New marker for mesothelioma: an immunoperoxidase study

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SUMMARY An antibody was raised against a protein isolated from the cytoplasm of mesothelioma cells. It was subsequently used in an immunoperoxidase procedure on formalin fixed, paraffin embedded tissue sections. A representative sample of benign and malignant tumours from all the systems of the human body was examined. All the tumours derived from coelomic surfaces (mesotheliomas of pleura, peritoneum, and ovary, and adenomatoid tumours of epididymis) reacted with the antibody. No other tumour tested in this study expressed the protein. These findings indicate that the antibody may be useful in the identification of mesothelioma cells in both histological and cytological diagnostic routine practice when morphological interpretation is in doubt.

Several cell markers have been claimed to be of value in the differential diagnosis between mesothelioma and adenocarcinoma affecting serosal surfaces. Among these are carcinoembryonic antigen (CEA), keratin, factor VIII, secretory component, milk fat globule related antigen (HMFG), and a specific antimesothelial cell serum.

We recently reported a protein found in the cytoplasm of reactive and malignant mesothelial cells. This protein has a high content of glutamic acid, serine, and glycine, and a molecular weight of 200,000.

In our preliminary immunohistological tests with a polyclonal antibody against this protein only mesotheliomas of epithelial type (epithelial mesodermomomas according to our classification), adenomatoid tumours of epididymis expressed the protein, but a restricted sample of other benign and malignant human tumours did not.

In this study we provide a broader report on isolation and purification procedures of the protein; and we present further results on our studies, aimed at detecting the protein in sections of routinely processed formalin fixed, and paraffin embedded human tumour tissues using an immunohistochemical procedure.

Material and methods

ANTIGEN ISOLATION AND PURIFICATION Specimens of serous effusions of various aetiology (transudates, benign exudates, malignant mesotheliomas, and metastatic carcinomas) were collected without adding anticoagulant and immediately stored at −30°C until purification.

First step
Centrifugation Specimens were thawed at 4°C and appeared slightly cloudy with differing yellowish tonalities. Two hundred and fifty ml of each of the fluids was centrifuged at 9000 g for one hour at 4°C, using a Sorval centrifuge. The precipitate was discarded and the clear supernatant collected.
Ultrafiltration The Amicon filtering system consisted of a fluid reservoir (five litres), coupled with a cell equipped with a magnetic stirrer, and a membrane for separating the liquid phase from solutes with molecular weight lower than 10,000 (Amicon membrane UM 10).

The retained supernatant, which contained a large fraction of solutes with molecular weight higher than 10,000, was concentrated to make a final volume of 4 ml by ultrafiltration with nitrogen gas (4 atoms) for 96 hours at 4°C. The dry weight of this material corresponded to 200–250 mg of lyophilised substances.

Second step
Gel filtration Fluid collected from ultrafiltration
was centrifuged at 40,000 g for 30 minutes at 4°C. The sparse precipitate was discarded. The fluid was completely filtered on a Sephadex G-100 fine column (Pharmacia, Uppsala, Sweden). This type of Sephadex meant that all the substances with molecular weights lower than 100,000 could be excluded from further purification, as the protein under study had a molecular weight higher than 100,000, according to our previous analysis by ultracentrifugation. Moreover, this technique allowed us to obtain a higher yield of the purified protein of about 2% of the entire protein amount (200 mg) processed by electrophoresis. The proteins were fractionated on a 15 × 800 mm Sephadex column, using 0.1M phosphate buffer, pH 7-0, as the eluent. Eluting velocity was 14 ml/hour, and the entire elution took 24 hours. Forty fractions, 8 ml each, were collected (TC 80 fraction collector, Gilson Electrophoresis). The column had been previously set using proteins of known molecular weight (β-galactosidase 116 000 mw; bovine serum albumin 66 200 mw; vegetable peroxidase 40 000 mw). The void volume was collected to obtain fractions of the native protein under study for characterisation. The void volume of the column contained a pool of proteins with molecular weights higher than 100,000. The peak was divided in two separate fractions that were then collected, concentrated by ultrafiltration, and finally analysed by electrophoresis to establish the native protein content. This procedure was devised to draw fractions with a lower impurity level and a higher enrichment of the protein under study.

Dialysis The final volume of about 2 ml was dialysed on a dialysis tube against distilled water for 24 hours, with two changes of the dialysing fluid to remove phosphate buffer before the proteins were lyophilised. As these proteins are stable when lyophilised they could be stored at 4°C before the preparative electrophoresis for final purification.

Third step

Antigen purification on PAGE electrophoresis For polyclarlamide gel electrophoresis the method of Laemmli was used. The samples were run in 7.5% polyacrylamide gel without SDS and with Trisglycine, pH 8.3, as electrode buffer (Bio-Rad Laboratories, Richmond, California). The separating gel size was 18 × 12 cm; the current was applied at 0.8 mA/cm for eight hours. When electrophoresis was performed at 1 cm large strip of gel was cut parallel with the migration front. Protein pattern (protidogram) was developed from this strip according to the following procedure in which reagents of commercial grade without further purification were used: (fixation) one hour of incubation with isopropanol 50% and trichloroacetic acid 10%; (staining) 12 hours of incubation with isopropanol 25% and trichloroacetic acid 10%; Comassie brilliant blue 0.1%; (decolouration) washing with methyl alcohol 30% and acetic acid 7%.

A single band was detected 0.5 cm below the upper edge of the gel without SDS. The protidogram was joined with the original gel, which had been kept at 4°C. At this point a second cut of the gel was made crosswise to the migration front, corresponding to the position of the previously developed band to extract the included protein. A polyacrylamide strip was minced in a glass jar using a china spatula. Distilled water (2 ml) was thus added and the preparation was kept for 12 hours at 4°C to dissolve the purified protein. This protein was water soluble, in accordance with our previous investigation.

