cytological diagnosis accounts for many of the diagnostic problems on purely technical grounds (Canti, 1981).

Second, due to human error in screening or to the morphologically benign-appearance of the malignant cells or

a combination of both of these factors, discrete malignant cells may not readily be identified among other nucleated cells in routine screening and this can lead to false-negative reports.

Third, the body fluid provides an ideal medium for cell proliferation. Apart from the iatrogenic changes due to radiotherapy or chemotherapy, it is this free proliferation in fluid medium that frequently results in the loss of the characteristic morphology of the cell by which it is normally identified in the light microscope. Moreover, in inflammatory conditions of the pleura, peritoneum and pericardium, the mesothelial cells often assume a strikingly abnormal appearance which makes them indistinguishable from tumour cells. In practice the number of cases which present such problems is small (probably 5 - 15% depending upon the particular cytology laboratory in question); the majority of malignant cells are easily recognisable. However, when the malignant cells are well-differentiated and appear 'benign-looking', they are often morphologically indistinguishable from the reactive mesothelial cells. In these cases, the specificity of the cytological method is preserved at the expense of the sensitivity of the technique by reporting these cases as benign, and it is this group of specimens with which this thesis is primarily concerned.

In order to increase the accuracy of the diagnosis of malignancy in serous effusions, numerous attempts have been made in the past to complement the cytological examination

by additional investigative techniques. Histochemical, electron microscopic, cytogenetic and biochemical techniques have been applied to specimens of effusions and their diagnostic efficiency evaluated. The place of these techniques in the diagnosis of malignancy of serous effusions is discussed in the following sections.

2.2 The histochemical approach

The first application of Periodic Acid Schiff (PAS)-Diastase staining technique to cytological smears of serous effusions was carried out by Spriggs (personal communication). As a primary screening technique to detect malignant cells in effusions, the sensitivity of the Periodic Acid Schiff - Diastase method is low as it only detects the mucin-secreting adenocarcinoma cells (Spriggs and Boddington, 1968). Therefore, although a positive stain for mucins invariably indicates the presence of malignant cells in the fluid, the technique is rarely used on a routine basis in the cytology laboratory. More often it is used to confirm the presence of adenocarcinoma cells in the fluid when this has been suspected on morphological grounds.

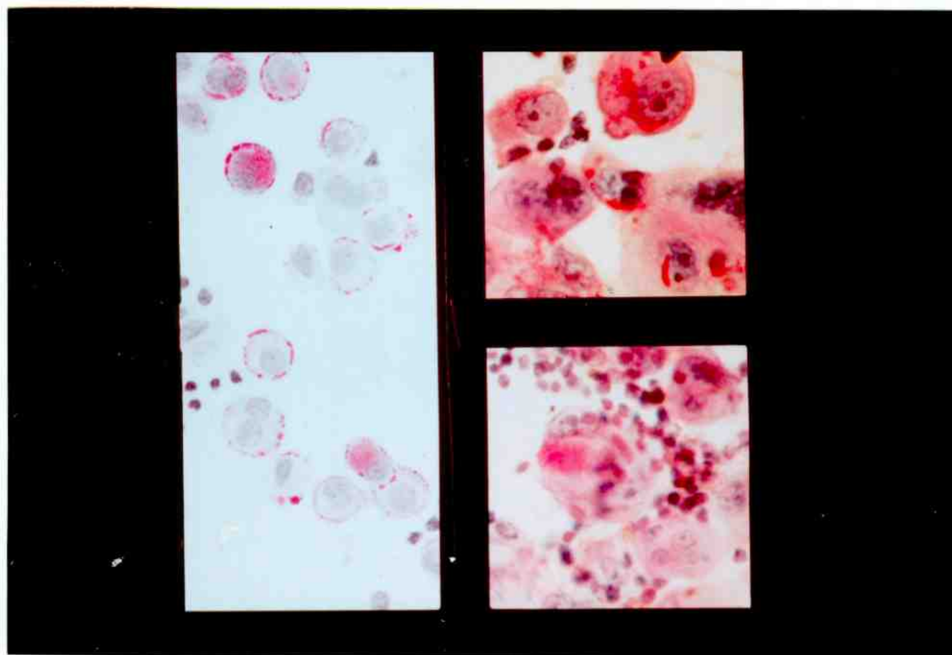
The Periodic Acid Schiff technique alone stains both glycogen and mucins. Whereas glycogen appears as bright red granular substances most often present at the periphery of the cytoplasm and surrounding the nucleus, mucins usually assume a diffuse appearance concentrated towards one pole of

the cell. To experienced cytologists, glycogen and mucins may be distinguished by their morphology. Nevertheless, there are cases in which this distinction is difficult (Figure 2.1) and can best be established by diastase digestion. Foot (1959) and Mavrommatis (1962) suggested that the presence of glycogen may be taken to indicate the cells in question are of mesothelial origin. This view however is not shared by other workers and in fact it has been clearly demonstrated by Canti that glycogen can be present in malignant as well as in mesothelial cells (Figure 2.1) (Spriggs and Boddington, 1968 - Figure 39).

Alcian blue is another histochemical staining technique for mucins. It differs from the PAS-Diastase method in that it stains the acidic form of mucins, whereas the PAS-Diastase method demonstrates the presence of neutral mucins which account for the vast majority of mucin-secreting adenocarcinoma cells in serous effusions. In the experience of Canti, who has used the Alcian blue technique on a routine basis for many years, positive stain of acidic mucins in effusions is a rare finding (personal communication). Other histochemical methods, for example, the Masson Fontana's silver or the methyl-green pyronin techniques, may be used as an objective test to confirm respectively the presence of melanoma cells or plasmacytoma cells, but effusions from patients with these diseases are very infrequent indeed.

2.3 The electron microscopic approach

Figure 2.1



Left - Periodic Acid - Schiff stained reactive mesothelial cells from a patient with alcoholic cirrhosis; two types of glycogen were observed, one typical and the other, which without diastase digestion, may be misinterpreted as mucins. (X130)

Top Right - Periodic Acid - Schiff stained adenocarcinoma cells from a patient with carcinoma of ovary; the malignant cells before diastase digestion showed both glycogen and mucins. (X130) (Courtesy of Dr G Canti)

Bottom Right - Periodic Acid - Schiff stained adenocarcinoma cells from the same specimen after diastase digestion showing mucins only. (X130) (Courtesy of Dr G Canti)

One of the earliest reports of the application of the Transmission Electron Microscope (TEM) to the study of the cells in effusions was published in 1956 by Luse and Reagan, and in this early study the feature of numerous microvilli present on both mesothelial and malignant cells was noticed. Since then, other specific intracytoplasmic features have occasionally been observed in particular types of tumour cells by the TEM techniques. In a study carried out by Woyke _et al_ (1972), the presence of numerous lamellar osmiophilic bodies was demonstrated in the cytoplasm of alveolar carcinoma cells. Similarly, the presence of neuro-secretory granules in oat cell carcinoma may be a useful diagnostic feature. However, as noted by Domagala and Woyke (1975), no special intracytoplasmic features were found in the majority of cancers which frequently metastasize to the serous cavities, i.e. ovary, breast, lung and stomach.

While transmission electron microscopic examination is based upon the ultrastructural studies of the cytoplasmic and nuclear details in ultrathin sections, the entire surface configuration of the cell can be clearly revealed by Scanning Electron Microscopy (SEM). One of the earliest reports of SEM investigation of cells in serous effusions was published in 1975 by Domagala and Woyke. They suggested that, (although not found in oat cell carcinoma of the lung), the presence of numerous microvilli on the surface of a cell in an effusion may be considered as a diagnostic feature for malignancy and the SEM technique could be regarded

as a useful tool in cases where the distinction between benign and malignant cells was difficult. However, Spriggs and Jerromo (1976) disagreed with this conclusion and demonstrated by the TEM technique the presence of numerous microvilli on the surface of mesothelial cells. They pointed out that the presence of abundant microvilli on the surface of a cell in an effusion was not specific for malignancy, although it was more likely for such a cell to be found in specimens full of malignant cells from adenocarcinoma and mesothelioma. In a recent publication by Domagala and Koss (1981), the principal differences between the surface configuration of cancer cells and mesothelial cells were summarised as shown in Table 2.1. Basically, while numerous microvilli could be found on the cell surface of both mesothelial and cancer cells, in the former the microvilli were orderly arranged but in the latter a denser and irregular pattern was observed.

The application of scanning electron microscopy to the diagnosis of malignancy in effusions has its disadvantages. First, the interpretation of SEM pictures is rather subjective. Second, there has been no specific and objective feature that can be used as a consistent distinguishing characteristic for malignant cells. Third, neither the SEM nor the TEM approach is practical as a screening technique to be used in a routine cytology laboratory.

Table 2.1

Principal differences between the surface configuration of malignant and mesothelial cells

(Domagala & Koss, 1981)

	Surface features	Malignant cells	Mesothelial cells
i)	Microvilli		
	Regular	+	+++
	Irregular	+++	±
	Density/unit area	High	Low
ii)	Blebs	±	+++
iii)	Ridges	-	±

Symbols: ± very rare; + rare; +++ common.

2.4 The cytogenetic approach

The presence of viable cells in effusions offers an opportunity of studying the karyotypes of malignant cells and reactive mesothelial cells with the aim of finding out whether differences in chromosomal constitution between these cells can be of diagnostic value in clinical practice. A study carried out by Spriggs et al (1962) demonstrated the presence of cells with abnormal karyotypes in effusions containing malignant cells. The karyotypic abnormalities observed ranged from hypodiploidy to gross hyperdiploidy. In addition marker chromosomes were frequently present. It was also observed that, while similar abnormal karyotypic patterns were present in the cells of the same specimen, there was no significant correlation between a particular type of chromosomal aberration and the histological origin of the tumour. On the other hand, it was also demonstrated that cells analysed from benign effusions had a normal diploid karyotype. These observations have also been reported by Ishihara et al (1961, 1963) and more recently by Korsgaard (1979).

In an attempt to improve the accuracy of the diagnosis of malignant cells in serous effusions, a number of authors carried out combined cytological and cytogenetic studies on specimens of serous effusions. Goodlin (1963) found the addition of cytogenetic analysis a simple complementary technique to the usual cytological procedures.

Benedict and Porter (1972) reported that, in most cases, cytogenetic analysis of effusions is unnecessary as positive cytology is usually adequate to make a diagnosis of malignancy. However, in patients with an effusion with an equivocal cytological pattern, cytogenetic evaluation can be helpful in making the diagnosis of malignancy. Dewald *et al* (1976) found that the combination of standard cytologic and chromosome analysis correctly identified 83% of the neoplasms, a result significantly better than that with either technique alone (cytology: 65%, cytogenetic analysis:71%). Korsgaard (1979) found the chromosome analysis of effusions a simple and rapid diagnostic method and an excellent complement to routine cytology, particularly in cases where cytological examination does not yield a definite diagnosis. A similar combined cytologic/cytogenetic study on serous effusions (Watts *et al*, in press) was carried out at St Mary's Hospital, London W2 and the results were largely in agreement with the earlier findings of the above authors.

The cytogenetic criteria used to identify malignant cells in serous effusions differ according to different authors, but basically demonstration of clonal evidence of hyperdiploidy, pseudodiploidy or marker chromosomes in not less than three metaphases was generally accepted as an indication of the malignant nature of the fluid in question (Clarke, personal communication). The presence of hypodiploid cells as evidence of malignancy was rarely accepted, as loss of chromosomes is known to occur during preparation and can cause erroneous interpretation. The necessity of demonstrating

the clonal nature of the tumour cells is important. As pointed out by Spriggs and Boddington (1968), the diagnostic validity of karyotypic analysis in the identification of malignant cells in effusions depends upon the demonstration of new clone formation. Neither inconsistent chromosomal aberrations, nor of course a consistent congenital cytogenetic abnormality, could be regarded as significant. In 1968, however, the former feature of inconsistent chromosomal aberrations was not recorded in effusions in the literature except for random chromosome loss during preparation. Recently, in a cytogenetic study carried out at St Mary's Hospital, London W2, both random chromosomal aberrations and marker chromosomes were demonstrated in ascitic fluids from patients with alcoholic cirrhosis (To et al, 1981). Similar findings of abnormal karyotypes in a benign effusion from a patient with acute rheumatoid lung disease was reported by Dewald et al (1976). Korsgaard (1979) also observed abnormal karyotypes in effusions from patients with benign pulmonary disease. These findings clearly indicate that cytogenetic studies must be interpreted with caution.

The cytogenetic analysis of serous effusions is an extremely laborious method and freshly-aspirated specimens are absolutely essential if dividing cells are to be obtained for analysis. As routine specimens of serous effusions are rarely fresh when delivered to the laboratory, a short-term culture method was developed at the cytology/ cytogenetics unit at St Mary's Hospital, London W2 (Watts et al, in press). This approach permitted the retrospective

selection of specimens for chromosome analysis and these specimens were selected on the basis of equivocal cytological findings in patients in whom there was strong clinical suspicion of malignant disease.

The role of cytogenetics in the cytodiagnosis of serous effusions was described in a recent publication by Spriggs (1981), who found that the demonstration of abnormal karyotypes could indeed establish an objective evidence of malignancy provided the preparations are of good quality. However, he also pointed out that, whereas the time-consuming nature of the exercise and the expertise required are the technical drawbacks, the majority of cases in which spontaneous metaphases could be demonstrated, are exactly those which could be recognised on morphology alone. Therefore it is these factors that together make chromosomal analysis of serous effusions an impractical investigation to include as part of a routine diagnostic service in a cytology laboratory.

2.5 The histological approach

A number of reports have been published comparing the diagnostic sensitivity and accuracy between cytological examination and pleural biopsy (Salyer *et al*, 1975; Frist *et al*, 1979; Winkelmann and Pfitzer, 1981). These studies showed that, in malignant cases, the limitation of blind pleural biopsy lies in the fact that only a very small area of the serosal surface is examined and obviously false-

negative diagnosis can frequently result (Von Hoff and LiVolsi, 1975). In contrast, however, in effusions secondary to tuberculosis, for example, pleural biopsy is a more accurate method than the cytological examination. The efficacy and fallacy of both techniques were described by Koss (1979). By referring to the study of Frist et al (1979) in which he collaborated, he found that the cytological approach is a much more accurate method than a pleural biopsy for the diagnosis of malignancy in serous effusions. However, in infectious disease especially in granulomatous disorders such as tuberculosis, the histological approach is superior to the cytological method in establishing the aetiological basis of the disease.

2.6 The biochemical approach

The classic differentiation of serous effusions into transudates and exudates is based on measurements of the total protein content and the specific gravity of the fluids (Koss, 1979). The transudates are mostly fluids with the total protein content below 3g/100ml and a specific gravity below 1.015. The exudates are characterised by a comparatively higher protein content and hence a higher specific gravity. While it is true that the majority of transudates are due to non-neoplastic cause, it is by no means an absolute finding (Storey et al, 1976)

A number of biochemical substances have also been measured in effusions for their possible diagnostic signif-

icance. Examples of some of these substances that have been individually measured in effusions are given in Table 2.2. While a substance may have a specific value if a particular disease is suspected (e.g. amylase in acute pancreatitis), the only substance appearing to be of some value, so far as the diagnosis of malignancy is concerned, is the Carcinoembryonic Antigen (CEA).

A number of studies have been carried out to determine the diagnostic value of CEA measurement in effusions. One of these studies was reported by Vladutiu et al (1979) who investigated the CEA content in effusions from 105 patients by a radioimmunoassay technique. The results of this investigation showed that a CEA concentration above 11ng/ml was found only in fluids from patients with carcinoma. Leowenstein et al (1978) and Basta et al (1975) also found a raised CEA level in effusions from cancer patients only. Another study reported by Rittgers et al (1978) on 191 serous effusions showed that, using a cut-off level of 12 ng/ml, 24 out of 70 effusions from patients with cancer had a CEA assay value higher than 12ng/ml, while only 1 out of 103 effusions from patients without malignant disease was over 12ng/ml (18ng/ml). Asseo and Tracopoulos (1982) reported a study of concurrent CEA measurement on effusions and on sera from 53 patients with malignant disease and 54 without malignancy. Using an enzyme-linked immunosorbent assay (Elisa) technique with a cut-off level of 5.0ng/ml, higher values were found only in effusions and in serum from patients with malignant disease. Furthermore,

Table 2.2

Examples of biochemical substances that have been measured in effusions

Storey et al (1976)	Booth et al (1977)	Vladutiu et al (1979)	Asseo and Tracopoulos (1981)
Lactic dehydrogenase	Carcino-embryonic Antigen	Carcino-embryonic Antigen	Orosomucoid
Amylase	Pregnancy-associated $alpha_2$ glycoprotein	$Beta_2$ micro-globulin	$Alpha_2$ macro-globulin
Cholesterol	Normal serum proteins	$Beta_2$ macro-globulin	Immuno-globulins
Triglyceride		Ceruloplasmin	
Complement		Orosomucoid	
Glucose		Lysozyme	
		Hexosaminidase	

it was also noticed that, when the CEA concentration was raised, it was generally higher in the fluid than in the serum, suggesting a difference in CEA metabolism between the two (e.g. local secretion by the tumour cells in the fluid and/or more rapid clearance of CEA in the serum). In a similar study of 141 patients reported by Nystrom et al (1977), only 1 'false-positive' result was obtained with effusion CEA measurement, which was also found to be a more sensitive method than serum CEA assay for the diagnosis of malignancy in the patients. In another study by Booth et al (1977) on 51 effusions and 45 corresponding sera CEA measurement, 3 'false-positive' results were obtained in 29 effusions from patients with benign disease. It was also found that the serum CEA assay failed to discriminate cancer and non-cancerous disease in the patients. Lastly, when the CEA level was raised in association with malignant disease, it was always higher in the fluid than in the serum. In another study by Stanford et al (1978), a CEA concentration as high as 245 ng/ml was found in a pleural effusion from a patient with inflammatory lung disease, and both serum and effusion CEA assay failed to discriminate malignant from benign conditions. However, it was noted that, in agreement with the observation made by Asseo and Tracopoulos (1982) and Booth et al (1977), when the CEA level was raised the higher value was always in the effusion.

In view of the discrepancy in the results of these different investigations, a CEA radioimmunoassay study on

supernatants of serous effusions was included in this thesis, details of which will be described in chapter 7.

The results of CEA measurement in effusions have to be interpreted with caution, especially when they are related to the cytological examination of the cell content of the fluids. The object of the cytological examination of serous effusions is to determine the presence of malignant cells in the fluids, while the result of a CEA assay can only point to whether an effusion occurs in a patient with or without malignant disease regardless of the cell content of the fluid. Presence of malignant disease in the patients may not indicate the presence of malignant cells in the fluids. This is because effusions caused by tumours can be of two types (Spriggs and Boddington, 1968). In the first type, it is caused directly by the infiltration of metastatic malignant cells on the serosal surface leading to the formation of the effusion with proliferation of the malignant cells in the fluid. In the second type, the effusion is caused only indirectly by the presence of the tumour elsewhere in the body, for example, by a lymphatic obstruction. In either type of effusion, a raised CEA level in the fluid may be expected with a CEA secreting tumour. Therefore, a positive result with a CEA assay does not necessarily indicate the presence of malignant cells in the effusion and consequently the test cannot be used to assist the cytological screening for malignant cells in the fluids.

Chapter 3

Tumour markers in medical oncology

3.1 Introduction

3.2 Method of detection - Radioimmunoassay

3.3 Method of detection - Immunocytochemistry

3.4 Applications of radioimmunoassay

3.5 Applications of immunocytochmeistry

3.6 Limitations of current tumour markers

3.7 The Carcinoembryonic Antigen (CEA)

3.8 The Epithelial Membrane Antigen (EMA)

3.9 Monoclonal antibodies

3.1 Introduction

The term 'tumour markers' include a group of cellular substances such as hormones, enzymes, immunoglobulins and oncofetal antigens, that have been used to characterise tumours for diagnostic and/or prognostic purposes in laboratory medicine and in clinical practice (Neville and Cooper, 1976; Dearnaley and Coombes, 1981; Buckman, 1982). The first of such markers to be identified was probably the Bence-Jones protein in myeloma patients, discovered in the 19th century. Since then, and with the development of various immunological techniques, this field of oncological science has greatly expanded. In general, tumour markers can be divided into those that are derived from the tumour cells or their stroma and those that are produced by other tissues in response to the tumours or their products. Those markers that are tumour-derived may be further classified into 'appropriate' or 'inappropriate' according to the histogenesis of the tumours although not all of them exhibit biological activity. Tumour markers may be detected in body fluids by the sensitive radioimmunoassay techniques and on surgical specimens at the cellular level by the immunocytochemical methods. Table 3.1 summarises some common tumour markers and their potential uses in tumour pathology.

3.2 Method of detection - Radioimmunoassay

Table 3.1

Examples of some common tumour markers and their potential uses in tumour pathology

Markers	Tumours
Beta-subunit of Human Chorionic Gonadotrophin (β-HCG)	Choriocarcinoma
Alpha-fetoprotein (AFP)	Hepatoma/Yolk sac tumours
Carcinoembryonic Antigen (CEA)	Colorectal Carcinomas
Calcitonin	Medullary Thyroid Carcinoma/Oat Cell Carcinoma
Paraproteins	Myeloma
Prostatic Acid Phosphatase	Prostatic Tumours
Epithelial Membrane Antigen (EMA)	Breast Carcinomas
B-Cell Markers	Lymphomas

The technique for radioimmunoassay utilizes the specific reaction of antibody and antigen to provide quantitative information of the antigen present in a test material (Roitt, 1980; Mishell and Shiigi, 1980; Johnstone and Thorpe, 1982). A number of procedural variations of the assay system has been developed. Recently, alternative labels such as enzymes (e.g. peroxidase or alkaline phosphatase) or fluorochromes in place of radioisotopes have been used. Basically, however, the underlying principle of the different systems is essentially the same and the assay procedure can be broadly divided into two types, the competitive and the non-competitive.

In the competitive assay, the amount of antigen (labelled) required to saturate a limited amount of antibody is determined. The binding of the labelled antigen to this limited amount of antibody can be partially inhibited by the competitive binding of the unlabelled antigen present in a test material. The reduction in the amount of the labelled antigen required to saturate the same amount of antibody is used to estimate the quantity of antigen present in the test material. The assay can be carried out in liquid phase or in solid phase. In the liquid phase method, the bound labelled antigen antibody complexes can be separated from the free antigen by adsorption of the free antigen on dextran coated charcoal; alternatively, the bound antigen may be separated by binding to ion-exchange resins, by precipitation with ammonium sulphate (the Farr technique) or

with anti-immunoglobulin. In the solid phase method, the antibody is initially coated onto a solid surface such as plastic. Antigen that is not bound to the antibody is removed by thorough washing.

In the non-competitive method, unlabelled antibody is coated onto a solid surface. The test material is added; after washing, an excess of labelled antibody is added. The degree of radioactivity of the bound, labelled antibody, is used as an index to estimate the quantity of antigen present in the test material. Depending upon the particular assay system, the second antibody may have the same, or different, specificity for the antigen as the first antibody. The non-competitive method is advantageous over the competitive method when either the purification of the antigen is difficult or radiolabelling the antigen would alter the specificity of the antigen.

The precision of an immunoassay is based upon the sensitivity and specificity of the system. The sensitivity of the system depends upon the careful calibration and standardisation of the optimal concentration of the reagents for the antibody antigen reaction. The specificity of the system depends upon the preparation and selection of the appropriate specific antibody which shows a high affinity for most preparations of the specific antigens but minimum cross-reactivity with other antigens. In this respect the use of monoclonal antibody may represent a considerable advance to that of conventional antiserum.

3.3 Method of detection - Immunocytochemistry

The introduction of antibody conjugated with a chromogenic tag was first reported by Coons et al (1941). This was the basis for the development of immunofluorescent microscopy, later followed by the immunoperoxidase technique pioneered by Nakane, Avremeas and Sternberger (Taylor, 1978b). The transition from the use of fluorescent tags to that of stable enzyme conjugates represented several advances in the field. The immunofluorescent method had some drawbacks. The impermanence of the stain and poor morphology of the preparation, together with the requirement for fresh tissues and specialised microscopy, limited its use in routine diagnostic histopathology. In contrast, the ease with which many cellular substances can be demonstrated on formalin-fixed, paraffin-embedded sections, the permanence of the peroxidase/diaminobenzidine (DAB) reaction, the simultaneous excellent morphological details with conventional histological counterstain on the same preparation, made the immunoperoxidase method the technique of choice in pathological investigations.

A number of variations of the immunoperoxidase technique have been developed on the basic method, in which the enzyme tag is directly conjugated onto the specific primary antibody (the 'direct' method). In order to increase the sensitivity as well as versatility of the method, the multi-step techniques were introduced. The two most commonly used multi-step methods

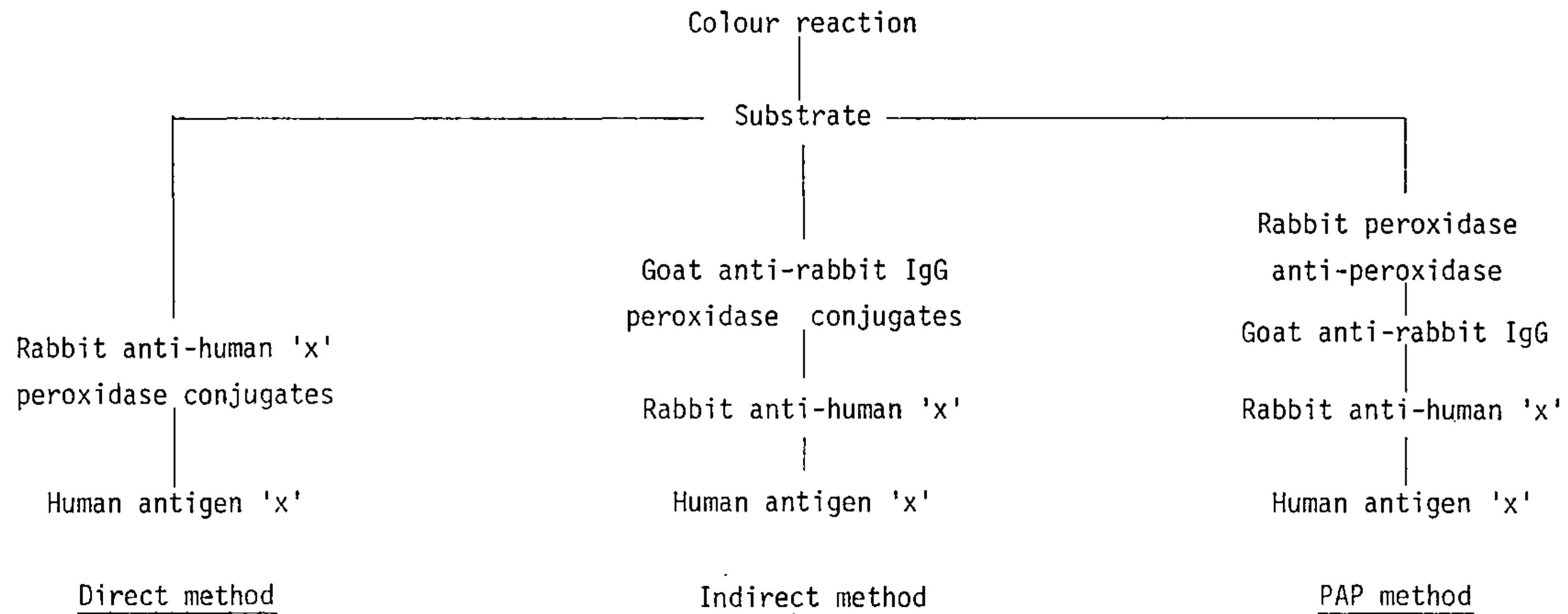
are the 'indirect' and the 'peroxidase anti-peroxidase' (PAP) techniques. In the indirect technique, the first antibody is not labelled and the second antibody, which is raised against the immunoglobulins of the species used to raise the first, is conjugated to the enzyme. In the PAP method, both the first and the second antibodies are unlabelled and the third step uses an immune complex of peroxidase anti-peroxidase raised in the same species in which the first antibody is raised. The second antibody, which is directed against the immunoglobulins of the species used to raise the first and third antibodies, is applied in excess to bridge the first and the third antibodies. The procedures for the direct, indirect and the PAP techniques are outlined in Figure 3.1.

The multi-step techniques have several advantages over the direct method. First, there is only need for one enzyme conjugate per species. Second, the multi-step methods allow the technique to be subject to various specificity controls (see below). Third, since several of the conjugates can bind onto a single first antibody, the colour of the enzyme/substrate reaction becomes much stronger. This explains the increased sensitivity of the multi-step methods.

The PAP method (Sternberger et al, 1970) was originally designed to enhance sensitivity, and as a non-conjugate procedure, to eliminate the process of conjugating the enzyme directly onto the antibody. The direct conjugation process can occasionally result in the denaturation of the antibody, or inactivation of

Figure 3.1

Principles of the direct, indirect and peroxidase anti-peroxidase (PAP) immunocytochemical techniques



of the enzyme, or residual unconjugated antibody and enzyme. However, with advances in the conjugation techniques, these effects have been circumvented (Avrameas and Ternynek, 1971). Indeed, commerically available enzyme (peroxidase or alkaline phosphatase) conjugates of excellent quality are available. Furthermore, the indirect technique has been reported to show a comparable degree of applicability when compared with the widely used PAP method (Heyderman, 1979; Sinclair _et al_, 1981).

The principle of the immunocytochemical technique is comparatively straightforward; the essence lies in proving that what one has stained is what one claims to have stained. When affinity-purified antiserum is not available, 'the complete abolition of the positive staining after absorption of the antibody with the antigen under test is the most useful specificity control' (Heyderman, 1979). To confirm that this loss of activity after the absorption is specific, two further controls can be used. First, the primary antiserum can be absorbed with an unrelated antigen (inappropriate antigen control), in which no loss of activity should take place in the primary antiserum. Second, the antigen under test can be absorbed against unrelated, non-cross-reacting antibodies (inappropriate antibody absorption control), in which the activity of the unrelated antibodies should not be lost. This system of controls is outlined as follows:

(a) Normal application

The 'primary' antiserum + the antigen under test

→ positive staining.

(b) Absorption control

Objective - To prove the specificity of the positive staining obtained in (a).

(bi) The 'primary' antiserum + the antigen under test

→ 'antibody-free' serum + 'antibody/antigen' complex.

(bii) The 'antibody-free' serum + the antigen under test

→ no staining.

(c) Inappropriate antigen control

Objective - To prove the specificity of the reaction obtained in (b).

The 'primary' antiserum + unrelated, non-cross-reacting antigen

→ no loss of activity in the 'primary' antiserum.

(d) Inappropriate antibody absorption control

Objective - To prove the specificity of the reaction obtained in (b).

Unrelated, non-cross-reacting antibodies + the antigen under test

→ no loss of activity in the unrelated, non-cross-reacting antiserum.

Other controls, for example, use of substrate alone, omission of the specific antiserum and substitution with buffer or non-immune serum, comparison with other antisera or with radioimmunoassay, immunodiffusion data, blocking with antibodies from another species, are unsatisfactory. The reasons are given as follows:

Use of substrate alone

This is only necessary when one is testing an antiserum against a new antigen, in which case preliminary experiments have to be carried out to determine that the antigen under test is insensitive to the endogenous enzyme blocking reagent.

Omission of the primary antiserum and replacement by buffer or non-immune serum

The loss of positive staining when the primary antiserum is omitted only indicates that the previous positive staining is due to the primary antiserum; it does not demonstrate the specificity claimed.

Comparison with other antisera

Although differences in the distribution of positive staining with two antisera indicates the difference in the specificity of the two antisera, similarity of their staining distribution cannot be taken as an indication of identical specificity. For example, a EMA positive/CEA positive cell will stain identically positively with antisera to either markers, although the two markers themselves are clearly different.

Blocking with antiserum from another species

This is a system of negative control using antibodies raised in different species against the same immunogen. The rationale is such that, since both antisera are raised against the same immunogen, antibodies from one antiserum

can bind onto all the available sites of the antigen under test; if then the other antiserum is applied, there will be no immunological reaction. This system may not even be true even if one is testing one monoclonal antibody against another for comparison of their specificity, as a recent paper has discussed (Lane and Koprowski, 1982). With conventional polyclonal antiserum, different batches of immune sera (against the same immunogen) will most likely contain different mixtures of antibodies with different specificity and sub-specificity (e.g. antisera to CEA). The antibodies in these different antisera, even after affinity purification, may not block each other and for this reason a negative result will be even more difficult to interpret than a positive one.

3.4 Applications of radioimmunoassay

The presence of a marker in body fluids so that it can be measured by sensitive radioimmunoassay, is fundamental to most tumour marker trials for prognostic in clinical practice (Hobbs ,1978; Dearnaley and Coombes, 1981). However, because of the lack of specificity (true-negative rate) or sensitivity (true-positive rate) in most of the presently available markers, there are currently few examples to support the efficacy of body fluid marker assay as a method to detect the presence of localized malignant lesions in asymptomatic individuals. Nevertheless, in appropriate cases following the initial presentation of the malignant disease in the patients, a pre-operative assay may

indicate the tumour burden and a persisting level after surgical removal of the tumour mass may indicate residual tumour (taking into account the half-life of the marker and the possible release of the marker by necrotic tumour tissues after the institution of therapy). Similarly, consecutive assays may detect recurrence of the tumour and prompt early medical attention. Furthermore, in selective cases, a marker assay may be used to assess therapeutic effectiveness. This is carried out with the knowledge that the marker is being produced by the tumour throughout the period under investigation and the anabolism and the catabolism of the marker is characterised. The marker assay may allow rapid evaluation of a wide range of therapy and may act as a guide to tailor appropriate therapy for individual patients.

A good example of the use of circulating marker is the measurement of the Beta-subunit of Human Chorionic Gonadotrophin (β-HCG) in patients with choriocarcinoma (Kardana and Bagshawe, 1976; Dearnaley and Coombes, 1981). Although the marker reaches a peak in the first eight weeks of normal pregnancy, a persisting elevated level is highly suggestive of abnormal trophoblastic tissue. Moreover, β-HCG can be detected on a tumour mass of only 10^5 cells and its production also reflects the response of this chemosensitive tumour to treatment hence allowing accurate monitoring of this disease.

3.5 Applications of immunocytochemistry

The application of immunocytochemistry to the field of diagnostic histopathology is a new and useful approach. With the introduction of stable enzyme conjugates (e.g. peroxidase, alkaline phosphatase), the immunocytochemical techniques allow permanent demonstration of tumour-derived substances at the cellular level on conventional histological sections, thus providing pathologists with simultaneous information on the presence of such products as well as the histological details on the same preparation. This may be of value in aiding conventional diagnosis and pathological classification, in presenting a functional picture with respect to the true extent of metastatic infiltration, and in suggesting the appropriate marker for plasma assay in prognosis.

One of the classic examples of the use of immunocytochemistry in histopathology is the differentiation of monoclonal B-cell lymphoma from polyclonal reactive lymphoid proliferation, by demonstrating in the former one single class of immunoglobulin. Similarly, B-cell lymphoma may be distinguished from undifferentiated carcinoma by immunocytochemical staining for immunoglobulins (Taylor, 1978 Mason *et al*, 1980). Other examples, such as the demonstration of calcitonin in medullary thyroid and bronchial carcinomas, the demonstration of alpha-fetoprotein in hepatoma and yolk sac tumours, are all recognised as useful adjuvants to conventional diagnosis.

Tumour markers may be useful in the histological classification of neoplasia. As pointed out by Taylor et al (1978), the histopathological diagnosis of neoplasia by morphological criteria could at times be uncertain, partly due to conceptual difficulties related to the histogenetic basis of the tumours and partly due to the lack of other methods with which to confirm the morphological diagnosis. Using a combined morphological and immunocytochemical approach, Kurman et al (1977) and Taylor et al (1978) showed that germ cell tumours of the testis and ovary can be classified according to their production of alpha-fetoprotein (AFP) and HCG (see Table 3.2) and suggested that embryonal carcinoma may be the neoplastic progenitor of the other germ cell tumours. This observation is important for the correct interpretation of the results from radioimmunoassay for both AFP and β-HCG in patients with germ cell tumours. Discordant levels of the two markers after chemotherapeutic application may be explained by the presence of two clones of cells each producing one marker; only one of the clones responds to the therapy applied. Alternatively, a fall in both markers may take place after chemotherapy while there is no clinically measurable tumour shrinkage (e.g. by ultrasound scan). This may indicate a mixed germ cell tumour, with a clone of cells positive for both markers and responding to treatment, and another clone of cells producing no marker and not responding to the therapy applied (Perlin et al, 1976).

Table 3.2

Immunohistologic classification of germ cell tumours of the testes and ovary (Taylor *et al*, 1978)

Tumour	AFP	HCG
Germinoma (seminoma* and dysgerminoma)	-	-
Teratoma	-	-
Embryonal carcinoma	+	+
Endodermal sinus (yolk sac) tumour	+	-
Choriocarcinoma	-	+

*A case of AFP-positive seminoma has been described by Raghavan *et al* (1981).

This immunocytochemical approach to the classification of tumours may subsequently be of value in relating the difference in marker patterns between histologically and clinically similar tumours to their subsequent behaviour. For example, Pizzolo *et al* (1980) found that two out of 22 lymphoid tumours failed to stain with a specific anti-human leycocyte monoclonal antibody (2D1) and both of these tumours had aggressive behaviour. This observation is being further investigated.

One recent aspect of tumour marker investigation in laboratory medicine has focused towards the detection of micrometastasis with an aim to increase the sensitivity of histological examination (Sloane *et al*, 1980a). A study was recently reported by Sloane *et al* (1980b) on the value of immunocytochemical staining for the Epithelial Membrane Antigen (EMA) in detecting micrometastasis of breast carcinoma in conventional histological sections of liver, lymph nodes and bone marrow. This investigation showed that, in the study of liver and lymph node biopsies, a greater extent of metastatic infiltration was frequently revealed by the EMA staining, often due to single malignant cells which were unrecognised with conventional stains. In the study of marrow aspirates, the actual number of positive cases was increased by the addition of EMA staining. This latter finding was further investigated by Dearnaley *et al* (1981) on cytological smears of bone marrow aspirates from patients with primary carcinoma of

the breast. The results of this study showed that a combination of cytological examination and EMA staining improved the sensitivity of detecting bone micrometastasis. Moreover, EMA staining also detected a small number of malignant cells in marrow of patients in whom there was no other evidence of metastatic involvement.

3.6 Limitations of current tumour markers

Progress in the field of tumour marker research has been obstructed by 3 major factors, all concerning the markers (Von Kleist, 1980). First, there is the lack of absolute tumour specificity. At present, research in the identification of tumour specific markers has resulted in disappointingly few such markers being found. The presently available markers must be regarded as substances associated with malignancy in greater amounts than with normal tissues. This quantitative difference in the expression of a marker is the basis of its use in differential diagnosis. Second, there is the problem of immunological cross-reaction. Conventional antisera contain a mixture of polyclonal antibodies of different specificity and sub-specificity, of different affinity, of different classes and sub-classes and with cross-reactivities. This makes it difficult to compare results obtained with different reagents. Also, the presence of cross-reaction will interfere with the precision of the test. Third, there is the poor correlation between the quantity of a

a marker produced and the stage of the neoplastic disease. With the currently available therapy, detection of a particular marker at its first appearance in a patient with malignant disease can rarely affect the outcome of the disease, as by the time the marker is detected metastasis has often occurred. Therefore, there is an obvious need to evaluate the limitation of the relationship between the quantity of product and the tumour size. Furthermore, the sensitivity of a marker test depends upon its production in a defined range of tumours. For example, not all carcinomas produce CEA and its production seems to be related to the stage of differentiation of the tumours as well as the tumour type. Hence it is also important to characterise the kinetics of production of a particular marker and to define its distribution.

3.7 The carcinoembryonic antigen (CEA)

Among the markers investigated, the Carcinoembryonic Antigen (CEA) has been widely studied. The antigen, first reported by Gold and Freedman (1965a, 1965b), was identified by an antiserum raised against pooled saline extracts of colonic adenocarcinoma. The antiserum, after absorption with normal tissue extracts, gave a single line by the Ouchterlony double diffusion method with the original tumour extracts. Reaction with extracts of normal mucosa was negative. It was also found that only primary tumours of the gastro-intestinal tract or their metastases expressed

CEA. Malignant lesions of other sites or benign tissues gave no reaction with the antiserum. Further tissue distribution studies by the double diffusion method indicated that an immunologically identical substance reactive with the antiserum was also present in foetal liver, pancreas and the alimentary canal in the first 2 to 6 weeks of gestation. However, the specificity of CEA for colonic carcinoma was only as good as the sensitivity of the method of detection. With the development of very sensitive radioimmunoassay techniques, CEA was demonstrated to be present in small quantities in normal epithelial tissues and in the circulation of normal healthy individuals. Raised serum CEA levels were also found in cigarette smokers, in patients with a variety of inflammatory conditions, such as ulcerative colitis and acute pancreatitis as well as in epithelial-derived malignant lesions other than those of the gastro-intestinal tract (Gold et al, 1978; Neville, 1981).

The present accumulated evidence has indicated that CEA is a glycoprotein with a molecular weight of approximately 200,000 daltons. Chemical studies have indicated that there are at least five chemically distinct antigenic determinants; three are composed of protein and two are carbohydrate. One of the carbohydrate determinants cross-reacts with the normal cross-reacting antigen (NCA) of 60,000 daltons (Ormerod, 1978). Other research has also indicated the existence of a second antigen (NCA2) which

cross-reacts with CEA (Burtin *et al*, 1973,1977). Any polyclonal antiserum to CEA will probably react with several different antigenic determinants and the relative proportions of antibodies to each of these will vary from one antiserum to the next depending on the immunogenicity of the respective determinants which in turn are dependent upon the particular preparation of CEA used to raise the antiserum. This molecular heterogeneity of CEA was clearly demonstrated by an experiment using four different 'standard CEA preparations' and two different anti-CEA antisera (Goldenberg *et al*, 1978). The results indicated the presence of significant antigenic differences between the four CEA preparations. This accounts for the difficulty in standardising CEA radioimmunoassays used in different laboratories.

Plasma CEA level is raised in patients with a wide variety of histogenetically different tumours as well as with numerous inflammatory and non-neoplastic disorders. A summary of the incidence of raised plasma CEA level in various conditions is shown in Table 3.3 (Neville and Cooper, 1976). In cancer patients, the plasma levels are related to the site and in some cases to the tumour burden; in others, discordance between plasma CEA levels and the tumour mass can occur. Because of the high incidence of false positive and negative results, it is clear that plasma CEA radioimmunoassay is of no value in detecting primary tumours. Nevertheless, when the presence of CEA positive tumour is confirmed, a serial quantitative serum

Table 3.3

Incidence of raised plasma CEA levels in various neoplastic and non-neoplastic disorders (Neville and Cooper, 1976)

Disorders	Incidence (%) of raised plasma CEA levels
Neoplastic	
Carcinoma of	
Colon and rectum	73
Pancreas	92
Liver	67
Bronchus	72
Breast	52
Uterus	53
Ovary	36
Non-neoplastic	
Ulcerative colitis and Crohn's disease	21
Cirrhosis and alcoholic liver disease	42
Chronic bronchitis and emphysema	25
Fibroadenosis	7

radioimmunoassay may be a valuable and non-invasive technique in surveillance, either to detect post-operative residual or recurrent tumours, or as an index to monitor changes in tumour mass in patients with advanced malignant disease not measurable by other means (Neville, 1981).

Goldenberg _et al_ (1978) reported a detailed immunocytochemical distribution of CEA in 950 formalin-fixed, paraffin-embedded surgical specimens of various malignant and benign tumours as well as inflammatory and normal tissues. This investigation indicated that adeno- or squamous carcinomas of various sites frequently stained positively with CEA. In ovarian malignancies, mucinous cystadenocarcinomata always stained positively with CEA, in contrast to serous cystoadenocarcinomata which were mostly negative. In non-malignant tissues, only benign colonic tumours, some inflammatory conditions of the colon, and normal mucosae adjacent to colonic neoplasm were CEA positive. It was suggested that in the latter cases the CEA demonstrated in the normal mucosae probably represents absorption from the adjacent neoplasm and not _de novo_ CEA synthesis. Goldenberg _et al_ (1978) were unsuccessful in the attempt to stain carcinoma of the breast for CEA, in contrast to the results of Heyderman and Neville (1977). This may have been due to the different reagents used. It was also pointed out that the discrepancy in the degree of 'tumour specificity' for CEA between data from radioimmunoassay and from immunocytochemical staining is probably due to the different sensitivity of the two techniques.

3.8 The epithelial membrane antigen (EMA)

In 1979, a new human epithelial marker designated the Epithelial Membrane Antigen (EMA) was reported by Heyderman *et al* and later in 1981 by Sloane and Ormerod. This marker was identified using an antiserum raised against the human milk fat globule membrane. These milk fat globules were secreted by lactating mammary cells by budding from the cytoplasm, through the luminal surface membrane, to the lumen of the breast acini. In the process of budding the globules were bound by the luminal plasma membrane. By defatting human cream, the membrane component of the fat globules was extracted and was used to raise the antiserum. The immunised whole serum was purified (Heyderman *et al*, 1979; Sloane and Ormerod, 1981). The resulting purified antiserum was used to define the histological distribution of the epithelial membrane antigen by indirect immunocytochemical staining on formalin-fixed, paraffin-embedded sections of a wide variety of human tissues.

The studies of Heyderman *et al* (1979) and of Sloane and Ormerod (1981) demonstrated that EMA had a wide distribution on normal and neoplastic human epithelium but was not expressed by haemopoietic, lymphoid, osseous and connective tissues. In normal or inflammatory conditions, the antigen was mostly present on the cell membrane and was only weakly and inconsistently expressed on the

mesothelium. Increased expression of the antigen was observed in most neoplasms of epithelial and mesothelial origins, frequently being localised in the cytoplasm as well as on the cell membrane. EMA staining was also found to be related to the histogenesis and state of differentiation of the tumours. For example, adenocarcinoma always stained strongly positive for EMA on their luminal surface, in contrast to hepatocellular carcinoma, embryonal carcinoma and carcinoid tumours, in which the luminal surface was consistently negative. Also, EMA staining appeared to be more consistent in well-differentiated carcinoma than in poorly-differentiated ones.

The histological distribution of EMA, together with the finding that the antigen could be demonstrated on conventional formalin-fixed, paraffin-embedded sections, suggested that EMA may have a valuable role to play in diagnostic pathology as a marker of epithelial differentiation. Positive EMA staining has been found useful in the discrimination between tumours such as anaplastic carcinoma and malignant lymphoma or between spindle cell carcinoma and sarcoma, which may be difficult to distinguish on morphological grounds. This immunocytochemical approach is of value in clinical practice as the correct diagnosis is a prerequisite for effective patient management. The relationship between the expression of EMA and the histogenesis of carcinoma has also been explored. An observation by Sloane and Ormerod (1981) has shown that a

proportion of oat cell carcinomas is positive for EMA. This finding is being studied to determine if histogenetic sub-types of oat cell carcinomas can be identified by immunocytochemical methods.

Another antiserum raised against the human milk fat globule membrane was reported by Ceriani et al (1977). In an immunofluorescent staining study on viable cells, the antiserum was shown to stain only normal mammary epithelial cells and cell lines derived from breast carcinomas; other types of epithelial cells (from kidney, lung and colon), fibroblasts and haemopoietic cells did not stain. Thus, this mammary cell-specific marker as defined by the antiserum appeared to differ from EMA in its specificity.

Three monoclonal antibodies to human milk fat globule membrane were reported by Taylor-Papadimitriou et al (1981). An immunohistochemical study of two of these three monoclonal antibodies was carried out by Arklie et al (1981) on formalin-fixed, paraffin-embedded sections of a variety of normal and malignant human tissues. This study showed that both of the monoclonal antibodies stained weakly with epithelial cells in resting breast, while in lactating breast both stained the luminal surface of the epithelial cells strongly. Also, well-differentiated carcinomas of the breast stained stronger than poorly-differentiated ones. The two monoclonal antibodies also

stained adenocarcinomas of the lung, uterus and ovary; carcinomas of the gastro-intestinal tract, cervix, naso-pharynx and liver were negative. In normal tissues, the monoclonal antibodies were shown to stain the luminal surface of epithelial cells in exocrine glands, in collecting tubules of kidney and in bronchioles of the lung. These two monoclonal antibodies appeared to have a less wide histological distribution than EMA.

3.9 Monoclonal antibodies

The technique for the production of hybridoma that makes monoclonal antibody to an antigen of choice was pioneered by Kohler and Milstein (1975, 1976). In this technique, mutants of malignant mouse myeloma cells lacking the enzyme hypoxanthine phosphoribosyl transferase are fused with individual mouse plasma cells obtained from the spleen of a donor mouse immunized with the antigen of interest. The fusion is carried out in the presence of polyethylene glycol (PEG) and the hybrids are selected on the HAT medium (medium containing hypoxanthine, aminopterin and thymidine). Unfused benign plasma cells from the immunised mouse will not continue to grow in culture and unfused myeloma cells mutants cannot grow in the HAT medium. This is because the myeloma mutants lack the enzyme and hence cannot utilise the exogenous hypoxanthine to synthesise purine and the endogenous nucleic acid biosynthetic pathway is blocked

by aminopterin. Consequently only the hybrids, which are the wild-type, can survive and grow. Since each individual plasma cell produces only one type of antibody, and since malignant mouse myeloma cells can be propagated in culture for an unlimited period, each of such hybrids, when successfully generated, is immortal and can provide an unlimited supply of homogeneous (monoclonal) antibody.

The success of this technique depends to a large extent on the immunogenicity of the antigenic determinants of interest and on the screening system employed to identify antibody of the specificity required (Diamond et al, 1981). Whilst it is true that impure immunogen may be used in the technique, the production of antibody-forming plasma cells depends upon the immunogenicity of the immunogen used. A weak immunogen provokes fewer plasma cells hence correspondingly fewer hybrids can be formed. Moreover, in the case of whole cell immunogen, not only is the host's immunological response directed against the antigenic determinants of interest, but also against all other possible antigenic sites present on the cells thus reducing the amount of the possible plasma cells producing the antibodies wanted.

The screening system used to identify wanted antibody-producing hybrids is also related to the initial immunogen used. When pure immunogen is used, radioimmunoassay is

often the technique of choice. When impure immunogen is used, the screening system would first be that which identifies those hybrids that are producing antibody specific for the impure immunogen (e.g. by radiobinding assay), followed by further selective screening with known positive and negative controls. When the aim has been to raise monoclonal antibodies for the purpose of histopathological diagnosis, immunocytochemical staining may be appropriate to use as a screening tool. However, a problem associated with the immunocytochemical approach is the fixation of the cells. The fixation process may destroy the antigenic sites against which the monoclonal antibodies are directed. In an attempt to eliminate this problem, fixed immunogen has been used. This step might ensure that the antigenic sites for the subsequently raised monoclonal antibodies will survive the fixation process (Edwards, 1981). As a single fusion can produce hundreds of hybrids all growing at different rates and producing immunoglobulins of different types, it is most important to design a rapid and sensitive screening system.

The impact of the development of the hybridoma technique on clinical pathology is currently being felt and its potential is being realised (Sikora, 1982). For example, in conventional polyclonal antisera the majority of antibodies will be directed against antigenic sites with the strongest immunogenicity, and the sites of weak

immunogenicity may not be detected. Since each hybridoma produces one type of antibody specific for one particular epitope, it is possible to obtain antibody to the antigenic determinant of weak immunogeneity. The detection of such a site by monoclonal antibody, increases the sensitivity as well as selectivity of the immunological reaction. Moreover, a characterised hybridoma will produce an unlimited supply of homogeneous antibody of defined specificity, affinity and biological activity (e.g. complement-fixing). The availability of immunological reagents of such quality allows world-wide standardisation, and hence reproducibility, of various assays and tests.

Attempts are being made using the hybridoma technique to produce antibody to discriminate between malignant and normal cells. For example, three hybridomas that produced monoclonal antibodies highly specific for a small proportion of human melanomas were reported by Yeh et al (1979). Similarly, monoclonal antibodies from two hybridomas shown to have specific anti-colorectal carcinoma activity were reported by Herlyn et al (1979). Furthermore, since impure immunogen can be used to raise monoclonal antibody to a component of interest, the monoclonal antibody thus obtained can then be used in turn to purify this component from the impurities (e.g. by immunoabsorption). An example of this novel approach is the purification of interferon in significant quantity, which is being explored on an industrial scale (Milstein, 1980).

The use of monoclonal antibodies has its drawbacks (Edwards, 1981). For example, they do not usually form precipitating immune-complexes (except with polymeric antigen) and hence cannot be used in precipitation assays (e.g. immuno-diffusion). This however may be overcome by mixing similar monoclonal antibodies. Also, some immunoglobulins do not fix complement and when this property is required, another monoclonal antibody of different class or sub-class would have to be generated. Lastly, the use of monoclonal antibodies can be a serious disadvantage when a particular hybridoma is directed against a common sequence (or structural similarities) between biologically unrelated molecules, in which complete cross-reactions will take place.

Finally, the development of *in vivo* immunolocalisation technique (Goldenberg, 1980) has added a new dimension to tumour marker technology. Although the technique is still at an early stage of development, its potential seems enormous. One aspect in which cytology may have an important role to play, is the combination of *in vivo* immunolocalisation of tumour deposits with cytological fine-needle aspiration. It is hoped that basic experiments, such as the work described in this thesis, may provide grounds for further research in this field.

Chapter 4

Optimisation of immunocytochemical staining for EMA and CEA on cytological smears of serous effusions

4.1 Introduction

4.2 Pilot study: Determination of the pattern of immunocytochemical staining on conventional 95% ethanol fixed smears of serous effusions.

4.3 Procedures for improving the quality of the smear preparation: Determination of the effect of elimination of protein and red blood cells on the smear preparation.

4.4 Factors affecting the immunocytochemical staining for EMA and CEA on smears stained by the indirect immunoalkaline phosphatase method:

i. Determination of the effect of 10 minutes 20% acetic acid treatment on endogenous alkaline phosphatase activity.

ii. Determination of the effect of 10 minutes 20% acetic acid treatment on the antigenic expression of EMA and CEA.

iii. Determination of the effect of storage of the smears on the stability of EMA and CEA.

iv. Determination of the effect of different fixatives on the antigenic expression of EMA and CEA.

4.1 Introduction

A series of preliminary experiments was necessary before the actual research, which formed the basis for this thesis, could be undertaken. Cytological smears of serous effusions were prepared by the conventional method of making smears directly from cell sediment of the fluids after centrifugation, and fixed in 95% ethanol. At the beginning of this research, immunocytochemical staining for EMA and CEA was carried out on cytological smears prepared by this method. Both the indirect immunoperoxidase technique (Heyderman and Neville, 1977) and the indirect immunoalkaline phosphatase method were evaluated for their capacity to demonstrate the markers. These two techniques had been routinely used on histological sections at the Ludwig Institute for Cancer Research and the Institute of Cancer Research at Sutton. However, the techniques had not been tried on cytological smears.

The quality of the immunocytochemical staining by both techniques was unsatisfactory and a series of experiments was necessary to resolve the problems in the staining and to identify the staining method of choice. The series of experiments described in this chapter relates to this objective.

4.2 Pilot study

Determination of the pattern of immunocytochemical staining on conventional 95% ethanol fixed smears of serous effusions

Materials and methods

95% ethanol fixed cytological smears prepared directly from contrifuged deposits, from a minimum of twelve serous effusions, were used. Smears from each specimen were stained respectively for EMA and CEA by the indirect immunoperoxidase technique (see Appendix 2) and by the indirect immunoalkaline phosphatase method (see Appendix 2). The staining on the smears was examined under the light microscope.

The anti-EMA serum used was a rabbit serum supplied by Dr M G Ormerod. The antiserum was raised against defatted human cream suspended in complete Freund's adjuvant and absorbed with human plasma, 3M KCl extracts of liver and kidney, normal cross-reacting antigen, lactoferrin and substances in the molecular weight range of 50,000 - 100,000 daltons in human milk. The anti-EMA specificity of the antiserum was confirmed by staining known EMA-positive human breast carcinoma sections and smears of known EMA-positive malignant cells from serous effusions with the antiserum which had been absorbed with a preparation of EMA. The staining activity was completely abolished.

The anti-CEA serum used was a rabbit serum supplied by Dr M G Ormerod. The antiserum was raised against the perchloric acid extract of a liver metastasis of carcinoma of the colon and absorbed with human plasma and the normal cross-reacting antigen. The anti-CEA specificity of the antiserum was confirmed by staining known CEA-positive human colonic carcinoma sections and smears of known CEA-positive malignant cells from serous effusions with the antiserum which had been absorbed with a preparation of CEA. The staining activity was completely abolished.

The goat anti-rabbit IgG peroxidase and alkaline phosphatase conjugates were supplied by Dr M G Ormerod. Later in the course of study, as large amounts of alkaline phosphatase conjugates were continuously required, it was purchased from the Sigma Chemical Limited. Every batch of conjugates was tested for immunological non-specific binding on known negative controls.

The dilutions of the antisera and the enzyme conjugates used in the experiments described in this chapter had been determined by prior experiments in the laboratory at Sutton on histological sections of breast tumours (for EMA) and colonic tumours (for CEA).

Results

Immunoperoxidase staining: Malignant cells which expressed EMA or CEA stained positively by the indirect

immunoperoxidase method. Occasionally pale EMA staining was observed on the mesothelial cells. Neither leucocytes nor red blood cells stained. The morphology of all cells was frequently indistinct. Non-specific dark brown background staining was present in every smear. In the smears prepared from blood-stained effusions, the presence of red blood cells obscured the morphological examination of the nucleated cells. In many smears, the cellular content appeared to have been lost during the staining process.

Immunoalkaline phosphatase staining: A similar staining pattern was observed on the smears. A red background stain was frequently present. However, in contrast to the immunoperoxidase stained smears, no cell loss was observed when this method was used.

Discussion

It was thought that the non-specific background staining on the smears could be due to the deposits of protein from the effusions. It was not generally appreciated that, because the protein content of serous effusions is frequently far higher than that of blood and can be well over 20g/100ml, not only does the increased viscosity of the fluid inhibit the flattening of cells in the smears, but the background staining of the protein obscures the morphological details and interferes with the microscopic

analysis. Furthermore, most specimens of serous effusions contain red blood cells. The presence of red blood cells on cytological smears may obscure the nucleated cells. For these reasons, further experiments were undertaken to improve the quality of the smear preparation and these are described in the following section. These involved attempts to minimise the amount of protein and red blood cells on the smears.

The deleterious effect on the smear preparation in the immunoperoxidase staining was due to the endogenous enzyme blocking agent used in the indirect immunoperoxidase method (Heyderman and Neville, 1977). In this technique, hydrogen peroxide was used to bleach the acid haematin of the red blood cells followed by sequential treatments with periodic acid and fresh sodium borohydride solution to block the endogenous peroxidase activity. However, while the hydrogen peroxide had the advantage of bleaching acid haematin in blood-stained smears, hydrogen bubbles were often produced by the borohydride which frequently disrupted the smear preparation. Since the cytological smears cannot be mounted on adhesive such as egg albumin, the production of hydrogen bubbles rendered the smears unsuitable for immunocytochemical staining. For this reason the immunoperoxidase method was abandoned and all subsequent experiments were carried out using the indirect immunoalkaline phosphatase technique.

4.3 Procedures for improving the quality of the smear preparation

Determination of the effect of elimination of protein and red blood cells from smear preparation

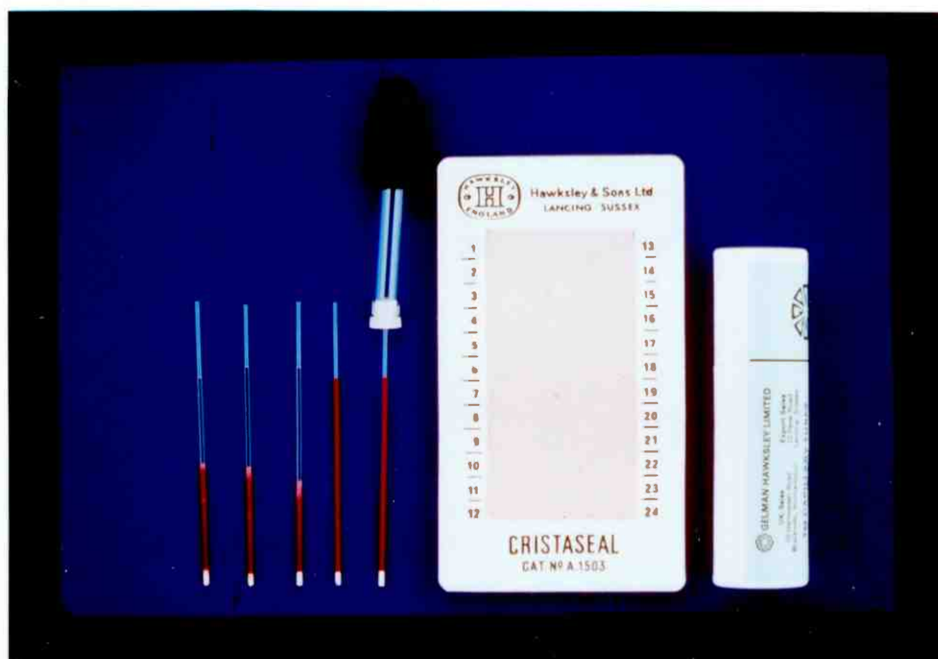
Materials and methods

A minimum of twelve blood-stained effusions were used as the test materials. These fluids were centrifuged at 300 g for five minutes and several smears prepared directly from part of the cell deposits from each specimen. The remaining cell deposits of the respective fluids were washed with PBS, recentrifuged and the supernatants were removed. The purpose of washing the cells was to get rid of the protein present in the centrifuged cell deposits. An attempt to remove the red blood cells and to concentrate mesothelial and malignant cells was then made by treating the sediments in one of two ways:

(a) The sediments were drawn up into several capillary tubes, sealed at one end with Cristaseal (Gelman-Hawksley Limited, England) and centrifuged at 700 g for ten minutes; the cells collected in the buffy layer were used for making smears (Figure 4.1);

(b) Alternatively, the sediment was layered onto a lymphoprep medium (Nyegard and Co, Oslo) and centrifuged at 300 g for 20 minutes, at which time the red cells and some

Figure 4.1



'Capillary/buffy coat' technique -

A cell suspension of the centrifuged deposit from a blood stained effusion is drawn into the capillary tubes which are sealed and centrifuged; the nucleated cells collect at the interface between the packed red blood cells and the saline to form the buffy coat.

polymorphonuclear cells had formed a pellet leaving the majority of tumour and mesothelial cells together with lymphocytes at the interface (Figure 4.2). These were aspirated and washed in PBS and subsequently used to make smears.

All the smears were fixed in 95% ethanol. The smears were stained by the Papanicolaou method and by the indirect immunoalkaline phosphatase technique for EMA and CEA. The quality of the Papanicolaou and the immunocytochemical staining for EMA and CEA on the smears prepared directly from the cell sediments of the blood-stained effusions was compared with those prepared from the same cell deposits after the nucleated cells had been washed and the red blood cells removed. Details of the method of preparing the smears are given in Appendix 1 and are recorded in the publication To *et al* (Acta Cytologica, in press).

Results

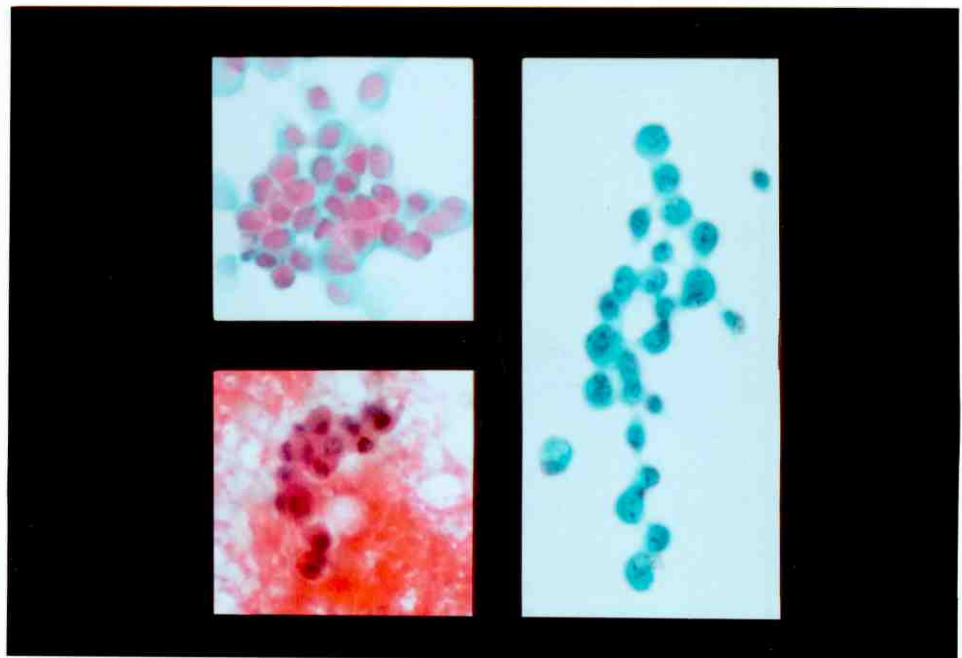
The results showed that the background staining of protein on the conventional smears was largely reduced by the washing process and most of the red blood cells on the conventional smears were removed by the 'capillary/buffy coat' or the 'lymphoprep' methods. The quality of the cytological preparation and consequently the degree of clarity obtained in the immunocytochemical staining, was improved by employing the additional techniques (Figures 4.3 and 4.4).

Figure 4.2

'Lymphoprep' gradient technique -

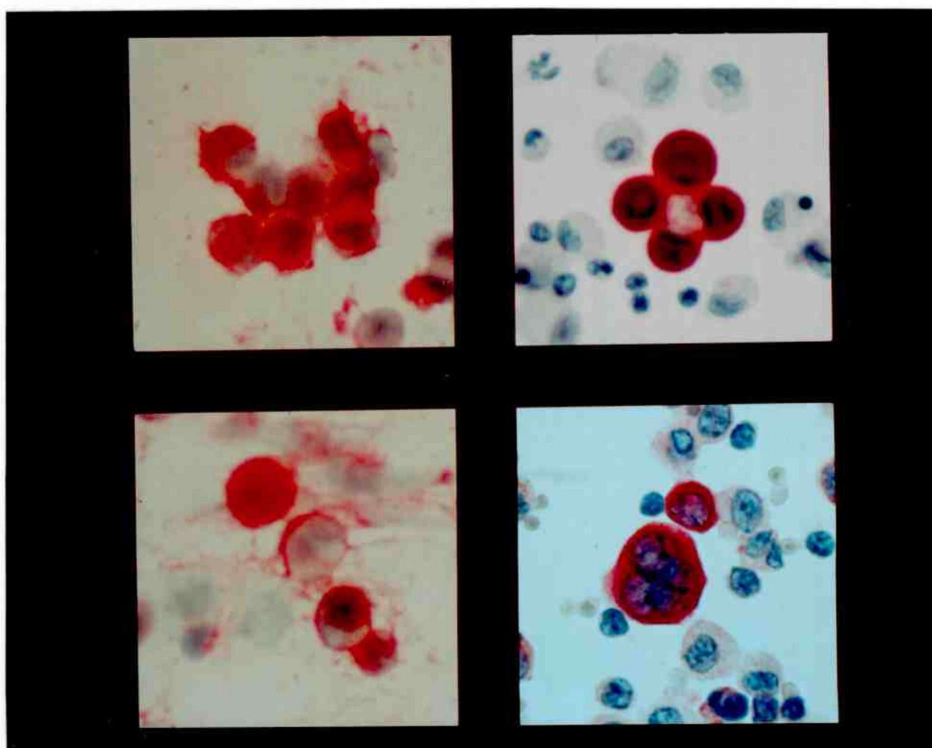
A cell suspension of the centrifuged deposit from a blood stained effusion is layered onto 'Lymphoprep'; red blood cells descend to the bottom of the medium leaving the nucleated cells at the interface (the white layer).

Figure 4.3



Top Left	-	Effect of air-drying on the Papanicolaou stained cells. (X130)
Bottom Left	-	Papanicolaou staining of reactive mesothelial cells from a benign effusion showing the effect of protein and red blood cells on the mesothelial cells. (X130)
Right	-	Elimination of protein and red blood cells from the same specimen has resulted in a Papanicolaou stained preparation with improved clarity.(X130)

Figure 4.4



Top and Bottom Left	-	Indirect immunoalkaline phosphatase stained smears prepared directly from centrifuged cell deposits showing poor cell morphology and background staining. (X130)
Top and Bottom Right	-	Indirect immunoalkaline phosphatase stained smears prepared from centrifuged cell deposits after elimination of protein and red blood cells showing improved clarity of the preparations. (X130)

Discussion

The washing process and the red blood cell elimination were included as routine procedures in the preparation of all the smears used in this study. A further point of importance concerned the fixation of the smears. It was noted that rapid fixation of the smears was essential for good morphological preservation (Figure 4.3). This rapid fixation was especially important with washed cells as there was no protein present to protect the cells from the increased osmolarity of the fluid as it dried. It was found that tilting the slide with one hand while smearing the cells on the slide with another slide facilitated the act of smearing and dropping into the fixative in one movement, thereby achieving rapid fixation in every case.

4.4 Factors affecting the immunocytochemical staining for EMA and CEA on smears stained by the indirect immunoalkaline phosphatase method

i. Determination of the effect of 10 minutes 20% acetic acid treatment on endogenous alkaline phosphatase activity

Materials and methods

A number of smears were prepared from a minimum of twelve effusions after removal of protein and red blood

cells as described in section 4.3. Some of the smears from each specimen were incubated with the chromogenic substrate for alkaline phosphatase (see Appendix 2) for one hour at room temperature. Other smears from the same specimens were incubated with the substrate after pretreatment with 20% acetic acid for ten minutes.

Results

Smears prepared from fluids in which the cells exhibited no endogenous alkaline phosphatase activity showed no staining.

Smears prepared from fluids in which the cells contained endogenous enzyme stained positively in those cases where the smears had not been pretreated with acetic acid. Smears from the same fluids showed no staining after treatment with acetic acid.

Conclusion

Pretreatment of the smears with 20% acetic acid for ten minutes completely removed endogenous alkaline phosphatase activity.

ii. Determination of the effect of 10 minutes 20% acetic acid treatment on the antigenic expression of EMA and CEA

Materials and methods

Smears of known EMA positive/CEA positive malignant cells from a minimum of twelve serous effusions were used. The smears were prepared after the nucleated cells had been washed and red blood cells removed. The cells in the smears were shown not to contain endogenous alkaline phosphatase activity by incubation of the smears with the chromogenic substrate alone. The smears of each specimen were stained for EMA and CEA by the indirect immunoalkaline phosphatase technique with and without the ten minutes 20% acetic acid treatment and the staining compared.

Results

The results showed that the acetic acid treatment had no effect on the antigenic expression of EMA and CEA, as the staining intensity on the treated and untreated smears of each specimen was comparable.

Discussion

The indirect immunoalkaline phosphatase technique was adopted as the staining method of choice. A further advantage of alkaline phosphatase conjugates over the peroxidase was that the former is a waterbased stain and the same smear could be used for retrospective Papanicolaou staining for morphological examination (see Chapters 5 and 6). This was carried out by removing the cover slip of an immunoalkaline

phosphatase stained smear with hot tap water, and re-staining by the Papanicolaou method. The permanent brown product of peroxidase/diaminobenzidine (DAB) meant that the same smear could not be used for retrospective Papanicolaou staining and this was a disadvantage where 'one-off' smears were concerned. However, electron microscopy cannot be carried out on alkaline phosphatase stained cells and this is the drawback of the alkaline phosphatase technique when compared to the peroxidase method.

iii. Determination of the effect of storage of the smears on the stability of EMA and CEA

Materials and methods

95% ethanol fixed smears containing EMA positive/CEA positive cells (confirmed by immunocytochemical staining) from a pleural effusion were used. The smears were prepared after the nucleated cells had been washed, the red blood cells removed and kept respectively in one of the following ways:

(a) at room temperature sprayed with carbowax

(b) at 4°C sprayed with carbowax

(c) at -20°C sprayed with carbowax

(d) in 95% ethanol at room temperature.

The smears kept by the respective methods were stained at monthly intervals for EMA and CEA by the indirect immuno-alkaline phosphatase technique and the staining results were compared with the original staining of the freshly-prepared smears prior to storage.

Results

Staining of the smears that were kept at room temperature or at 4°C diminished after several weeks. Staining of the smears that were kept in 95% ethanol at room temperature, or at -20°C, were comparable with the original staining up to a year at the time of writing.

Discussion

Since over four thousand smears were used in the work for this thesis, keeping the smears in ethanol was clearly impractical in terms of laboratory space. All the smears prepared for the study were therefore kept at -20°C, stacked in slide boxes.

In contrast to formalin-fixed, paraffin-embedded histological sections in which the expression of EMA and CEA is retained for many years, careful storage is required for the smears. This was realised when, later in the course of study, it was noted that 95% ethanol fixed smears failed to stain for either EMA or CEA although smears from the same specimens previously stained positively with both the markers. In this respect CEA deteriorated more rapidly than EMA. Therefore, proper storage of the smears is important as it allows smears to be used for retrospective immunocytochemical staining.

iv. Determination of the effect of different fixatives on the antigenic expression of EMA and CEA

Materials and methods

Smears of known EMA positive/CEA positive cells from a pleural effusion were used and were all prepared after the nucleated cells had been washed and the red blood cells removed. The smears were fixed respectively by one of the following methods:

(a) 100% ethanol for five minutes at room temperature.

(b) 95% ethanol for five minutes at room temperature.

(c) 75% ethanol for five minutes at room temperature.

(d) 50% ethanol for five minutes at room temperature.

(e) 1:1 95% ethanol/ether for five minutes at room temperature.

(f) Buffered formol saline (10% of 36% w/w formaldehyde) for five minutes at room temperature.

(g) Carnoy's solution (10 mls glacial acetic acid, 30 mls chloroform, 60 mls absolute ethanol) for five minutes at room temperature.

(h) Bouin's solution (5 mls glacial acetic acid, 25 mls 36% w/w formaldehyde, 75 mls saturated aqueous picric acid) for five minutes at room temperature.

(i) Methacorn (10 mls glacial acetic acid, 30 mls chloroform, 60 mls methanol) for five minutes at room temperature.

(j) Formol calcium (11 mls 36% w/w formaldehyde, 9 mls 1M $CaCl_2$, 0.5 grams $CaCO_3$, 100mls distilled water) for five minutes at room temperature.

(k) Buffered formol acetone (Mason <u>et al</u>, 1975) (20 mg Na_2HPO_4, 100mg KH_2PO_4, 45 mls acetone, 25 mls

36% w/w formaldehyde, 30mls distilled water) for 30 seconds at room temperature.

After fixation the smears were washed in distilled water and stained for EMA and CEA by the indirect immuno-alkaline phosphatase technique. The staining intensity and the morphological preservation of the cells fixed by the respective methods were compared.

Results and conclusion

All of the fixatives tested preserved the expression of EMA and CEA, but the staining intensity was strongest on cells fixed in buffered formol saline and almost as strong in formol calcium, buffered formol acetone, 100% ethanol, 95% ethanol and 1:1 95% ethanol/ether. The best morphological preservation was obtained with 100% and 95% ethanol and 1:1 95% ethanol/ether. As 95% ethanol is used routinely in most cytology laboratories it was chosen as the method of fixation for this research.

Chapter 5

Evaluation of Epithelial Membrane Antigen staining as a method of identifying malignant cells in serous effusions

5.1 Materials and methods

5.2 Results

5.3 Discussion

5.1 Materials and methods

309 serous effusions from 246 patients were investigated. 182 effusions were received from 151 patients with proven malignant disease and 127 fluids from 95 patients in whom there was no clinical evidence of malignancy. Table 5.1 summarises the source of specimens used in this study. The clinical diagnoses of the patients are shown in Table 5.2 and have been obtained from the patients' notes. In most cases of malignant disease, the diagnosis of malignancy had been established by histological examination of tumour tissues. In other cases, the clinical progress of the patients or the post-mortem findings were taken as acceptable evidence of malignant disease.

The anti-EMA serum and the alkaline phosphatase conjugates used in this study were the same materials described in Chapter 4. For every batch of primary or secondary antiserum used in this study, the dilution was determined by a standard chequerboard experiment. The working dilution of the primary antiserum was the dilution which did not stain mesothelial cells at all or stained them only weakly, while the staining on the malignant cells remained strong. The working dilution of the conjugates was selected on the basis that it gave maximum staining. This was achieved by testing two-fold dilutions of both the primary antiserum and the conjugates on smears from a minimum of three benign and three malignant effusions

Table 5.1

Source of specimens

Effusions from patients with malignant disease					
Hospital	No. of patients	No. of effusions	Type of specimens		
			Pleural	Ascites	Pericardial
St Mary's Hospital	52	67	41	26	0
St Bartholomew's Hospital	52	58	29	28	1
Royal Marsden Hospital	47	57	32	24	1
Total	151	182	102	78	2
Effusions from patients with benign disease					
Hospital	No. of patients	No. of effusions	Type of specimens		
			Pleural	Ascites	Pericardial
St Mary's Hospital	67	99	69	30	0
St Bartholomew's Hospital	28	28	20	8	0
Total	95	127	89	38	0
Overall Total	246	309	191	116	2

Table 5.2

Clinical diagnoses of 246 patients

Malignant Diseases		Non-Malignant Diseases	
Carcinoma of		Cardiac failure	26
Breast	47	Pulmonary disease	25
Ovary	29	Hepatic cirrhosis	17
Lung squamous	9	Renal failure	5
Lung adeno	7	Other	4**
Lung oat cell	4	Unknown cause	18
Gastro-intestinal tract	15		
Other sites	13*		
Unknown sites	11		
Other malignancies			
Mesothelioma	2		
Lymphoma	11		
Myeloma	2		
Leukaemia	1		
Total	151	Total	95

*Pyriform fossa 1; Stomach 2; Pancreas 3; Kidney 4; Bladder 1; Fallopian tube 1; Cervix 1.

**Rheumatoid arthritis 1; Ovarian fibroma 1; Acute pancreatitis 1; Acute pericarditis and pleurisy 1.

known to contain, respectively, benign mesothelial cells and malignant epithelial cells.

The specimens were delivered to the cytology laboratory usually within 24 hours after aspiration, and most of them were processed immediately upon arrival. A few were kept overnight and these were stored at 4°C. For each specimen, a minimum of five smears were prepared using the method described in Appendix 1. One smear was air-dried and stained by the standard Giemsa method (Appendix 3). The others were fixed in 95% ethanol. Two of the ethanol-fixed smears were stained by the Papanicolaou method (Appendix 3), one by the Periodic Acid Schiff-Diastase technique (Appendix 3) and the remaining one was stained for EMA by the indirect immunoalkaline phosphatase technique as described in Appendix 2. A known EMA-positive control was included in every batch of slides for immunocytochemical staining. The cytological assessment of the smears was based on the morphological studies of the Papanicolaou and Giemsa stained cells. Second opinion of cytologically difficult cases was sought from Dr G Canti of the cytology unit at St Bartholomew's Hospital, London. The PAS-Diastase staining was used to confirm the presence of adenocarcinoma cells. The results of the EMA staining were correlated with those of the morphological studies, the PAS-Diastase staining and the clinical diagnoses of the patients.

5.2 Results

Three patterns of EMA positive staining were observed. In some cells a 'rim' of positive staining was present around the edge of the cells (Figure 5.1). In other cells, a 'diffuse' staining of the cytoplasm could be detected (Figure 5.1). The third pattern of staining was characterised by an intense positive staining throughout the cytoplasm (Figure 5.2). 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 the 'strong' pattern of 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 (Figure 5.2). Whenever the strong staining was present it was recorded as the pattern of staining for that particular specimen.

164 serous effusions from 137 patients with carcinoma or malignant mesothelioma were investigated. A further 18 effusions from 14 patients with non-epithelial malignancies were examined. The clinical diagnoses of these patients and the results of the cytological examination and the EMA staining of their specimens is given in detail in Table 5.3 and summarised in Table 5.5 (Table 5.5 is placed in page 107).

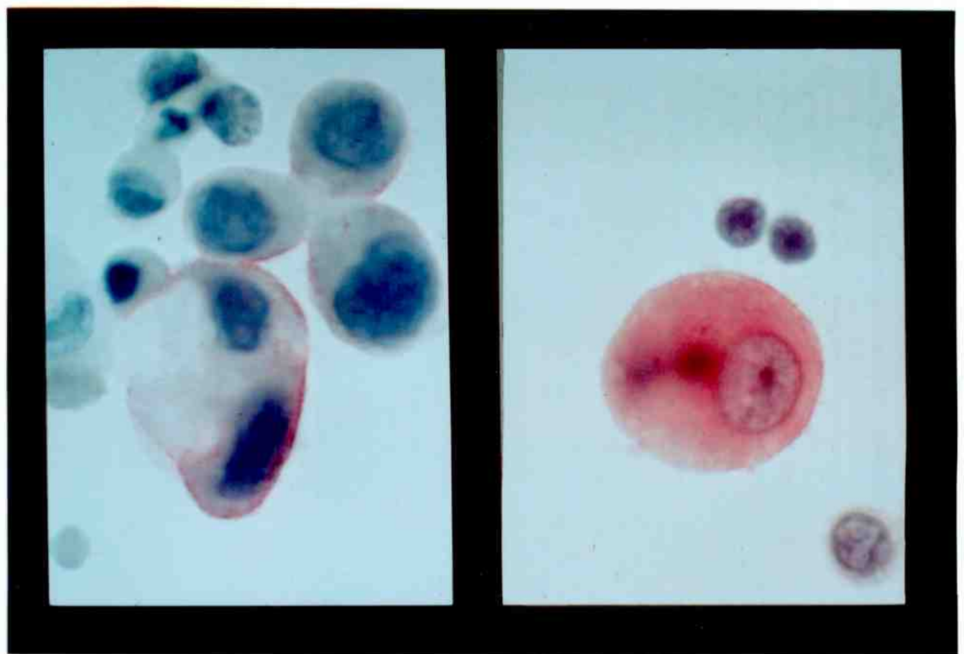
Table 5.3

Correlation of EMA staining, cytological and clinical diagnoses of 182 effusions from 151 patients with proven malignant disease

No. of effusions	Clinical Diagnoses	Cytology positive EMA staining			Cytology suspicious EMA staining			Cytology negative EMA staining		
		Strong	Weak	Negative	Strong	Weak	Negative	Strong	Weak	Negative
	Carcinoma or mesothelioma									
58	Ca breast	18(13)	17(7)	7(2)	3	2	0	1	2	8([5])
35	Ca ovary	18(8)	11(6)	1	0	0	0	0	0	5([3])
10	Ca lung squamous	3	3	0	1	2	0	0	0	1
7	Ca lung adeno.	5(2)	1(1)	0	0	1	0	0	0	0
4	Ca lung oat cell	0	1	3	0	0	0	0	0	0
16	Ca G-I tract	6(4)	1	2	0	2	2	0	0	3
15	Other epithelial malignancies	7(4)	1	1	1	0	0	0	0	5([2])
17	Primary unknown	5(3)	3(2)	0	0	0	1	2	2	4([1])
2	Mesothelioma	1	1	0	0	0	0	0	0	0
164	Total no. of effusions	63(34)	39(16)	14(2)	5	7	3	3	4	26([11])
	Non-epithelial malignancies									
15	Lymphoma	0	1	11	0	0	0	0	0	3
2	Myeloma	0	2	0	0	0	0	0	0	0
1	Leukaemia	0	0	1	0	0	0	0	0	0
18	Total no. of effusions	0	3	12	0	0	0	0	0	3

( ) = mucin secretion ([ ]) = leucocytic effusions

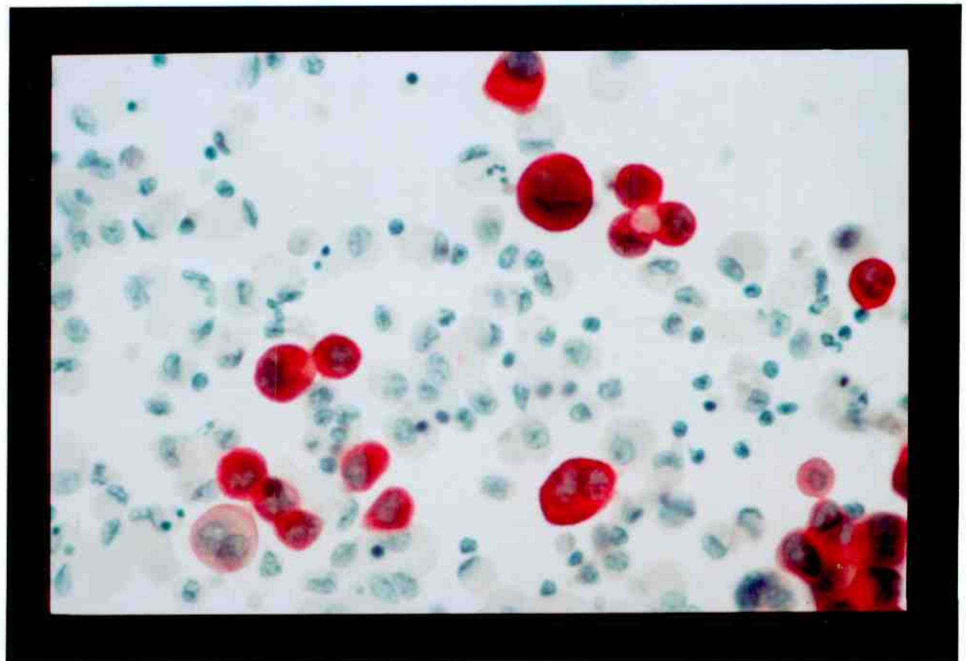
Figure 5.1



Left - Indirect immunoalkaline phosphatase staining of a malignant cell in a smear from a patient with primary carcinoma of ovary showing a 'rim' pattern of weak EMA staining. (X320)

Right - Indirect immunoalkaline phosphatase staining of a malignant cell in a smear from a patient with primary carcinoma of breast showing a 'diffuse' pattern of weak EMA staining. (X320)

Figure 5.2



Indirect immunoalkaline phosphatase staining of malignant cells in a smear from a patient with primary carcinoma of ovary showing both strong and weak EMA staining. (X320)

A firm cytological diagnosis of malignancy was made on the morphological appearance of the exfoliated cells in Papanicolaou and Giemsa stained smears in 116 of the 164 effusions (71%) from patients with carcinoma or malignant mesothelioma. Mucin secretion was demonstrated in 52 of the 116 cytologically positive effusions (45%) by the Periodic Acid Schiff-Diastase method. Strong EMA staining was shown by the exfoliated cells in 63 of the 116 cytologically positive effusions (54%) and weak EMA staining in 39 of the 116 fluids (34%).

15 of the 164 effusions (9%) were observed to contain cells showing changes suggestive but not conclusive of malignancy and a cytologically suspicious report was given. Strong EMA staining was found in five of these 15 cases (33%) and weak staining in seven of the 15 specimens (47%).

The remaining 33 of these 164 effusions (20%) were given a cytologically negative report as no evidence of malignancy was seen, and 11 of these specimens contained only leucocytes. However, strong EMA staining was observed in three of these 33 cases (9%) and weak EMA staining in four of the 33 specimens (12%).

Malignant cells were seen in 15 of the 18 effusions (83%) from patients with non-epithelial malignancies (lymphoma, leukaemia, myeloma). None of these effusions gave strong EMA staining,

but weak EMA staining was observed in three of the 15 cytologically positive cases (20%).

127 serous effusions were received from 95 patients with no clinical evidence of malignancy. The clinical diagnoses of these patients and the results of the cytological examination and the EMA staining of their specimens is given in detail in Table 5.4 and summarised in Table 5.5.

No evidence of malignancy was observed in Papanicolaou and Giemsa stained smears in 121 of these 127 effusions (95%). Strong EMA staining was observed in two of these 121 cytologically negative cases. One was obtained from a patient with a clinical diagnosis of acute viral pericarditis and pleurisy and the other from a patient with a histologically confirmed diagnosis of alcoholic cirrhosis. Weak EMA staining was found in 20 of the 121 cytologically negative cases (17%).

Six of the 127 effusions were seen to contain cells on which a differential cytodiagnosis could not be made and a cytologically suspicious report was given. Strong EMA staining was found in one of these six cases; the specimen was from an elderly female with pneumonia. Weak EMA staining was observed in two of the six cytologically suspicious cases.

5.3 Discussion

The object of this investigation was to determine if an

Table 5.4

Correlation of EMA staining, cytological and clinical diagnoses of 127 effusions from 95 patients with no clinical evidence of malignancy

No. of effusions	Clinical Diagnoses	Cytology suspicious EMA staining			Cytology negative EMA Staining		
		Strong	Weak	Negative	Strong	Weak	Negative
36	Lung disease	1	1	0	1	6	27
24	Liver disease	0	0	0	1	4	19
8	Renal disease	0	0	0	0	4	4
35	Heart disease	0	1	3	0	5	26
21	Unknown etiology	0	0	0	0	1	20
3	Others	0	0	0	0	0	3
127	Total number of effusions	1	2	3	2	20	99

Table 5.5

Summary of EMA staining in 309 effusions from 246 patients

Clinical Diagnoses (No. of patients)	Cytological Diagnoses	Effusion with EMA stain classified as			Total
		Strong	Weak	Negative	
Epithelial Malignancy (137 patients)	Malignant	63 (34)	39 (16)	14 (2)	116 (52)
	Suspicious	5	7	3	15
	Non-Malignant	3	4	26	33
					164
Non-Epithelial Malignancy (14 patients)	Malignant	0	3	12	15
	Non-Malignant	0	0	3	3
					18
Non-Malignant (95 patients)	Suspicious	1	2	3	6
	Non-Malignant	2	20	99	121
					127

( ) : mucin secretion

immunocytochemical stain for EMA can be used for diagnostic purposes to detect malignant cells in serous effusions. The results of this investigation showed that weak EMA staining occurred in 22 specimens from patients without malignant disease as well as in 53 specimens from patients with malignant disease. It is therefore clear that weak EMA staining cannot be used as a criterion for malignancy.

In contrast, of the 74 effusions with strong EMA staining, 71 of them occurred in specimens from patients with proven carcinoma. A summary of the distribution of strong EMA staining in the 309 specimens investigated in this study is shown in Table 5.6. 63 specimens with strong EMA staining were observed among the 116 cytologically-positive malignant effusions of epithelial origin (54%). The proportion of malignant cells that stained for EMA varied in different specimens, and there did not appear to be a preferential sensitivity towards a particular tumour type. In the remaining 53 cytologically positive specimens which did not demonstrate strong EMA staining, it should be noted that the specificity of the EMA staining was increased by increasing the dilutions of the primary antiserum in order to detect the difference in the expression of EMA between malignant and mesothelial cells. Consequently the sensitivity of the technique was reduced. In these effusions many of the malignant cells might have stained strongly if a less dilute antiserum had been used.

Table 5.6

Summary of 309 effusions with strong EMA staining

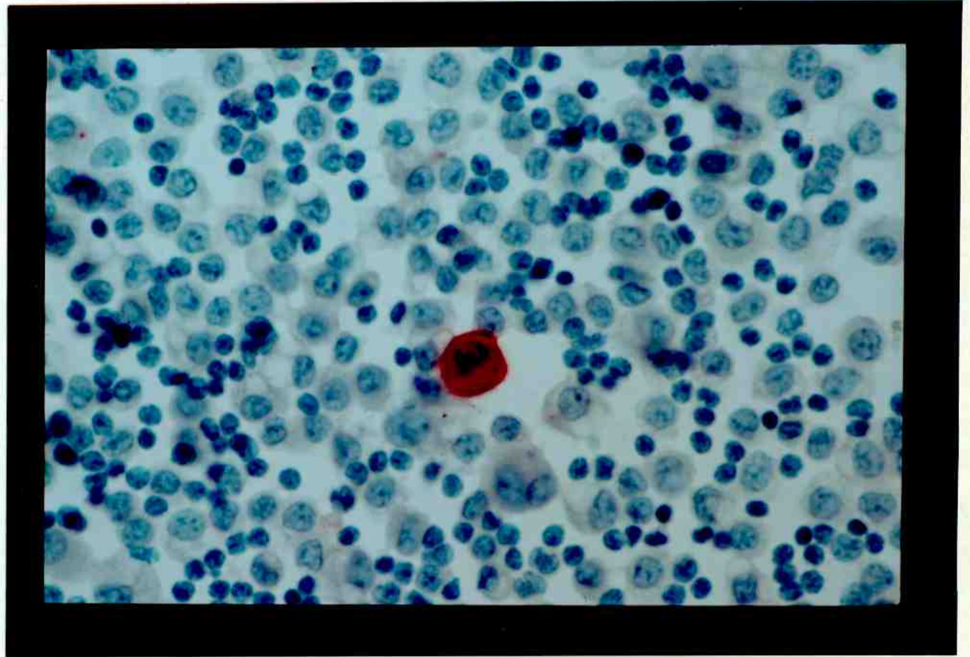
	Patients with carcinoma or mesothelioma (164 effusions)	Patients with non-epithelial malignancies (18 effusions)	Patients with malignant disease (127 effusions)
EMA strong +ve/cytology positive	$^{63}/_{116}$	$^{0}/_{15}$	$^{0}/_{0}$
EMA strong +ve/cytology suspicious	$^{5}/_{15}$	$^{0}/_{0}$	$^{1}/_{6}$
EMA strong +ve/cytology negative	$^{3}/_{33}$	$^{0}/_{3}$	$^{2}/_{121}$

Eight specimens with strong EMA staining were present in 48 specimens (17%) from patients with proven epithelial malignancies but where the cytology was reported as suspicious or negative. In three cases where the malignant cells were not identified in routine screening, the few cells that showed strong EMA staining were restained by the Papanicolaou method and were identified as malignant cells by retrospective morphological examination (Figures 5.3 and 5.4). Therefore, strong EMA staining would be of value in the detection of discrete malignant cells which were missed in routine screening.

Eighteen specimens were obtained from patients with tumours of non-epithelial origin. As histological studies have shown that EMA is not expressed by connective, lymphoid, haemopoetic or osseous tissues, the malignant cells in these cases were not expected to stain for the marker. EMA staining on malignant cells could therefore be used to indicate an epithelial/mesothelial derivation.

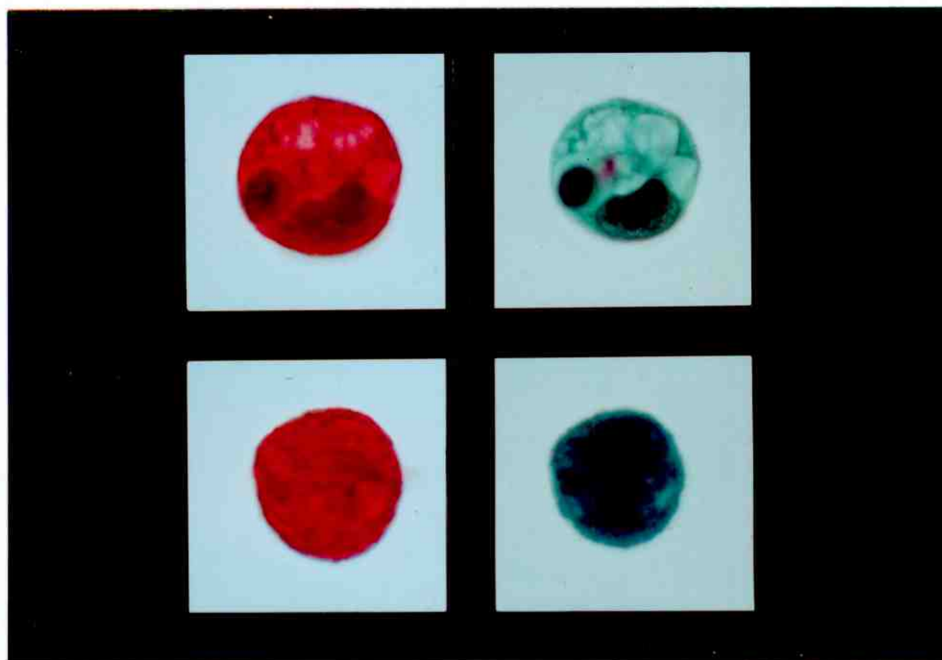
Three of the 127 (2%) effusions from 95 patients in whom no evidence of malignancy was found, showed strong EMA staining. One of the three was from a patient with pneumonia, whose effusion was classified cytologically as suspicious. Another came from a patient with viral pericarditis and pleurisy, and the third from a patient with alcoholic cirrhosis. In this specimen, random chromosomal abnormalities were found. This cytogenetic feature had

Figure 5.3



Indirect immunoalkaline phosphatase staining for EMA of a discrete malignant cell among numerous reactive mesothelial cells in a smear from a patient with primary carcinoma of breast. (x130)

Figure 5.4



Top Left - Indirect immunoalkaline phosphatase staining for EMA of a discrete malignant cell in a pleural effusion reported negative on routine cytological examination. (X320)

Top Right - Same cell restained by the Papanicolaou method showing obvious malignant features. (X320)

Bottom Left - Indirect immunoalkaline phosphatase staining for CEA of a discrete malignant cell in a pleural effusion reported negative on routine cytological examination. (X320)

Bottom Right - Same cell restained by the Papanicolaou method showing obvious malignant features. (X320)

previously been reported in benign effusions from patients with alcoholic cirrhosis (To _et al_, 1981). It could be speculated that strong EMA staining may be related to the abnormal karyotypes occurring in benign effusions. Chromosomal aberrations were found to be significantly more frequent in the peripheral lymphocytes of chronic alcohol users (Mitelman and Wadstein, 1978). Mutagenic activity has recently been demonstrated in the urine of patients with alcoholic cirrhosis who were non-smokers (Gelbart and Sontag, 1980). The possibility that these atypical mesothelial cells could result from the direct or indirect mutagenic effect of alcohol in the supernatant of the effusions was investigated. A pilot study of six effusions from alcoholic cirrhotics were evaluated for the presence of mutagenic activity by means of the Ames test, which was carried out in collaboration with Dr A Boobis and Mr S Plummer at the Royal Postgraduate Medical School, London. However, no such activity was demonstrated.

Thus the usefulness of EMA staining was limited by the few false-positive results obtained. Nevertheless, the overall results indicate that a high concentration of EMA in a cell from a serous effusion is strongly suggestive of malignancy. In conjunction with the clinical data, this may be of value in the further characterisation of cells which on morphological grounds are of indeterminate origin. There are two further applications. First, in an effusion containing morphologically malignant cells of

unknown site of origin, expression of EMA would identify an epithelial/mesothelial derivation. Second, some effusions from patients with malignant disease contain very few or no recognisable tumour cells. In this situation EMA staining may pick out small numbers of potentially malignant cells whose identity can be confirmed by a subsequent Papanicolaou stain.

Chapter 6

Evaluation of Carcinoembryonic Antigen staining as a method of identifying malignant cells in serous effusions; Correlation with EMA staining

6.1 Materials and methods

6.2 Results

6.3 Discussion

6.4 Correlation with EMA staining

6.1 Materials and methods

182 specimens of serous effusions from 146 patients were investigated in this study. They were derived from the 309 specimens used for the EMA staining in the last chapter. The source of these 182 specimens is shown in Table 6.1 The clinical diagnoses of the 146 patients is shown in Table 6.2. The anti-CEA serum and the enzyme conjugates used in this study were the same materials described in Chapter 4. The protocol for this study was identical with that of the EMA staining and similarly the results of the CEA staining were correlated with the cytological examination of the Papanicolaou and Giemsa stained smears. It was also correlated with the results of the 182 PAS-Diastase staining of the specimens and the clinical diagnoses of the patients. In addition, the results of the CEA staining on the 182 specimens were compared with those of the EMA staining.

6.2 Results

Three patterns of CEA staining (strong, rim and diffuse) were observed. The patterns were similar to those obtained after EMA staining and were recorded as strong and weak, as before. The results of the CEA staining, the cytological examination of the Papanicolaou and Giemsa stained smears, the PAS-Diastase staining of the 182 specimens and the clinical diagnoses of the patients is given in detail in Table 6.3 and summarised in Table 6.4.

Table 6.1

Source of specimens

Effusions from patients with malignant disease					
Hospital	No. of patients	No. of effusions	Type of specimen		
			Pleural	Ascites	Pericardial
St Mary's Hospital	24	30	21	9	0
St Bartholomew's Hospital	34	40	17	22	1
Royal Marsden Hospital	23	29	13	15	1
Total	81	99	51	46	2

Effusions from patients with benign disease					
Hospital	No. of patients	No. of effusions	Type of specimen		
			Pleural	Ascites	Pericardial
St Mary's Hospital	39	57	35	22	0
St Bartholomew's Hospital	26	26	19	7	0
Total	65	83	54	29	0
Overall Total	146	182	105	75	2

Table 6.2

Clinical diagnoses of 146 patients

Malignant Diseases		Non-Malignant Diseases	
Carcinoma of		Cardiac failure	18
Breast	25	Pulmonary disease	15
Ovary	15	Hepatic cirrhosis	11
Lung squamous	4	Renal failure	3
Lung adeno	5	Other	4**
Lung oat cell	4	Unknown cause	14
Gastro-intestinal tract	5		
Other sites	7*		
Unknown sites	6		
Other malignancies			
Lymphoma	7		
Myeloma	2		
Leukaemia	1		
Total	81	Total	65

*Pancreas 2; Stomach 2; Kidney 3.

**Rheumatoid arthritis 1; Ovarian fibroma 1; Acute pancreatitis 1; Acute pericarditis and pleurisy 1.

Table 6.3

Correlation of CEA staining, cytological and clinical diagnoses of 182 effusions from 146 patients

No. of effusions	Clinical Diagnoses	Cytology positive CEA staining			Cytology suspicious CEA staining			Cytology negative CEA staining		
		Strong	Weak	Negative	Strong	Weak	Negative	Strong	Weak	Negative
	Carcinoma									
32	Ca breast	3(2)	5(3)	16(3)	0	1	1	0	1	5([3])
17	Ca ovary	2(2)	1(1)	13(4)	0	0	0	0	0	1
4	Ca lung squamous	0	1	1	0	0	1	0	0	1
5	Ca lung adeno.	2	2(2)	0	0	0	1	0	0	0
4	Ca lung oat cell	0	0	4	0	0	0	0	0	0
6	Ca G-I tract	1	0	1	0	0	1	0	0	3
8	Other epithelial malignancies	1	2	1	0	0	0	0	0	4([2])
11	Primary unknown	1	1(1)	1	0	0	1	1	1	5([1])
87	Total no. of effusions	10(4)	12(7)	37(7)	0	1	5	1	2	19([6])
	Non-epithelial malignancies									
9	Lymphoma	0	0	7	0	0	0	0	0	2
2	Myeloma	0	0	2	0	0	0	0	0	0
1	Leukaemia	0	0	1	0	0	0	0	0	0
12	Total no. of effusions	0	0	10	0	0	0	0	0	2
83	Non-malignant disease	0	0	0	0	0	0	0	0	83

( ) = mucin secretion ([ ]) = leucocytic effusions

Table 6.4

Summary of CEA staining of 182 effusions from 146 patients

Clinical Diagnoses (No. of patients)	Cytological Diagnoses	Effusion with CEA stain classified as			Total
		Strong	Weak	Absent	
Epithelial Malignancy (71 patients)	Malignant	10 (4)	12 (7)	37 (7)	59 (18)
	Suspicious	0	1	5	6
	Non-Malignant	1	2	19	22
					87
Non-Epithelial Malignancy (10 patients)	Malignant	0	0	10	10
	Non-Malignant	0	0	2	2
					12
Non-Malignant (65 patients)	Non-Malignant	0	0	83	83

( ) : Mucin secretion

87 effusions were obtained from 71 patients with proven epithelial malignancies. A firm cytological diagnosis of malignancy was made on the morphological appearance of the exfoliated cells in Papanicolaou and Giemsa stained smears in 59 of these 87 specimens (68%). Mucin secretion was demonstrated in the malignant cells in 18 of the 59 cytologically positive effusions (31%) by the Periodic Acid Schiff-Diastase technique. 22 of these 59 cytologically positive specimens (37%) stained with CEA, 10 strongly and 12 weakly.

Six of the 87 effusions from patients with proven epithelial malignancies were observed to contain cells showing changes suggestive but not conclusive of malignancy and a cytologically suspicious report was given. One of these six cases (17%) stained weakly with CEA.

In the remaining 22 of the 87 effusions, a negative cytology report was given as no evidence of malignancy was seen. Six of these 22 cytologically negative specimens were leucocytic effusions. Three of these 22 cytologically negative effusions (14%) stained with CEA, one strongly and two weakly.

12 effusions were received from ten patients with proven non-epithelial malignancies (Lymphoma, myeloma, leukaemia). Ten fluids were reported as cytologically positive and the remaining two negative. None of these 12 cases stained with CEA.

83 specimens were obtained from 65 patients in whom there was neither clinical nor cytological evidence of malignancy. None of these 83 specimens stained with CEA.

6.3 Discussion

This CEA study has shown that, within the confined cell types found in serous effusions and at the dilutions of the antisera used in the study, both strong and weak CEA staining on the smears is specific for epithelial malignant cells. However, this high specificity is offset by a low sensitivity as only 22 of the 59 (37%) cytologically positive specimens stained for CEA. Nevertheless, one specimen which contained cells that were suspicious on morphological grounds stained with CEA. This specimen was subsequently discovered to come from a patient with metastatic carcinoma of the breast involving the serous membrane. This indicated that CEA staining may be of assistance in detecting the malignant cells which on morphological grounds cannot be classified as malignant unequivocally. There were in addition three effusions from patients with proven epithelial malignant disease in which malignant cells were not identified in conventionally stained smears. A few discrete cells in smears from these effusions were picked out by CEA staining. In these three cases, the cells that stained positively with CEA were re-examined and confirmed as malignant on morphological grounds by directly restaining the smears for Papanicolaou (Figure 5.4).

The epithelial-specificity of CEA is such that none of the non-epithelial malignant specimens stained with CEA. Therefore, the presence of CEA-stained tumour cells in an effusion can be taken to indicate an epithelial derivation and therefore may be useful in the differential diagnosis of tumour types. We have also observed that strong CEA staining is more frequently present on exfoliated single cells, in contrast to malignant cell clusters which were mostly negative or only occasionally stained. This characteristic appears to be in accordance with the kinetic findings that CEA synthesis varies inversely in relation to the cell growth (Drewinko and Yang, 1976; Ellison et al, 1977).

6.4 Correlation with EMA staining

All of the 182 specimens used in this CEA study were also stained for EMA. A comparison of the two staining results is shown in Table 6.5. Whilst it is clear that EMA has a much wider histological distribution than CEA, its diagnostic value in serous effusions is limited by the frequent non-specific weak staining and the 2% false-positive strong staining shown in Table 6.5 and also reported in the last chapter. The high sensitivity of EMA staining is therefore offset by a low specificity. In contrast, both weak and strong CEA staining have been shown to be specific for epithelial malignant cells in serous effusions yet this high specificity is offset by a

Table 6.5

Comparison of CEA/EMA staining of 182 effusions from 146 patients

No. of effusions	Clinical Diagnoses	Cytology positive CEA/EMA staining			Cytology suspicious CEA/EMA staining			Cytology negative CEA/EMA staining		
		Strong	Weak	Negative	Strong	Weak	Negative	Strong	Weak	Negative
87	Epithelial Malignancy	10 (26)	12 (20)	37 (13)	0 (2)	1 (2)	5 (2)	1 (2)	2 (3)	19 (17)
12	Non-Epithelial Malignancy	0 (0)	0 (3)	10 (7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2)
83	Non-Malignant	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (2)	0 (13)	83 (68)
182	Total No. of effusions	10 (26)	12 (23)	47 (20)	0 (2)	1 (2)	5 (2)	1 (4)	2 (16)	104 (87)

( ) : EMA staining

low sensitivity. However, if strong EMA staining only is taken as a probable indication of neoplastic changes, the sensitivity of EMA staining approaches that of CEA staining. This is apparent in Table 6.6 which records the pattern of CEA staining (both strong and weak) and strong EMA staining in the 182 specimens used in this study.

The object of applying additional staining techniques to specimens of serous effusions is to assist the routine cytological examination. Table 6.7 shows the distribution of strong EMA staining, CEA and PAS-Diastase staining in the 87 specimens from patients with proven carcinomas. 59 of these 87 specimens (68%) were cytologically positive for malignancy. Of these 59 specimens, 14 stained for both EMA and CEA, 12 for EMA alone, eight for CEA alone and 25 did not stain for either marker. 18 of the 59 cytologically positive specimens stained for mucins, and four of these 18 cases did not stain for EMA or CEA. Therefore, altogether 38 of the 59 cytologically positive specimens (64%) were detected by these additional staining techniques.

In addition, in the six cytologically suspicious effusions, discrete malignant cells were present in one fluid which was detected by both EMA and CEA staining and in another case by EMA staining alone. Among the 22 cytologically negative effusions, six were leucocytic effusions. In the remaining 16 fluids, a further three cases were found to contain discrete malignant cells - two by both

Table 6.6

Comparison of positive CEA staining and strong EMA staining in 182 effusions from 146 patients

	Patients with carcinomas (87 effusions)	Patients with non-epithelial malignancies (12 effusions)	Patients without malignant disease (83 effusions)	
CEA positive / Cytology positive	22/59 (26/59) ((18/59))	0/10 (0/10)	0/0 (0/0)	EMA strong positive / Cytology positive
CEA positive / Cytology suspicious	1/6 (2/6)	0/0 (0/0)	0/0 (0/0)	EMA strong positive / Cytology suspicious
CEA positive / Cytology negative	3/22 (2/22)	0/2 (0/2)	0/83 (2/83)	EMA strong positive / Cytology negative

( ) : EMA staining

((18)) / ((59)) : Mucin secretion

Table 6.7

Distribution of strong EMA staining, CEA and PAS-Diastase staining in 87 effusions from 71 patients with proven carcinomas

Cytological Diagnoses	CEA+/EMA+	CEA+/EMA-	CEA-/EMA+	CEA-/EMA-	Total
Cytology Positive	14 (6)	8 (5)	12 (3)	25 (4)	59
Cytology Suspicious	1	0	1	4	6
Cytology Negative	2	1	0	19	22

( ) : mucin secretion

EMA and CEA staining and one by CEA staining alone. PAS-Diastase staining did not detect any case which was missed in routine cytological examination. Therefore, altogether five of the 26 cytologically suspicious and negative effusions (19%) were found to contain discrete malignant cells by EMA and/or CEA staining.

In conclusion, in the 87 effusions from patients with proven epithelial malignancy, the use of the additional staining techniques to routine cytological examination has increased the sensitivity of detecting malignant cells from 68% (59 cases by cytology alone) to 74% (64 cases). It is absolutely essential to realise that not all of these 87 effusions contained malignant cells. Some of them may have been caused only indirectly by the presence of tumours elsewhere in the body (e.g. by lymphatic obstruction) and did not contain malignant cells. Indeed six of the 87 specimens were leucocytic effusions. Thus the inclusion of additional staining techniques in the investigative procedure increased the sensitivity with which fluids caused by metastatic involvement of the serous membrane can be detected. It is likely that in most of the remaining 23 cases (26%) there were no tumour cells.

In an attempt to extend this multiple marker study, two other markers were investigated. A preliminary immunocytochemical study of the pre-keratin marker (Sun and Green, 1978)

was carried out on six benign and six malignant specimens of effusions known to contain, respectively, mesothelial cells and malignant cells from different primary sites. The results showed that the mesothelial cells in all of the six benign specimens were stained as strongly for the marker as the various malignant cells. Consequently this marker was not investigated further. Another identical preliminary immunocytochemical study was carried out with a prostatic-specific marker (Nadji et al, 1981). Apart from the positive staining obtained on a histological section of a prostatic tumour which was used as a positive control, all of the twelve effusions tested (six benign and six malignant) were negative. According to the report by Nadji et al (1981), this marker was found to be specific for prostatic gland epithelium. For this reason, this marker was not further investigated as effusions from patients with prostatic cancer are exceedingly rare.

Chapter 7

The diagnostic value of CEA radioimmunoassay on supernatants of serous effusions; Correlation with CEA staining

7.1 Materials and methods

7.2 Results

7.3 Discussion

7.4 Correlation with CEA staining

7.1 Materials and methods

151 serous effusions from 125 patients were investigated in this study. 88 specimens were obtained from 75 patients with malignant disease and 63 fluids were obtained from 50 patients in whom there was no evidence of malignancy on clinical grounds. Table 7.1 indicates the source of specimens used in this study. The clinical diagnoses of the 125 patients is shown in Table 7.2 The CEA assay was carried out on the supernatants of the 151 effusions and the results were correlated with the cytological examination of the Papanicolaou and Giemsa stained smears and the clinical diagnoses of the patients.

In addition, 92 of the 151 effusions used in this study were specimens included in Chapter 6 on which CEA staining was carried out. Although different anti-CEA sera were used for the radioimmunoassay and for the staining, an attempt was made to correlate the results of the CEA staining on the malignant cells in the fluids with those of the CEA radioimmunoassay on the supernatants of these 92 effusions.

The CEA radioimmunoassay was carried out by Mr Hugh Mitchell in the Department of Medical Oncology at Charing Cross Hospital, London W6, with the permission of Professor K D Bagshawe. Specimens of the supernatants of serous effusions were kept at -20°C before the assay. In the

Table 7.1

Source of specimens

Effusions from patients with malignant disease					
Hospital	No. of patients	No. of effusions	Type of specimen		
			Pleural	Ascites	Pericardial
St Mary's Hospital	31	36	36	0	0
Royal Marsden Hospital	44	52	23	28	1
Total	75	88	59	28	1
Effusions from patients with benign disease					
Hospital	No. of patients	No. of effusions	Type of specimen		
			Pleural	Ascites	Pericardial
St Mary's Hospital	49	62	41	21	0
St Bartholomew's Hospital	1	1	0	1	0
Total	50	63	41	22	0
Overall Total	125	151	100	50	1

Table 7.2

Clinical diagnoses of 125 patients

Malignant Diseases		Non-Malignant Diseases	
Carcinoma of		Cardiac failure	11
Breast	35	Pulmonary disease	14
Ovary	9	Hepatic cirrhosis	12
Lung squamous	4	Renal failure	3
Lung adeno	4	Other	3**
Lung oat cell	1	Unknown cause	7
Gastro-intestinal tract	8		
Other sites	4*		
Unknown sites	6		
Other malignancies			
Myeloma	1		
Lymphoma	1		
Melanoma	1		
Leukaemia	1		
Total	75	Total	50

*Pyriform fossa 1; pancreas 1; kidney 1; bladder 1.

**Rheumatoid arthritis 1; ovarian fibroma 1; acute pericarditis and pleurisy 1.

assay, duplicate samples of 100µl of each specimen were incubated at 37°C overnight with 50µl of PBS, 50µl of primary anti-CEA goat antiserum (PK1G (D2)) at the appropriate dilution (1 in 4,400) and 50 µl of radiolabelled CEA antigen. The next morning, 50ul of secondary horse anti-goat IgG antiserum at the appropriate dilution (1 in 40), 50µl of polyethylene glycol (PEG) and 50µl of human plasma were added. The mixed suspension was incubated for 2 - 4 hours at room temperature and then filtered using the 'Kemtek' automated radioimmunoassay system (Bagshawe, 1975), in which the degree of radioactivity in the precipitant was estimated. All the reagents used in the assay were the standard reagents used in routine CEA assay in Professor Bagshawe's Department.

7.2 Results

151 specimens of serous effusions from 125 patients were investigated. The range of the radioimmunoassay values lay between 0 to 20,000 ng/ml. 111 specimens had an assay value less than 20 ng/ml, 23 had an assay value between 20 - 100 ng/ml and 17 specimens had an assay value greater than 100 ng/ml respectively. These results were correlated with the cytological diagnoses made on the cells of the effusions and the clinical diagnoses of the patients, and are given in detail in Table 7.3 and summarised in Table 7.4. A scatter diagram showing the CEA radioimmunoassay values with the cytological examination is given in Figure 7.1.

Table 7.3

Correlation of CEA radioimmunoassay values (ng/ml), the cytological and clinical diagnoses of 151 effusions from 125 patients

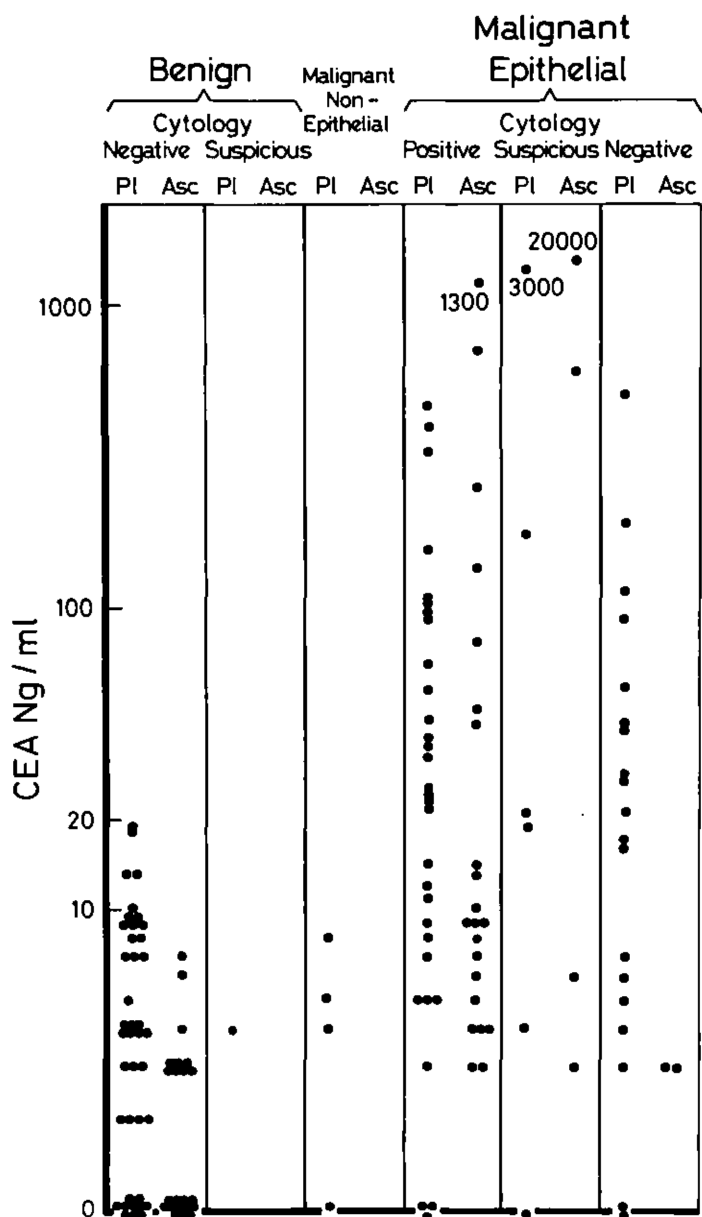
No. of effusions	Clinical Diagnoses	Cytology positive CEA levels			Cytology suspicious CEA levels			Cytology negative CEA levels		
		<20 ng/ml	20-100 ng/ml	>100 ng/ml	<20 ng/ml	20-100 ng/ml	>100 ng/ml	<20 ng/ml	20-100 ng/ml	>100 ng/ml
	Carcinoma									
43	Ca breast	16	12	1	1	1	1	4	5	2
9	Ca ovary	8	0	0	0	0	1	0	0	0
4	Ca lung squamous	1	0	1	2	0	0	0	0	0
4	Ca lung adeno.	1	1	2	0	0	0	0	0	0
1	Ca lung oat cell	1	0	0	0	0	0	0	0	0
9	Ca G-I tract	0	1	4	1	0	2	1	0	0
4	Other epithelial malignancies	1	0	1	0	0	0	2	0	0
10	Primary unknown	0	1	1	1	0	0	4	2	1
84	Total no. of effusions	28	15	10	5	1	4	11	7	3
4	Non-epithelial malignancies	1	0	0	0	0	0	3	0	0
	Non-malignant disease									
14	Heart disease	0	0	0	0	0	0	14	0	0
17	Lung disease	0	0	0	1	0	0	16	0	0
16	Liver disease	0	0	0	0	0	0	16	0	0
4	Renal disease	0	0	0	0	0	0	4	0	0
6	Others	0	0	0	0	0	0	6	0	0
6	Unknown etiology	0	0	0	0	0	0	6	0	0
63	Total no. of effusions	0	0	0	1	0	0	62	0	0

Table 7.4

Summary of CEA radioimmunoassay values (ng/ml) in 151 effusions from 125 patients

Clinical Diagnoses (No. of patients)	Cytological Diagnoses	CEA levels (ng/ml)			Total
		<20ng/ml	20-100 ng/ml	>100ng/ml	
Epithelial Malignancy (71 patients)	Malignant	28	15	10	53
	Suspicious	5	1	4	10
	Non-Malignant	11	7	3	21
					84
Non-Epithelial Malignancy (4 patients)	Malignant	1	0	0	1
	Non-Malignant	3	0	0	3
					4
Non-Malignant (50 patients)	Suspicious	1	0	0	1
	Non-Malignant	62	0	0	62
					63
	Total	111	23	17	

Figure 7.1



Correlation of CEA radioimmunoassay values (ng/ml), cytological and clinical diagnoses of 151 effusions from 125 patients.

44 of the 84 effusions from patients with epithelial malignancies had CEA assay values less than 20 ng/ml. In 23 fluids the assay values lay between 20 - 100 ng/ml and in 17 specimens the assay values were greater than 100 ng/ml. In the 44 specimens which had CEA assay values of less than 20 ng/ml, the cytological smears were reported as positive for malignancy in 28, suspicious in five and negative in 11. Among the 23 specimens which had CEA assay values between 20 - 100 ng/ml, the cytological smears were reported as positive for malignancy in 15, suspicious in one and negative in seven. Of the 17 specimens which had assay values greater than 100 ng/ml, the cytological smears were reported as positive for malignancy in ten, suspicious in four and negative in three.

The CEA assay value of the four effusions from four patients with non-epithelial malignant disease (one myeloma, one lymphoma, one melanoma and one leukaemia) were all less than 10 ng/ml. One specimen was reported as cytologically positive for malignancy and the other three were reported as negative.

The value of the CEA assays of the 63 effusions from 50 patients who had no evidence of malignant disease was less than 20 ng/ml in every case. The highest assay value was 19 ng/ml and this specimen (a pleural effusion) was from a patient with a clinical diagnosis of spinal abscess. The cytological smears from all but one of these

63 effusions was reported as negative for malignancy. This one specimen was reported as suspicious for malignancy on morphological grounds and was later found to come from a patient with pulmonary embolus. The CEA assay value of this specimen was 8 ng/ml.

7.3 Discussion

The results of this study indicated that CEA radio-immunoassay values greater than 19 ng/ml were found only in fluids from patients with epithelial malignant disease. This finding was consistent with the results obtained by Basta *et al* (1975), Leowenstein *et al* (1978), Vladutiu *et al* (1979) and Asseo and Tracopoulos (1982), who also found raised CEA levels only in effusions from patients with epithelial malignancy. In view of this finding it may be appropriate to use this test to screen patients who develop effusions of an unknown cause. As pointed out by Spriggs and Boddington (1968), effusions due to malignant disease can be of two types. In the first type, the fluid is caused directly by infiltration of malignant disease on the serosal surface. In the second type, the effusion is caused only indirectly by the presence of tumour elsewhere in the body, for example, by a lymphatic obstruction. Therefore, effusions of the second category may account for a substantial number of cytologically negative specimens from patients with metastatic malignant disease. In these cases, a high CEA assay value may

be taken as an indication of the presence of epithelial malignant disease in the patients and it would be in this group of patients that the assay could prove to be of maximum value. It must, however, be made absolutely clear that although the CEA assay can suggest the presence of epithelial malignant disease in the patient, an elevated CEA level does not necessarily indicate the presence of malignant cells in the fluids. In this respect cytology and CEA assay represent two different approaches to the diagnosis of malignancy in patients with effusions.

However, not all workers who investigated the value of CEA assay on effusions have found it to be a reliable indicator of malignancy. Nystrom _et al_ (1977), Rittgers _et al_ (1977) and Booth _et al_ (1977) indicated that, although the test may be of some value in detecting the cause of effusions, all found a small number of 'false-positive' results in effusions from patients with inflammatory conditions. Stanford _et al_ (1978) found raised CEA levels both in effusions from patients with malignant disease or with inflammatory conditions and failed to find a statistical difference between the two groups of patients with the CEA assay. In view of these conflicting reports a multi-centre trial of this test may be merited.

Other markers have been measured in effusions (see Table 2.2); none has proved to be useful for the diagnosis

of malignancy. In this research, the level of Orosomucoid (Asseo and Tracopoulos, 1981) on 135 effusions from 81 patients with malignant disease and 54 patients with benign disorder was measured. Raised levels were found both in benign and in malignant conditions. A scatter diagram of the results is shown in Figure 7.2, showing a contrast with the CEA levels presented in Figure 7.1.

7.4 Correlation with CEA staining

Among the 151 effusions used in this CEA radioimmunoassay study, there were 92 specimens on which CEA staining had been carried out (see last Chapter). 49 of these 92 specimens were obtained from 40 patients with epithelial malignancy; one was from a patient with myeloma and the remaining 42 fluids were from 33 patients with benign disease. The results of the CEA radioimmunoassay, CEA staining and the cytological examination of these 92 specimens are shown in Table 7.5.

Despite the fact that different anti-CEA sera were used for the assay and for the staining, analysis of the 49 specimens from patients with epithelial malignant disease proved to be of interest in two contrasting ways. This is shown in the scatter diagram in Figure 7.3. Six out of 10 specimens (60%, 9 cytologically positive and one negative) with strong CEA staining were found in effusions with assay values greater than 100 ng/ml. Six

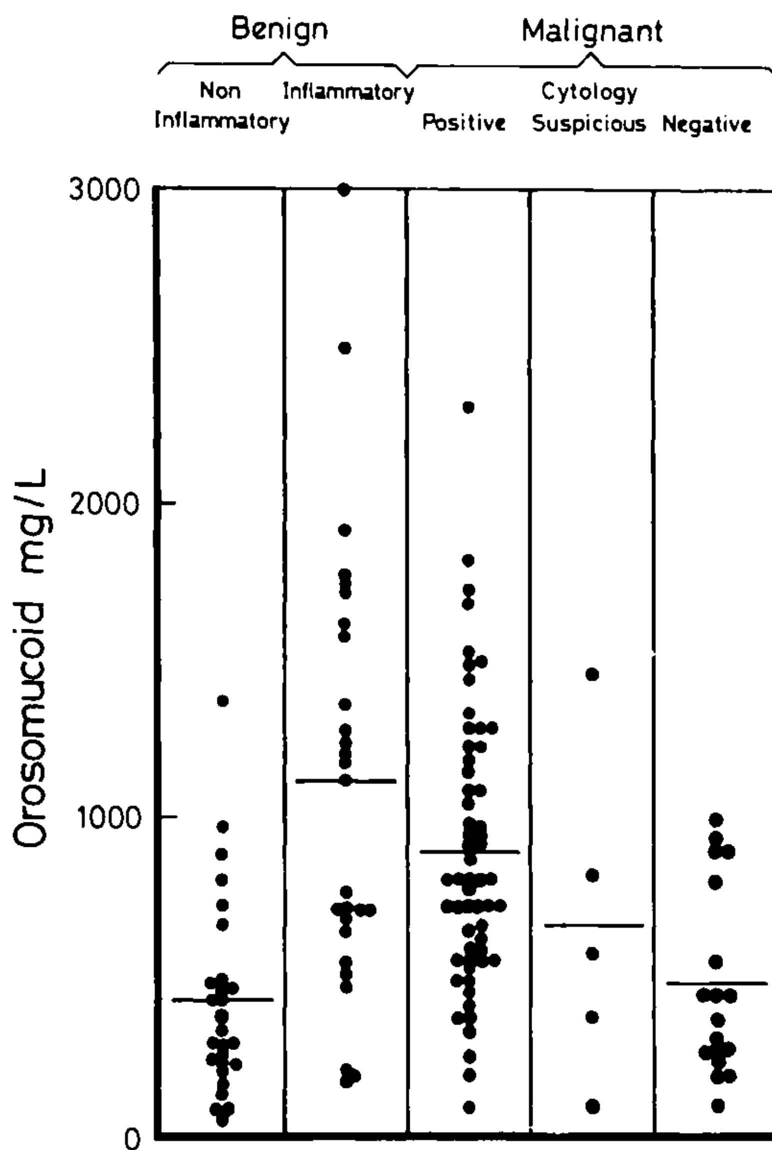
Table 7.5

Comparison of CEA staining and CEA radioimmunoassay of 92 effusions from 74 patients

Clinical Diagnoses (No. of patients)	Cytological Diagnoses	CEA Staining	CEA levels		
			<20 ng/ml	20-100 ng/ml	>100 ng/ml
Epithelial Malignant Disease (40 patients)	Positive	Strong	2	1*	6
		Weak	1	5	0
		Negative	14	3	0
	Suspicious	Strong	0	0	0
		Weak	0	0	1
		Negative	3	0	1
	Negative	Strong	0	1	0
		Weak	0	1	0
		Negative	7	3	0
Non-Epithelial Malignant Disease (1 patient)	Positive	Negative	1	0	0
Non-Malignant Disease (33 patients)	Negative	Negative	42	0	0

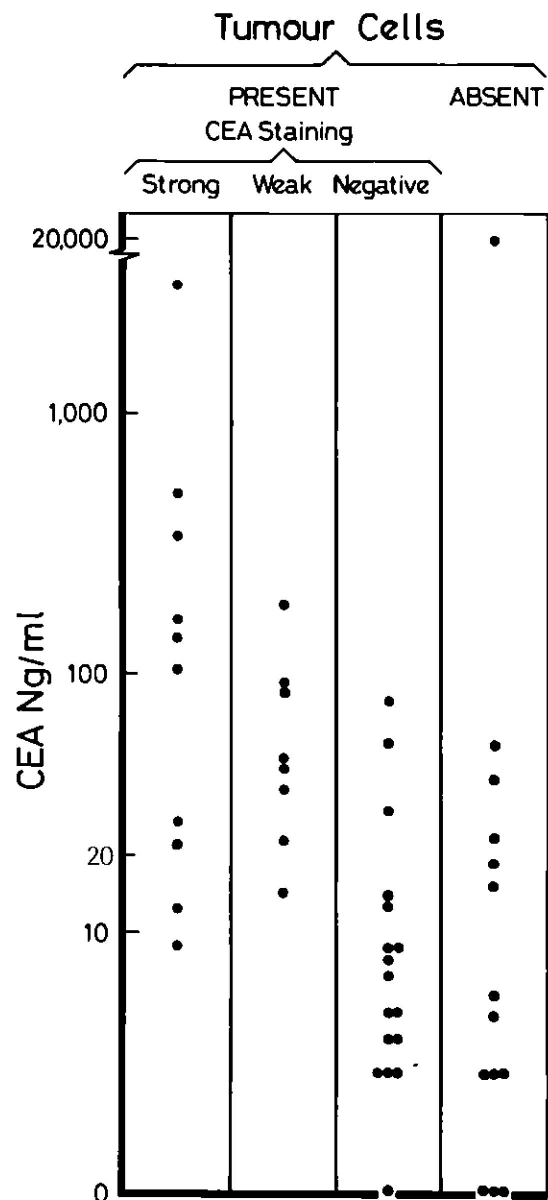
*Pericardial effusion

Figure 7.2



Correlation of Orosomucoid levels, cytological and clinical diagnoses of 135 effusions from 135 patients.

Figure 7.3



Correlation of CEA radioimmunoassay values (ng/ml) and CEA staining in 49 effusions from 40 patients with epithelial malignancy.

out of eight specimens (75%, six cytologically positive, one suspicious and one negative) with weak CEA staining were present in fluids which had assay values between 20 - 100 ng/ml. 14 out of the 17 cytologically positive specimens (82%) in which no staining was present had assay values less than 20 ng/ml. It seems therefore that the CEA staining pattern correlated well with the CEA assay values in indicating a positive relationship between active cellular CEA synthesis by the tumour cells in effusions and a high level of CEA in the fluids. However, three cytologically positive specimens with assay values between 20 - 100 ng/ml did not stain for CEA thus indicating that the levels of CEA in effusions were not merely dependent upon the CEA production by the malignant cells in the fluids. It is likely that, in effusions due to CEA-positive tumours metastasizing to the body cavity, both plasma infiltration across the serous membrane as well as local production of CEA by the tumour cells in effusions, play a role in the concentration of CEA in the fluids. This observation supported the finding made by Booth et al (1977), Stanford et al (1978) and Asseo and Tracopoulos (1982), that when CEA level was raised the higher value was always in the effusion rather than in the serum.

Chapter 8

Monoclonal antibodies against benign mesothelial cells

8.1 Immunization of mice and selection for fusion

i. Preparation of immunogen.
ii. Immunization schedule
iii. Detection of antibody activity in mice after immunization by radioactive cell binding assay.

8.2 Cell fusion and isolation of hybrids

i. Fusion procedure and cloning in soft agar.
ii. First screening for antibody activity by radioactive cell binding assay.
iii. Second screening for antibody activity by radioactive cell binding assay and by immunocytochemical staining.
iv. Recloning the hybrids by limiting dilutions.
v. Third screening for antibody activity by immunocytochemical staining.

8.3 Production and screening of ascites

i. Passaging the hybrid cells in mice to produce ascites.
ii. Screening of ascitic fluids by immunocytochemical staining.

8.4 Discussion

8.1 Immunization of mice and selection for fusion

i. Preparation of immunogen

Nine effusions containing a large number of cells, mainly of mesothelial origin, were used as the source of immunogen. The specimens were obtained from patients with benign disorders. Details of these specimens are shown in Table 8.1. The cells in the pooled samples were collected by centrifugation and the mesothelial cells were concentrated by the lymphoprep method (see Appendix 1) which removed most of the red blood cells and some of the polymorphonuclear cells. The cells were then fixed in 10% formalin for half an hour, washed twice in PBS and finally suspended in 5 mls PBS.

ii. Immunization schedule

Balb/C mice 1 and 2 were injected intraperitoneally with 1×10^6 formalin-fixed cells in 0.2 ml PBS on three occasions at approximately five-week intervals. A final boost of the same dosage was given after 15 weeks, and followed by fusion three days later.

iii. Detection of antibody activity in mice after immunization by radioactive cell binding assay

Preparation of target cells

10% formalin-fixed and unfixed mesothelial cells prepared from a pooled sample of benign effusions were used.

Table 8.1

Effusions used for the preparation of immunogen

Patients	Clinical Diagnoses	Pleural/Ascites	Percentage of mesothelial cells	Density of mesothelial cells per litre
1	Cirrhosis	A	70%	2.8×10^8
2	Lung abscess	P	90%	3.6×10^8
3	Alcoholic cirrhosis	A	80%	5.0×10^8
4	Heart failure and cirrhosis	A	80%	2.4×10^8
5	Alcoholic cirrhosis	A	95%	3.2×10^8
6	Alcoholic cirrhosis	A	100%	4.2×10^8
7	Cirrhosis	A	85%	1.3×10^8
8	Chronic cardiac failure	P	94%	1.9×10^8
9	Renal failure	A	95%	0.9×10^8

The cells in PBS suspension were plated out into individual wells of a microtitre plate at 10^5 cells/well. The plate was centrifuged at 700 g for five minutes and the supernatants removed. The cells were then washed once in 300µl of 0.5% BSA in PBS.

Preparation of test solution

A blood sample was taken from the two immunized mice. The samples were allowed to clot and the sera diluted two-fold in PBS from 1/20 to 1/1280.

Assay procedure

200µl of the test solution (in this case doubling dilutions of the sera) were added to the cells in the wells and incubated for 1½ hours at room temperature. After the incubation period the plate was centrifuged, the supernatants removed and the cells resuspended in 300µl of 0.5% BSA in PBS. This washing process was repeated three times. The cells were then incubated for another 1½ hours with 200 µl I^{125} labelled goat anti-mouse Ig, and then washed again three times in 300µl of 0.5% BSA in PBS. The supernatants were removed and individual wells were cut from the plate and placed in counting tubes. The radioactivity of the bound antibody in each well was measured using a gamma counter. The assay was carried out in duplicate for each dilution.

Control

For each microtitre plate used, a minumum of six negative controls (the blanks) were included. The full assay procedure, except for the addition of test solution, was applied to the controls.

Results

The results of this experiment are given in Table 8.2. The increase in dilution of the sera was directly related to the decrease in the bound radioactivity count on the fixed cells in each case (mouse 1 and 2). This discrimination was not observed with the unfixed cells for either mouse. Mouse 1 was chosen for the cell fusion as the antibody activity was marginally higher.

8.2 Cell fusion and isolation of hybrids

i. Fusion procedure and cloning in soft agar

The cell fusion and cloning techniques used were the established methods at the Ludwig Institute for Cancer Research. The tissue culture work involved was carried out by Miss J Pelly of the Ludwig Institute. The whole procedure was carried out under sterile conditions and the methodology is given below.

Selective medium (prepared at Ludwig Institute)

Medium 1 : Leibovitz (L15) + 10% Foetal Calf Serum (FCS) + 1.0×10^{-4}M Hypoxanthine (H) +

Table 8.2

Detection of antibody activity in immunized mice

Dilution of serum	Estimated radioactivity (Standard I^{125} = 151,921)			
	Mouse 1 fixed benign mesothelial cells (Blank = 2227)	Mouse 2 fixed benign mesothelial cells (Blank = 1620)	Mouse 1 unfixed benign mesothelial cells (Blank = 1522)	Mouse 2 unfixed benign mesothelial cells (Blank = 1398)
1:20	20616	15701	5334	7355
1:40	11901	8318	5585	6149
1:80	8709	8762	6059	7473
1:160	8873	5280	5447	7075
1:320	5667	3103	4035	5629
1:640	4039	2395	4124	6305
1:1280	4006	2152	6404	4944

4×10^{-5}M Thymidine (T) + 100µl/ml Kanamycin (K).

Medium 2 : Medium 1 with 20% Foetal Calf Serum.

Medium 3 : 5% CO_2 buffered Dulbecco's Minimal Essential Medium (DMEM) + 10% Foetal Calf Serum + 1.0×10^{-4}M Hypoxanthine + 1.0×10^{-5}M Aminopterin (A) + 4×10^{-5}M Thymidine + 100µl/ml Kanamycin.

Medium 4 : Medium 3 without Aminopterin.

Preparation of agar-coated 24-well Costar plate

The day before fusion, two 24-well Costar plates were prepared by spreading 100µl of a 0.1% solution of Agar (Difco) in distilled water over the bottom of each well. The plates were left to set overnight at room temperature.

Preparation of myeloma cells (NS1)

The mouse myeloma cell line (NS1) was maintained *in vitro* at the Ludwig Institute. Three days before the fusion, 10 mls of the cells suspended in 5% CO_2 buffered DMEM + 20% FCS + Kanamycin (100µl/ml) were plated in each well of two six-well Costar plates at a density of 10^5 cells/ml. On the day of fusion, the cell density was

estimated using a haemocytometer. The volume of medium which contained 10^7 NS1 cells was taken and centrifuged. The supernatant was discarded and the cells were resuspended in 5mls of medium 1.

Preparation of spleen cells

On the day of fusion, the spleen was removed from the immunized mouse (Mouse 1). The spleen cells were dispersed on a grid and suspended in 10mls of medium 1. The cell density was estimated using a haemocytometer. The volume of medium which contained 10^8 spleen cells was centrifuged and the supernatant discarded. The cells were resuspended in 5mls of medium 1.

Preparation of thymocytes

On the day of fusion, a thymus was removed from a mouse, the thymus cells were dispersed on a grid and suspended in 10mls of medium 1. The density of the cells was estimated using a haemocytometer. The cell suspension was centrifuged and resuspended in medium 2 so that the final volume contained 10^6 cells/ml.

Fusion

5mls of the spleen cells and 5mls of the NSI cells suspension prepared above were mixed (to give a ratio of 10 spleen cells to one NSI cell) in a sterile round bottom test tube, centrifuged and the supernatant removed. The cell

pellet was agitated by gently tapping the tube and the tube was placed in a 37°C water bath. 0.8ml of 50% polyethylene glycol 1500 (PEG, made up in Leibovitz medium) was added slowly to the cell pellet over one minute. 10mls of serum-free Leibovitz medium in 1ml aliquots were gently added over the next six minutes. After six minutes, the cells were centrifuged and resuspended in 20mls of medium 2 containing thymocytes (10^6 cells/ml). The thymocytes were used as feeder cells.

Plating and cloning in agar

18mls of the 20mls cell suspension prepared after fusion were mixed with 9mls of 0.75% agar medium (10mls of DMEM with 2% agar + 16.6mls of medium 1) to give a final concentration of 0.25% agar cell suspension. The cell suspension was plated out at 0.5ml per well in the two agar-coated 24-well Costar plates prepared the day before fusion. The plates were left to set for two hours. Each well was then overlayered with 1.5mls of medium 3 and the plates were incubated at 37°C in an atmosphere of 5% CO_2 and air.

Plating without agar

The remaining 2mls of the 20mls cell suspension prepared after fusion was distributed between four wells of a 24-well Costar plate. The four wells, each containing 0.5ml of cell suspension, were made up to 2mls with

1.5mls of medium 3 and the plate was placed in 5% CO_2/air incubator at 37°C. These four non-agar wells were prepared in case a cell colony was not obtained in the agar wells and the cells in these four wells could then be used for cloning.

Maintenance of the cultures

The day after fusion, 1ml of medium was removed from each well and replaced with 1ml of fresh medium 3. Thereafter the wells were fed every other day with 1ml of fresh medium 4 in a similar way. The supernatants in the wells were screened for antibody activity when cell colonies became visible on gross examination. The colonies appeared in the form of white morulae suspended in the agar.

ii. First screening for antibody activity by radioactive cell binding assay

The first screening for antibody activity was carried out (in duplicate) on a pooled sample of 10% formalin-fixed mesothelial cells as a suspension in PBS. The binding assay was the same as described in section 3. Approximately 10^5 cells/well were distributed in a microtitre plate. Undiluted supernatants from the culture wells were used as the primary antibody (test solution) and I^{125} labelled goat anti-mouse Ig was used

as the second antibody. The radioactivity of the bound antibody in each well was estimated using a gamma counter.

The results from the first screening of all the wells is shown in Table 8.3. Wells in which the radioactivity obtained was more than twice that of the blank, were chosen for further sub-cloning and testing. 13 wells fell into this category. All of the 13 'positive' wells were agar wells. Individual cell colonies in the agar in these 13 wells were picked out with Pasteur pipettes, and plates out separately onto the wells of a fresh 24-well Costar plate containing 0.5ml of medium 1 with 10^6 thymocytes per well. A total of 72 wells were plated. Table 8.4 shows the distribution of these 72 wells in relation to the 13 positive wells from which they were derived. Each well was topped up to 2mls with medium 4 and the plates were incubated at 37°C in an atmosphere of 5% CO_2 and air. The supernatant in each well was screened for antibody activity when the cells had formed a pellet at the bottom of the wells on gross examination.

iii. Second screening for antibody activity by radioactive cell binding assay and by immunocytochemical staining

The second screening for antibody activity was first carried out using a radioactive cell binding assay on a pooled sample of 10% formalin-fixed mesothelial cells as a suspension in PBS. The method for the assay and

Table 8.3

Results of first screening by radioactive cell binding assay

Well number	Estimated Radioactivity (Standard I^{125} = 290,396)		Well chosen for further sub-cloning and testing
	Fixed mesothelial cell suspension (Blank = 2254)	Fixed mesothelial cell suspension (Duplicates) (Blank = 1815)	
1.1	<u>5566</u>	2562	+
1.2	2352	2484	
1.3	2258	2356	
1.4	2338	2896	
1.5	2526	2470	
1.6	2690	2122	
1.7	3062	<u>4362</u>	+
1.8	2981	<u>4284</u>	+
1.9	2364	3118	
1.10	3192	<u>4526</u>	+
1.11	3196	<u>4300</u>	+
1.12	2940	2476	
1.13	3194	<u>3964</u>	+
1.14	2858	<u>4140</u>	+
1.15	2484	<u>4172</u>	+
1.16	<u>9244</u>	<u>6998</u>	+
1.17	<u>4944</u>	2804	+
1.18	2770	2750	
1.19	2618	2092	
1.20	3386	2774	
1.21	<u>4848</u>	3086	+
1.22	2940	3302	
1.23	2888	2862	
1.24	2168	2744	
1.25	2644	<u>3906</u>	+

Well number	Estimated Radioactivity (Standard I^{125} = 290,396)		Well chosen for further sub-cloning and testing
	Fixed mesothelial cell suspension (Blank = 2254)	Fixed mesothelial cell suspension (Duplicates) (Blank = 1815)	
1.26	2792	2036	
1.27	3476	1654	
1.28	1760	1786	
1.29	2720	2608	
1.30	2728	1930	
1.31	2308	2670	
1.32	1626	2322	
1.33	2422	2268	
1.34	2298	2344	
1.35	2248	2220	
1.36	2492	2768	
1.37	2404	2310	
1.38	3942	3850	+
1.39	2274	2202	
1.40	2472	2496	
1.41	2948	2000	
1.42	2688	1756	
1.43	2238	2222	
1.44	2442	2686	
1.45	2466	2822	
1.46	2250	1896	
1.47	2062	2188	
Non-agar 1	1784	2636	
Non-agar 2	2000	1652	
Non-agar 3	3722	1878	
Non-agar 4	2106	1914	

Table 8.4

Number of cell colonies picked from the 13 positive wells after the first screening

Well number from first screening	Number of individual colonies picked
1.1	7
1.7	10
1.8	2
1.10	10
1.11	4
1.13	3
1.14	9
1.15	4
1.16	7
1.17	6
1.21	4
1.25	5
1.38	1
	Total = 72

criteria for selection were the same as those described in section 8.1.iii and 8.2.ii.

The results of the second screening are shown in Table 8.5. The radioactivity of the supernatants from 13 wells was more than twice that of the blanks. Fresh medium 4 was added to these 13 wells and the cultures were allowed to grow for a further 72 hours. The supernatants were re-screened for antibody activity by immunocytochemical staining after this.

For the immunocytochemical staining, 13 identical sets of six ethanol fixed smears (2 mesothelial, 1 mesothelioma, 2 Ca breast and 1 Ca ovary) were used. The staining was carried out using the indirect immunoalkaline phosphatase technique (see Appendix 2). Undiluted supernatants from the 13 wells were used as the primary antibody (200μl per smear) and alkaline phosphatase conjugates goat anti-mouse Ig as second antibody (300μl per smear).

The results of the staining are shown in Table 8.6. Five of the 13 wells (wells 1.7/2.8, 1.10/2.22, 1.14/2.38, 1.25/2.68, 1.25/2.70) were producing antibody which stained mesothelial cells strongly. Two of these five wells (wells 1.14/2.38 and 1.25/2.70) stained mesothelial cells only. Well 1.25/2.68 stained mesothelial cells as well as some monocytes. Well 1.7/2.8 stained mesothelial cells strongly

Table 8.5

Results of second screening

by radioactive cell binding assay

Origin from first screening	Well number	Estimated Radioactivity (Standard I^{125} = 28,076)		Well chosen for further testing
		Fixed mesothelial cell suspension (Blank=10222)	Fixed mesothelial cell suspension (Duplicates) (Blank=9126)	
1.1	1.1/2.1	5444	6887	
1.1	1.1/2.2	10885	10575	
1.1	1.1/2.3	9951	9977	
1.1	1.1/2.4	13621	15427	
1.1	1.1/2.5	30203	15867	+
1.1	1.1/2.6	13250	11261	
1.1	1.1/2.7	16630	9625	
1.7	1.7/2.8	27753	21005	+
1.7	1.7/2.9	12529	12884	
1.7	1.7/2.10	15909	13313	
1.7	1.7/2.11	14934	9817	
1.7	1.7/2.12	18217	10992	
1.7	1.7/2.13	16599	15466	
1.7	1.7/2.14	4175	9332	
1.7	1.7/2.15	7152	7976	
1.7	1.7/2.16	11091	11235	
1.7	1.7/2.17	14938	10483	
1.8	1.8/2.18	18151	10201	
1.8	1.8/2.19	16530	8650	
1.10	1.10/2.20	13465	9835	
1.10	1.10/2.21	9631	7496	
1.10	1.10/2.22	29265	23520	+

Origin from first screening	Well number	Estimated Radioactivity (Standard I^{125} = 28,076)		Well chosen for further testing
		Fixed mesothelial cell suspension (Blank=10222)	Fixed mesothelial cell suspension (Duplicates) (Blank=9126)	
1.10	1.10/2.23	15536	8905	
1.10	1.10/2.24	14594	8992	
1.10	1.10/2.25	14334	7330	
1.10	1.10/2.26	7461	12500	
1.10	1.10/2.27	5337	11446	
1.10	1.10/2.28	4878	12663	
1.10	1.10/2.29	7093	9211	
1.11	1.11/2.30	15938	5997	
1.11	1.11/2.31	10394	6821	
1.11	1.11/2.32	10011	6342	
1.11	1.11/2.33	12232	6609	
1.13	1.13/2.34	16210	12039	
1.13	1.13/2.35	2490	6129	
1.13	1.13/2.36	13346	12294	
1.14	1.14/2.37	15507	9630	
1.14	1.14/2.38	23887	20180	+
1.14	1.14/2.39	9067	14809	
1.14	1.14/2.40	8060	11957	
1.14	1.14/2.41	12470	11316	
1.14	1.14/2.42	25944	19231	+
1.14	1.14/2.43	4295	10117	
1.14	1.14/2.44	9346	10217	
1.14	1.14/2.45	9886	8601	
1.15	1.15/2.46	11365	7941	
1.15	1.15/2.47	9512	9106	
1.15	1.15/2.48	10706	9904	

Origin from first screening	Well number	Estimated Radioactivity (Standard I^{125} = 28,076) Fixed mesothelial cell suspension (Blank=10222)	Fixed mesothelial cell suspension (Duplicates) (Blank=9126)	Well chosen for further testing
1.15	1.15/2.49	11108	9021	
1.16	1.16/2.50	21968	14697	+
1.16	1.16/2.51	35546	50844	+
1.16	1.16/2.52	22748	25980	+
1.16	1.16/2.53	27708	26027	+
1.16	1.16/2.54	9437	16142	
1.16	1.16/2.55	26278	27613	+
1.16	1.16/2.56	26298	17964	+
1.17	1.17/2.57	11125	10704	
1.17	1.17/2.58	10612	12222	
1.17	1.17/2.59	9336	7185	
1.17	1.17/2.60	11096	16222	
1.17	1.17/2.61	11818	10740	
1.17	1.17/2.62	12745	14970	
1.21	1.21/2.63	6435	10284	
1.21	1.21/2.64	9675	12906	
1.21	1.21/2.65	7395	10051	
1.21	1.21/2.66	10385	9950	
1.25	1.25/2.67	9634	12219	
1.25	1.25/2.68	25536	22543	+
1.25	1.25/2.69	12595	13913	
1.25	1.25/2.70	23711	21941	+
1.25	1.25/2.71	9917	8826	
1.38	1.38/2.72	6850	8015	

Table 8.6

Results of second screening by immunocytochemical staining

Origin from first screening	Origin from second screening by cell binding assay	Second screening by immunocytochemical staining						Well chosen for re-cloning and further testing
		Lympho-cytes	Polymorpho-nuclear cells	Mono-cytes	Meso-thelial cells	Malignant cells	Malignant mesothe-lioma cells	
1.1	1.1/2.5	-	-	-	-	-	-	
1.7	1.7/2.8	-	$\underline{\pm}$	-	+	$\underline{\pm}$	-	chosen
1.10	1.10/2.22	+	+	+	+	+	+	
1.14	1.14/2.38	-	-	-	+	-	-	chosen
1.14	1.14/2.42	-	-	-	-	-	-	
1.16	1.16/2.50	-	-	-	-	-	-	
1.16	1.16/2.51	-	+	-	-	-	-	
1.16	1.16/2.52	-	-	-	-	-	-	
1.16	1.16/2.53	-	-	-	-	-	-	
1.16	1.16/2.55	-	+	-	-	-	-	
1.16	1.16/2.56	-	-	-	-	-	-	
1.25	1.25/2.68	-	-	+	+	-	-	chosen
1.25	1.25/2.70	-	-	-	+	-	-	chosen

Symbols: + positive staining; ± weak staining; $\underline{\pm}$ very weak staining; - negative

and a few malignant cells and polymorphonuclear cells stained very weakly. Well 1.10/2.22 stained mesothelial cells as well as all other types of cells in all specimens. In the remaining eight of the 13 wells, two stained strongly the polymorphonuclear cells only, and no staining was observed in six.

The cells from wells 1.7/2.8, 1.14/2.38, 1.25/2.68, 1.25/2.70, which stained mesothelial cells strongly, were selected for recloning.

iv. Recloning the hybrids by limiting dilutions

The density of the cells in the four positive wells (wells 1.7/2.8, 1.14/2.38, 1.25/2.68, 1.25/2.70) selected after the second screening were estimated using a haemocytometer. Three dilutions of 100 cells/ml, 20 cells/ml and 5 cells/ml were made from each culture in 5% CO_2 buffered DMEM + 10% FCS + Kanamycin (100µl/ml) + thymocytes (10^6 cells/ml). 200µl/well of each dilution was plated into 32 wells of a 96-well microtest plate. The plates were then incubated at 37°C in an atmosphere of 5% CO_2 and air.

Approximately one week later, the plates were scored for single clones. Four clones were obtained from well 1.7/2.8, 18 from well 1.14/2.38, 11 from well 1.25/2.68 and 22 from well 1.25/2.70. A total of 55 clones were obtained. This result is shown in Table 8.7. Each clone was picked up with a Pasteur pipette and transferred into a fresh well of a 24-well Costar plate containing 0.5ml

Table 8.7

Number of clones obtained after recloning of the hybrids

Origin from first screening	Origin from second screening	Number of clones obtained after recloning
1.7	1.7/2.8	4
1.14	1.14/2.38	18
1.25	1.25/2.68	11
1.25	1.25/2.70	22
		Total 55

medium L15 + 10% FCS + Kanamycin (100µl/ml) + 2 x 10^6 thymocytes. Each well was made up to 2mls with 5% CO_2 buffered DMEM + 10% FCS + Kanamycin (100µl/ml). The plates were incubated at 37°C in an atmosphere of 5% CO_2 and air. The cultures were fed every day by removing 1ml of medium from each well and replacing with 1ml of fresh DMEM medium with 10% FCS and Kanamycin (100µl/ml). The supernatants in the 55 wells were screened for antibody activity when a pellet of cells had grown in the wells.

v. Third screening for antibody activity by immunocytochemical staining

The third screening was carried out by immunocytochemical staining using undiluted supernatants of the 55 wells obtained after the recloning process as the primary antibody. The staining procedure was the same as described in section 8.2.iii. The supernatant from each well was applied to one smear only, which contained benign mesothelial cells.

The results of the third screening are shown in Table 8.8. All smears stained negatively with the supernatant from the 51 clones derived from wells 1.14/2.38, 1.25/2.68 and 1.25/2.70. Three of the four wells (wells 1.7/2.8/3.1, 1.7/2.8/3.3, 1.7/2.8/3.4) derived from well 1.7/2.8 in the second screening stained strongly positive with mesothelial cells, no staining was observed with the remaining well. The hybrids in the three positive wells were used to

Table 8.8

Results of third screening by immunocytochemical staining

Origin from first screening	Origin from second screening	Well number third screening	Staining on mesothelial cells
1.7	1.7/2.8	1.7/2.8/3.1	+
1.7	1.7/2.8	1.7/2.8/3.2	-
1.7	1.7/2.8	1.7/2.8/3.3	+
1.7	1.7/2.8	1.7/2.8/3.4	+
1.14	1.14/2.38	1.14/2.38/3.5	-
1.14	1.14/2.38	1.14/2.38/3.6	-
1.14	1.14/2.38	1.14/2.38/3.7	-
1.14	1.14/2.38	1.14/2.38/3.8	-
1.14	1.14/2.38	1.14/2.38/3.9	-
1.14	1.14/2.38	1.14/2.38/3.10	-
1.14	1.14/2.38	1.14/2.38/3.11	-
1.14	1.14/2.38	1.14/2.38/3.12	-
1.14	1.14/2.38	1.14/2.38/3.13	-
1.14	1.14/2.38	1.14/2.38/3.14	-
1.14	1.14/2.38	1.14/2.38/3.15	-
1.14	1.14/2.38	1.14/2.38/3.16	-*
1.14	1.14/2.38	1.14/2.38/3.17	-
1.14	1.14/2.38	1.14/2.38/3.18	-
1.14	1.14/2.38	1.14/2.38/3.19	-
1.14	1.14/2.38	1.14/2.38/3.20	-
1.14	1.14/2.38	1.14/2.38/3.21	-
1.14	1.14/2.38	1.14/2.38/3.22	-
1.25	1.25/2.68	1.25/2.68/3.23	-*
1.25	1.25/2.68	1.25/2.68/3.24	-*
1.25	1.25/2.68	1.25/2.68/3.25	-*
1.25	1.25/2.68	1.25/2.68/3.26	-

Origin from first screening	Origin from second screening	Well number third screening	Staining on mesothelial cells
1.25	1.25/2.68	1.25/2.68/3.27	-
1.25	1.25/2.68	1.25/2.68/3.28	-
1.25	1.25/2.68	1.25/2.68/3.29	-
1.25	1.25/2.68	1.25/2.68/3.30	-
1.25	1.25/2.68	1.25/2.68/3.31	-
1.25	1.25/2.68	1.25/2.68/3.32	-
1.25	1.25/2.68	1.25/2.68/3.33	-
1.25	1.25/2.70	1.25/2.70/3.34	-
1.25	1.25/2.70	1.25/2.70/3.35	-
1.25	1.25/2.70	1.25/2.70/3.36	-
1.25	1.25/2.70	1.25/2.70/3.37	-
1.25	1.25/2.70	1.25/2.70/3.38	-*
1.25	1.25/2.70	1.25/2.70/3.39	-
1.25	1.25/2.70	1.25/2.70/3.40	-*
1.25	1.25/2.70	1.25/2.70/3.41	-
1.25	1.25/2.70	1.25/2.70/3.42	-
1.25	1.25/2.70	1.25/2.70/3.43	-
1.25	1.25/2.70	1.25/2.70/3.44	-
1.25	1.25/2.70	1.25/2.70/3.45	-
1.25	1.25/2.70	1.25/2.70/3.46	-
1.25	1.25/2.70	1.25/2.70/3.47	-
1.25	1.25/2.70	1.25/2.70/3.48	-
1.25	1.25/2.70	1.25/2.70/3.49	-
1.25	1.25/2.70	1.25/2.70/3.50	-*
1.25	1.25/2.70	1.25/2.70/3.51	-
1.25	1.25/2.70	1.25/2.70/3.52	-
1.25	1.25/2.70	1.25/2.70/3.53	-
1.25	1.25/2.70	1.25/2.70/3.54	-
1.25	1.25/2.70	1.25/2.70/3.55	-

Symbols: + = positive staining. - = no staining

* = positively stained cytoplasmic substances are present in some of the mesothelial cells in these specimens

produce ascites in Balb/C mice for further testing.

8.3 Production and screening of ascites

i. Passaging the hybrid cells in mice to produce ascites

Three Balb/C mice were injected intraperitoneally with 0.2ml Pristane (2, 6, 10, 14 - tetramethylpentadecane) at least 24 hours before use. The hybrid cells from wells 1.7/2.8/3.1, 1.7/2.8/3.3 and 1.7/2.8/3.4 were prepared as individual cell suspensions in PBS. Respectively 10^7 hybrid cells from the three wells were injected intraperitoneally into the three mice and cells were allowed to grow as ascitic tumour cells. When the mice became sick, the ascitic fluid was aspirated, the cells were centrifuged and the supernatants were frozen at -40°C until used.

ii. Screening of ascitic fluid by immunocytochemical staining

Preliminary staining

The optimal dilutions of the three ascitic fluids used in the screening procedure were determined by staining one smear containing mesothelial cells and one containing malignant cells with various dilutions of the ascites. The ascitic fluids were diluted from 1 in 5 to 1 in 3000. It was found that at low dilutions, both

mesothelial and malignant cells were stained. At higher dilutions the staining on the mesothelial cells was retained while that on the malignant cells diminished. The optimal results were obtained at a dilution of 1 in 1500 for ascites 1.7/2.8/3.1 and 1.7/2.8/3.3 and at 1 in 500 for ascites 1.7/2.8/3.4. These dilutions were used for subsequent screening procedure.

Screening procedures

Identical sets of eight ethanol-fixed (4 mesothelial, one Ca lung, one Ca ovary, one mesothelioma and one myeloma) smears were prepared for this test. They were stained by the indirect immunoalkaline phosphatase technique using the three ascitic fluids at the appropriate dilutions. The results are shown in Table 8.9. All three ascites stained both mesothelial cells and malignant cells. As the staining patterns obtained with the three ascites were very similar, a more extensive study on a variety of benign and malignant specimens was carried out with one of the ascites (1.7/2.8/3.1) only.

95%-ethanol fixed smears were prepared from 13 different effusions containing mesothelial cells and 13 fluids containing malignant cells from a variety of primary sites. These smears were stained with ascites 1.7/2.8/3.1 by the indirect immunoalkaline phosphatase technique. The results are shown in Table 8.10. All of the smears containing mesothelial cells were stained

Table 8.9

Screening of the ascites by immunocytochemical staining

Clinical Diagnoses	Ascites 1.7/2.8/3.1 (Dilution 1 in 1500)		Ascites 1.7/2.8/3.3 (Dilution 1 in 1500)		Ascites 1.7/2.8/3.4 (Dilution 1 in 500)	
	Mesothelial cells	Malignant cells	Mesothelial cells	Malignant cells	Mesothelial cells	Malignant cells
Pneumonia	++		++		++	
Alcoholic cirrhosis	-		+		-	
Alcoholic cirrhosis	++		++		+	
Alcoholic cirrhosis	+++		+++		+++	
Ca Lung		++		++		++
Ca Ovary	++	±	++	±	++	±
Mesothelioma	-	-	-	-	-	-
Myeloma		++		++		+

Symbols: +++ very strong; ++ strong; + weak; - negative

Table 8.10

Further screening of the ascites 1.7/2.8/3.1 by immunocytochemical staining

Clinical Diagnoses	Ascites 1.7/2.8/3.1 (Dilution 1 in 1500)			
	Malignant cells	Mesothelial cells	White blood cells	Red blood cells
Pulmonary embolus		++	lym ++; poly ++	
Pulmonary embolus		++	lym ++; poly ++	+++
Pneumonia		++		++
Pneumonia		++		
Cardiac failure		+		
Cardiac failure		+		++
Cardiac failure		±		
Alcoholic cirrhosis		+++		
Alcoholic cirrhosis		+		++
Alcoholic cirrhosis		+++		
Alcoholic cirrhosis		++		
Renal failure		±		
Acute pericarditis and pleurisy		++		
Ca breast	±		lym +	
Ca breast	-		lym +; poly +	±
Ca lung	+++			
Sq Ca lung	++	++		
Oat cell Ca	++	++		
Ca colon	++	++		
Ca colon	++	+±		++
Ca pancreas	+±	+±	monocytes +++	
Ca ovary	±	++		
Ca unknown site	+	++		
Mesothelioma	++			
Mesothelioma	±			
Myeloma	++			

Symbols: +++ very strong; ++ strong; + weak; - negative

as were all smears containing malignant cells. In addition some staining of lymphocytes, polymorphonuclear cells and red blood cells was noted in some specimens.

8.4 Discussion

The success of the hybridoma technique for the production of a wanted monoclonal antibody depends to a large extent on the immunogenicity of the antigenic determinants of interest. There has been no previous research in this field and it was difficult to decide upon the most effective form of immunogen to use, which would stimulate the maximum production of wanted antibody-forming plasma cells. The selection of immunogen had to be decided mainly on theoretical grounds and four aspects were considered:

1) whether to use whole cells or cell fragments;
2) whether to use cultured or non-cultured cells;
3) whether to use fixed or unfixed cells;
4) if fixed cells were used, which fixation method was appropriate.

Whole cells, or cell fragments

The object of this research was to obtain monoclonal antibodies against component(s) of non-neoplastic mesothelial cells not present in malignant cells. Human cell membrane alone can be fragmented into over a hundred components. Although in theory it may be possible to identify

cell fragment(s) from mesothelial cells not shared by malignant cells, in practice this would involve a systematic long-term programme of research in itself. For this reason, whole mesothelial cells were used as immunogen. On the other hand, however ,it was realised that this approach would induce a very heterogenous population of antibody-forming plasma cells and would consequently reduce the amount of plasma cells producing antibody of interest. This would especially be a serious drawback if the antigenic determinant(s) of interest is of weak immunogenicity.

Cultured or non-cultured cells

One of the problems anticipated was that vast amounts of mesothelial cells would be needed both for immunization and for screening. The use of cultured mesothelial cells therefore represented a possible solution to this problem. However, the advantage of this approach was offset by the possibility that the antigenic status of mesothelial cells obtained *in vitro* may not correspond to those *in vivo*. Consequently mesothelial cells from a number of serous effusions were pooled.

Fixed or unfixed cells

The object was to obtain specific monoclonal antibody against non-neoplastic mesothelial cells to be used on fixed

cytological smears. Although live mesothelial cells (as the immunogen) might have caused a more rigorous immunological response in the host, it was also essential to obtain monoclonal antibody against the antigenic site(s) that could survive cell fixation. For this reason, fixed mesothelial cells were used as it was thought that this approach might increase the likelihood of obtaining such hybridomas.

Method of fixation

The choice of fixative presented considerable problems. The selection of 10% formalin as the fixative of choice was based on our previous experience with immunocytochemical staining of cytological smears for EMA and CEA. In Chapter 4, it was found that slightly stronger staining was obtained after formalin fixation of the cells than after alcohol fixation, indicating that the antigens were preserved better by the former method. It was noted that the morphology of the cells was poorly displayed after formalin fixation, but since the morphological preservation of the cells was not important for the hybridoma experiment (as it was for the immunocytochemical studies described in Chapter 4), formalin was selected as the method of fixation.

A very important factor in the successful application of the hybridoma technique is the effectiveness and efficiency of the screening system. In this research, the first and second screening for antibody activity were carried out

using a radioactive cell binding assay. This technique measured the amount of antibody bound to the pooled mesothelial cell mixture which was used as the target. In this respect, the specificity of the test was limited in that it did not discriminate between mesothelial cells and various blood cells which were invariably present in the pooled sample. Therefore, although the binding assay can detect antibody activity, the specificity of such activity cannot be assessed. For this reason, a decision was made after the second screening by binding assay to rescreen the wells for antibody specificity by immunocytochemical staining on fixed smears. By this approach it was possible to visualise exactly which cells were stained. However, when this cytological approach was used, it was noted that six of the 13 positive wells previously selected by the binding assay in the second screening failed to show any staining at all. There could be several reasons for this discrepancy. It was possible that the ten minute 20% acetic acid treatment used in the staining process destroyed the antigenic site(s) against which the antibodies in the supernatants were directed. Alternatively, discrepancy could arise because the assay was carried out on 10% formalin-fixed cells, whereas the staining was performed on 95% ethanol-fixed smears. Another possible explanation could lie in the fact that a few days passed between the assay and the staining. During that time it was likely that the wanted antibody-forming clones were overgrown by

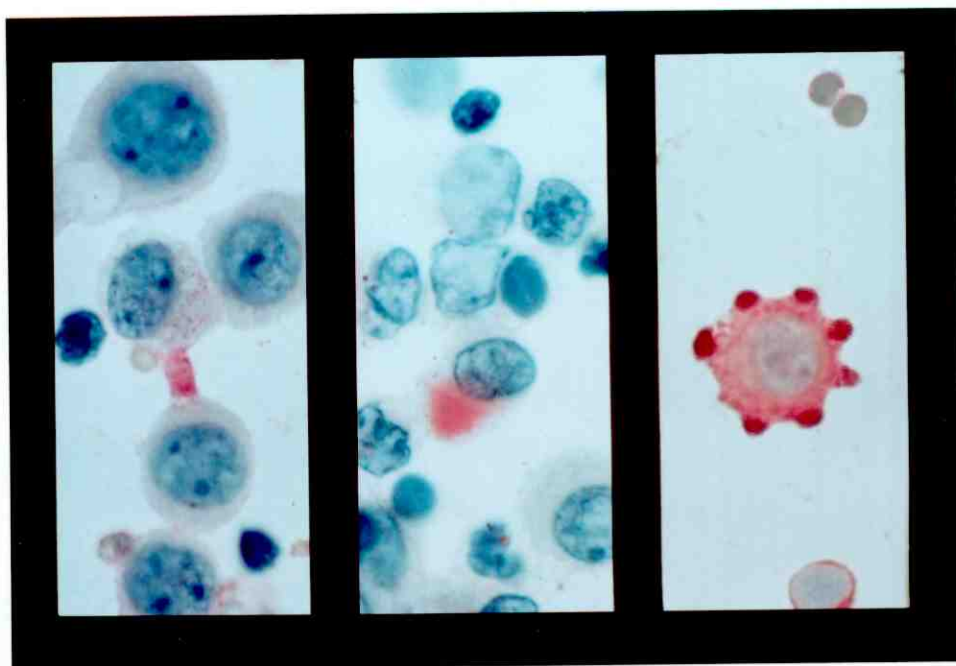
contaminating non-antibody producing cells or the wanted clones themselves were unstable and lost the ability to produce antibody as a result of somatic mutation(s) or chromosomal loss. Lastly, the supernatants may not contain sufficient antibody to be detected.

These findings emphasized three points. First, the screening system for the production and specificity of the antibodies by the hybrids, should be tested throughout the whole operation, by the method which is going to be utilised ultimately. Thus, immunocytochemical staining on fixed smears would be the screening method of choice. Second, wanted antibody-producing clones should be recloned as soon as possible. In retrospect, the hybrids in this research should have been recloned by the limiting dilution technique immediately after the binding assay in the second screening. As several clones could have been present in each positive well, early recloning was of prime importance. Third, for the clones in which the antibody activity was retained after the recloning process (clone numbers 1.7/2.8/3.1, 1.7/2.8/3.3, 1.7/2.8/3.4), a more extensive screening by immunocytochemical staining may have been beneficial before proceeding to ascites production. A full cytological screen of mesothelial and malignant cells may have been appropriate at this stage.

Although a specific monoclonal antibody against non-neoplastic mesothelial cells was not obtained, a point of

considerable interest was the pattern of immunocytochemical staining observed with the supernatants at various stages of cloning. In section 8.2.iii, it was noted that well 1.7/2.8 stained intercellular 'bridges' between benign mesothelial cells of a benign specimen (see Figure 8.1). In section 8.2.v, supernatants from seven of the 51 wells derived from well 1.14/2.38, 1.25/2.68 and 1.25/2.70 stained mucin-like substances in the cytoplasm of some mesothelial cells (see Figure 8.1). Whether or not this indicated inclusion bodies (by phagocytosis) or cellular substances already present in the cytoplasm of the mesothelial cells was unknown. In section 8.3.ii, surface 'capping' was observed on a few mesothelial cells from a benign effusion stained by ascite 1.7/2.8/3.1 (see Figure 8.1). These cellular features may be of general interest to the study of cells in effusions.

Figure 8.1



Left - Indirect immunoalkaline phosphatase staining of an intercellular 'bridge' between mesothelial cells of a benign effusion by supernatant from well 1.7/2.8 in the second screening. (X320)

Centre - Indirect immunoalkaline phosphatase staining of a mucin-like substance in the cytoplasm of a mesothelial cell of a benign effusion by supernatant from well 1.25/2.68/3.25 in the third screening. (X320)

Right - Indirect immunoalkaline phosphatase staining of surface 'capping' on a mesothelial cell from a benign effusion by ascite 1.7/2.8/3.1. (X320)

Chapter 9

General discussion and conclusion

9.1 Resumé of the significant conclusions from this research

Diagnostic cytology is based upon the subjective interpretation of the morphology of exfoliated cells. Its accuracy depends almost entirely upon the personal experience and skill of the individual cytologist, rather than on any objective criterion. The aim of this research was to try and develop an objective method for identifying malignant cells in effusions.

For this purpose, the immunocytochemical distribution of two markers was studied. The demonstration of CEA on the cells in effusions was shown to be specific for malignant epithelial cells, although the sensitivity of detection was low. The usefulness of the EMA staining was limited by the few false-positive results obtained. Nevertheless, in conjunction with the clinical data, the demonstration of EMA as well as CEA on the cells in effusions can be of value in characterizing cells for which a definitive diagnosis cannot be made on morphological grounds. Furthermore, the development of the method of restaining the cells which have been previously stained for a marker, by the Papanicolaou method, was particularly useful for retrospective morphological re-evaluation. In this way it was possible to identify discrete malignant cells that had been missed in routine screening.

The CEA assay on effusions was found to be of maximum value in patients in whom there was no evidence of malignancy either on clinical or on cytological grounds. Consequently, an elevated CEA level (> 19ng/ml) provided evidence of epithelial malignant disease in the patients under investigation. Although the assay represents a different approach to that of identifying malignant cells in effusions, the information is indirectly complementary to the cytological approach.

The attempt to raise monoclonal antibodies to non-neoplastic mesothelial cells was unsuccessful. The monoclonal antibodies obtained were not specific for these cells and also stained malignant cells. However, in the light of experience there are good grounds for repeating the attempt, with special emphasis being placed on the screening system. Immunocytochemical staining on a variety of smears containing malignant and mesothelial cells must be the screening method of choice in future to identify and substantiate the specificity of any monoclonal antibodies of interest.

9.2 The place of this research in relation to other immunocytochemical studies

Very little research into the application of immunocytochemistry to the field of cytology has been reported (Nadji, 1980). A similar study to this research was reported by O'Brien *et al* (1980), who investigated the

immunocytochemical localization of EMA, CEA and the Zinc Glycinate Marker (ZGM) on cell blocks of serous effusions; the levels of CEA in the fluids were also measured. The results of CEA staining and assay in the study of O'Brien *et al* are in agreement with those of this research. However, the cell block technique is a relatively insensitive method of diagnosing malignant cells in effusions compared with the smear preparation. Moreover, the determination of the dilution for the anti-EMA serum was not defined in the study of O'Brien *et al*. This could explain the total lack of specificity of the EMA staining in their study, compared to the present work in which EMA staining was comparatively more specific.

In this research EMA staining could not discriminate reactive mesothelial cells from malignant epithelial cells in every case. In contrast, Dearnaley *et al* (1981) showed the EMA staining was specific for malignant cells in smears of bone marrow aspirates. The explanation for this discrepancy between the two studies lies in the fact that in this study of effusions, EMA, a marker for epithelial differentiation, was used to discriminate between malignant epithelial cells and mesothelial cells, which may be EMA positive. This discrimination was based upon the quantitative difference in the expression of EMA. In the bone marrow study, the same marker was used to pick out epithelial cells among non-epithelial cells which are EMA negative. This distinction was established upon a qualitative basis. Therefore, the use of

EMA in these two studies illustrates one important point, which is that the value of a particular marker is not only dependent upon the specificity of the marker itself, but is also related to the type of specimens in which it is used.

This selective approach in choosing the correct marker for appropriate specimens has resulted in a number of useful applications with clinical significance. Wahlström *et al* (1979) showed that the immunocytochemical staining of CEA can be used to assist the differential diagnosis between endocervical and endometrial adenocarcinoma. The results of that study demonstrated that 86 out of 107 cases of endocervical adenocarcinoma investigated were CEA positive, whereas 112 cases of endometrial adenocarcinoma were all CEA negative. In another study of cervical cancer, Van Nagell *et al* (1979) showed that immunocytochemical staining of CEA could be used to discriminate between invasive carcinoma of the cervix and the normal cervix, which was CEA negative. In a study by Wang *et al* (1979), it was shown that immunocytochemical staining of CEA could be used to discriminate between mesothelioma and other lung cancers. Positive CEA staining indicated that the tumour cells in question were of bronchial epithelial origin.

9.3 Re-definition of the problems in this research

The prime object of the cytodiagnosis of effusions is

to determine the presence of malignant cells in the fluids. While the presence of malignant cells in effusions indicates the presence of malignant disease in the patients, the absence of malignant cells does not exclude the presence of malignancy. Consequently, although a test such as the CEA assay on effusions may indicate the presence of malignant disease in the patients, (and in this respect it is useful), it does not achieve the cytological objective.

This distinction between the presence of malignant cells in effusions and the presence of malignant disease in the patients presenting with an effusion has its clinical importance in patient management. For example, in cases in which a patient with a localised lung tumour and with an inflammatory effusion resulting from the obstruction and collapse of a lobe, surgical removal of the tumour mass may be the procedure of choice. However, if the lung tumour has metastasised to the pleural cavity, surgical intervention is non-contributory. The discrimination between the two situations can be difficult in clinical practice and this is precisely the area in which cytological examination of effusions represents a simple, quick and cost-effective investigative procedure. Accurate diagnosis of the presence of malignant cells in the effusion can provide the surgeon with the exact information upon which the correct decision can be made.

The basic problem in the search for a marker that is

present on various malignant cells and not on mesothelial cells is that at present there is no such a marker available which possesses both the specificity and sensitivity required for the purpose. At the time of the submission of this thesis, a new marker for human cancer cells designated the Ca antigen, and the Ca1 monoclonal antibody were reported by Ashali _et al_ (1982) and by McGee _et al_ (1982) which may have the necessary properties. In effusions, malignant cells are metastasing from numerous sites and it is difficult to find a 'cancer basic marker' which can fulfill both the specificity and sensitivity required. When the primary site is known and a marker is available for the tumour, the cells in effusions may be tested for the presence of the marker. However, until a marker becomes available which can be used to detect most types of malignant cells in effusions, the development of monoclonal antibody against non-neoplastic mesothelial cells represents a practical alternative approach.

9.4 Approach for future

It is generally believed, that the differentiation of a particular cell type, whether normal or malignant, may be associated with the expression of a particular substance(s) in the cell membrane. These markers may be organ-specific, tissue-specific or histogenetically stage-specific (The Lancet, 1982). In the past, these markers have been difficult to define due to the lack of appropriate techniques.

For example, a conventional antiserum raised against non-neoplastic mesothelial cells will contain a heterogenous population of antibodies against numerous components on the cell membrane. Some of these antibodies may be specific for mesothelial cells but the techniques for purifying these antibodies are lacking. The development of the hybridoma technique provides the opportunity for monoclonal antibodies to be raised to these previously unrecognised antigens.

It is possible that in the mesothelium, stage-specific (non-neoplastic mesothelium or mesothelioma) substance(s) exist and may be detected by the use of monoclonal antibodies. These monoclonal antibodies, if successfully generated, could be used for the diagnosis of serous effusions but the success of this approach lies in the sensitivity of the antibodies obtained. Not only do the antibodies have to be specific for mesothelial cells, they must also stain all the mesothelial cells in any one particular benign specimen for a truly negative diagnosis to be established. This would be especially the case with specimens in which the cells are characterised by isonucleosis. However, this problem of absolute sensitivity within a specimen might be resolved by mixing monoclonal antibodies to different antigenic determinants specific for the mesothelium.

Thus the limitations of the approach of raising monoclonal antibodies to non-neoplastic mesothelial cells lie in the fact that it requires a 100% sensitivity within a specimen and can only detect truly-negative cases.

Consequently the tumour marker approach remains the approach of choice. Provided the marker used is specific for malignant cells in effusions, the demonstration of the marker on some of the cells in a specimen would be sufficient for a positive diagnosis to be made. However, in this tumour marker approach, although a quantitative difference in the expression of a particular marker may be sufficient for the discrimination between mesothelial and malignant cells to be made, a qualitative difference is clearly preferred. A number of cytoplasmic and cell membrane markers should be tested, for example, the various isozymes of alkaline phosphatase (Benham et al, 1981) or various monoclonal antibodies to different antigens that have been reported (Herlyn et al, 1979; Colcher et al, 1981; Cuttitta et al, 1981). The aim is to have a panel of markers so that the malignant cells in effusions may be identified and typed. Moreover, the overall sensitivity of the tumour marker approach in detecting malignant cells in effusions may be improved by mixing a 'cocktail' of antibodies each specific for different malignant cells and not reactive with the mesothelial cells. In conclusion, it seems that the combined use of anti-mesothelium monoclonal antibodies and tumour marker studies would be the correct approach for the future.

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Appendix 1

Smear preparation methods

Method for preparation of the cytological smears

1. Centrifuge effusion at 300 g for five minutes in a plastic centrifuge tube.

2. Remove the supernatant and examine the deposit.

3. If the deposit is blood-stained, remove the red blood cells either by the 'Capillary/Buffy Coat' method or by the 'Lymphoprep' gradient technique (see below).

4. If the deposit is free from blood, resuspend in 20mls of phosphate buffered saline (PBS, pH 7.2) and centrifuge again. Repeat this washing process.

5. Remove the supernatant. Prepare smears from deposit by placing a drop on a grease-free slide and smear with a second slide as for a blood film. Fix immediately in 95% alcohol for a minimum of five minutes.

6. Store in alcohol or spray smear with carbowax fixative and store at -20°C.

'Capillary/Buffy Coat' technique for blood-stained effusions (Fig. 4.1)

1. Wash the centrifuged deposit with 20mls of PBS,

recentrifuge. Repeat this washing process.

2. Remove the supernatant and double the volume of deposit with PBS. The aim is to achieve a sufficiently heavy suspension of cells to provide a good buffy coat but at the same time sufficiently diluted to allow free movement within the capillary tubes during centrifugation.

3. Draw the cell suspensions into the capillary tubes (1 capillary tube per smear) by pipetting or tilting and seal one end of the tube with Cristaseal (Hawksley & Sons Ltd.).

4. Place the capillary tubes on rubber cushions in a test tube and centrifuge at 700 g for ten minutes.

5. Examine the tubes against the light and identify the buffy coat. Carefully cut the tubes with a diamond pencil at the interface between the buffy coat and the packed red blood cells.

6. Dot the buffy coat on a grease-free slide and smear.

7. Fix immediately in 95% alcohol for a minimum of ten minutes.

'Lymphoprep' gradient technique for blood-stained effusions (Fig. 4.2)

1. Wash the centrifuged deposit with 20mls of PBS, recentrifuge. Remove the supernatant.

2. Mix the centrifuged deposit with 5mls of PBS.

3. Layer the cell suspension onto 10ml of 'Lymphoprep' (Nyegaard ' Co. A/S, Oslo).

4. Centrifuge at 300 g for 20 minutes.

5. Remove the nucleated cells at the top of the medium with a Pasteur pipette and transfer to a clean 25ml plastic centrifuge tube.

6. Centrifuge at 300 g for five minutes and remove the supernatant.

7. Wash the centrifuged deposit with 20mls of PBS, recentrifuge.

8. Follow steps 5 and 6 in the 'Preparation of the Smear' section.

Appendix 2

Immunocytochemical staining methods

Indirect immunoalkaline phosphatase staining procedure

1. Immerse smears in 20% acetic acid for ten minutes to block endogenous alkaline phosphatase activity.

2. Wash well in tap water then in PBS. Tip off all PBS.

3. Flood the slides with 300µl of primary rabbit antiserum appropriately diluted in 5% non-immune goat serum in PBS.

4. Incubate in a moist chamber for 1½ hours at room temperature.

5. Wash with 0.5% bovine serum albumin (BSA) in PBS, followed by PBS containing 2 drops of detergent (Tween 80), then PBS.

6. Tip off all PBS and flood the slides with 300µl of secondary alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma) appropriately diluted in 5% non-immune goat serum in PBS.

7. Repeat steps 4 and 5, then wash in distilled water.

8. Prepare the chromogenic substrates as follows:

   Solution A: Mix 10ml of Veronal Acetate Buffer (pH 9.2) with 5mg of Brentamine Fast Red T R (Sigma). Filter.

Solution B: Suspend 5mg of Napthol AS:B1 phosphoric acid sodium salt (Sigma) in a few drops of Dimethyl Formamide just before use.

9. Mix solutions A and B just before use, and put 1ml onto each slide. Leave at room temperature.

10. Rinse in distilled water, then wash in tap water.

11. Counterstain with Mayer's Haemalum for 10 to 30 minutes and blue in saturated lithium carbonate solution, wash in tap water.

12. Mount in Glycerin Jelly.

13. If the slide is to be kept permanently the cover slip should be ringed with nail varnish or sealant when dry.

14. Result: sites of immunocytochemical activity - red, nuclei - blue.

Indirect immunoperoxidase staining procedure

1. To bleach acid haematin, immerse smears in 7.5% H_2O_2 in distilled water for five minutes, then wash well in tap water.

2. To block endogenous peroxidase activity, immerse smears in
   (i) 2.28% (0.1M) periodic acid in distilled water for five minutes, wash well in tap water; followed by

(ii) 0.02% fresh sodium borohydride in distilled water for two minutes, wash well in tap water.

3. Flood the slide with 300µl of primary rabbit antiserum appropriately diluted in 5% non-immune goat serum in PBS.

4. Incubate in a moist chamber for 1½ hours at room temperature.

5. Wash with 0.5% bovine serum albumin (BSA) in PBS, followed by PBS containing two drops of detergent (Tween 80), then PBS.

6. Tip off all PBS and flood the slides with 300µl of secondary peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma) appropriately diluted in 5% non-immune goat serum in PBS.

7. Repeat steps 4 and 5, then wash in distilled water.

8. Immerse smears in a freshly prepared chromogenic substrate solution (100mls PBS, 50mg Diaminobenzidine, 100µl 30% H_2O_2) for five minutes.

9. Counterstain with Mayer's Haemalum for ten to thirty minutes and blue in saturated lithium carbonate solution, wash in tap water.

10. Dehydrate, clear, and mount in DPX (Gurr).

11. Result: site of immunocytochemical activity - dark brown, nuclei - blue.

Appendix 3

Cytological staining methods

Papanicolaou stain (modified) for 95% ethanol fixed smears

1. ½ minute in 95% alcohol.
2. ½ minute in 70% alcohol.
3. ½ minute in distilled water.
4. 5 minutes in Harris' Haematoxylin.
5. 1 minute in tap water.
6. 10 seconds in acid alcohol.
7. ½ minute in tap water.
8. ½ minute in tap water.
9. ½ minute in Scott's Tap Water Substitute.
10. ½ minute in tap water.
11. ½ minute in 70% alcohol.
12. ½ minute in 95% alcohol.
13. 1 minute in O.G.6.
14. ½ minute in 95% alcohol.
15. ½ minute in 95% alcohol.
16. 2 minutes in E.A. 50.
17. 3 changes in absolute alcohol (½ minute each).
18. 3 changes in Xylene (or CNP 30) (½ minute each).
19. Mount in DPX.

Giemsa staining technique for air-dried smears

1. Fix in methanol for 10 minutes.

2. Place slide in staining solution (12 mls Giemsa stain + 48 mls Sorensen's buffer, pH6.8) for 20 minutes.

3. Differentiate in methanol for 3 seconds.

4. Place slide in Sorensen's buffer (pH 6.8) for 2 minutes.

5. Remove slide and allow to dry.

6. Mount in DPX.

<u>Periodic Acid Schiff-Diastase technique for 95% ethanol fixed smears (positive control:histological sections of liver and small intestine for glycogen and mucins respectively)</u>

1. Cover the slide with diastase (Sigma) solution for five minutes (1mg diastase in 10 mls distilled water), wash in tap water.

2. Treat with 1% periodic acid for five minutes, wash in tap water for five minutes then rinse in distilled water.

3. Place slide in Schiff's reagent (Raymond Lamb) for five to 15 minutes, rinse in distilled water then wash in tap water.

4. Counterstain in haematoxylin for three minutes, wash in tap water, differentiate in acid alcohol for a few seconds, wash again in tap water.

5. Dehydrate, clear, and mount in DPX (Gurr).

6. Site of mucins - red, nuclei - blue.