The sample was then filtered on a Jena-2 porous glass filter under a water pump to separate the solid polyacrylamide from the protein solute. This solution was dialysed once again against distilled water at 4°C for 12 hours with two changes of water. It was then frozen at −30°C. The purified protein was lyophilised (Delta Christ Lyophilizer, West Germany) and weighed to obtain the total yield (2–6 mg of protein).

Chemical and physical characteristics of the protein were taken into account in the purification procedure as the isoelectric point had been found to be about 8.3, which coincided with the pH of the electrophoretic buffer, pH 8.3. Nevertheless, its slight migration in the gel allowed us to obtain a single band near the upper edge that had been isolated by the above mentioned procedure.

Analysis of the purity Analytical electrophoresis was carried out according to the method of Laemmli using SDS 7.5% polyacrylamide gel. Electrophoresis lasted four hours, with a 0.8 mA current/cm. Fifty μg of lyophilised protein from transudates, mesothelium, and metastatic effusions, respectively, were compared with 20 μg of purified protein.

Molecular weight determination Twenty μg of pure protein and small amounts of the protein from mesothelium effusions under purification processing were compared with standard protein of known molecular weight (myosine 200 000; β-galactosidase 116 000; bovine serum albumin 66 200; vegetable peroxidase 40 000), using PAGE analytical electrophoresis (molecular weight protein standards for SDS-Gel electrophoresis. Bio-Rad Laboratories, Richmond, California).

Preparation of the antibody Polyclonal antiserum was generated by immunising a New Zealand white rabbit with the protein isolated, as previously described. One hundred and fifty μg of protein in Freud's complete adjuvant (Difco, Detroit, Michigan, USA) was injected four times at weekly intervals. Seven days later a subcutaneous booster
injection was given. The antiserum was collected seven days after the booster. Before use antiserum was absorbed with human serum of equal volume for two hours at 37°C and then with extract of human liver and pancreas. It was subsequently clarified by centrifugation at 100,000 g for one hour.

**IMMUNODIFFUSION**

The Ouchterlony technique was applied, using 1% agar gel. The thickness of the agar was 0.5 mm and well size 20 μl. The distance between wells was 8 mm from edge to edge, and the diffusion time was two to three days in a humid chamber at room temperature. The central well was filled with 10 μl of the antiserum. The pure protein was diluted with 100 μl of phosphate buffered saline, and the six peripheral wells were filled with the protein solution at increasing dilution (1; 1/2; 1/4; 1/8; 1/16; 1/32). The same procedure was applied using raw fluids from mesothelioma, transudate, and metastatic carcinoma tested against the antiserum.

**IMMUNOHISTOCHEMICAL PROCEDURE**

The antibody was used in an immunohistochemical procedure (Universal Dako Pap Kit system for rabbit antibody, Dakopatts) to detect the antigenic protein in benign and malignant tumours from all the bodily systems. Sections (5 μm) were prepared from biopsy and necropsy specimens, fixed in neutral buffered formalin for no more than 24 hours and processed routinely to paraffin wax at 55–60°C. The table shows the tumours examined.

Sections were warmed overnight at 40°C and then placed in an oven at 56°C for 30 minutes. Slides were immersed in three xylene baths (10 minutes each). Rehydration of sections was performed according to the following schedule: 99% alcohol-xylene, for five minutes; 99% alcohol (three baths, three minutes each); 95% alcohol for three minutes; 70% alcohol for three minutes; 45% alcohol for three minutes; distilled water for three minutes.

Staining procedure was performed according to the Dako Pap Kit system that uses Sternberger's peroxidase-antiperoxidase (PAP) technique. Two steps of this procedure were modified: incubation with 3% hydrogen peroxidase was protracted to 15 minutes at room temperature; and incubation with the primary antibody was protracted to 45 minutes at room temperature.

Negative controls consisted of duplicate sections, with phosphate buffered saline or Tris buffer substituted for the primary antibody, which consistently showed no staining.

<table>
<thead>
<tr>
<th>Tumours</th>
<th>Binding of antibody</th>
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<tbody>
<tr>
<td>Mesothelioma of pleura and peritoneum</td>
<td>+</td>
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<tr>
<td>Adenomatoid tumour of epididymis</td>
<td>+</td>
</tr>
<tr>
<td>Urothelial carcinoma of bladder</td>
<td>-</td>
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<tr>
<td>Adenocarcinoma of stomach</td>
<td>-</td>
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<tr>
<td>Adenocarcinoma of small bowel</td>
<td>-</td>
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<tr>
<td>Adenocarcinoma of large bowel</td>
<td>-</td>
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<td>Carcinoma of prostate</td>
<td>-</td>
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<tr>
<td>Myxoid liposarcoma</td>
<td>-</td>
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<tr>
<td>Squamous cell carcinoma of lung</td>
<td>-</td>
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<tr>
<td>Adenocarcinoma of lung</td>
<td>-</td>
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<tr>
<td>Oat cell carcinoma of lung</td>
<td>-</td>
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<tr>
<td>Bronchioloalveolar carcinoma of lung</td>
<td>-</td>
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<tr>
<td>Carcinoma of kidney</td>
<td>-</td>
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<tr>
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<td>-</td>
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<tr>
<td>Serous and mucinous cystadenoma and</td>
<td>(serous cells)</td>
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<tr>
<td>cystadenocarcinoma of ovary</td>
<td>+</td>
</tr>
<tr>
<td>Pleomorphic rhabdomyosarcoma</td>
<td>-</td>
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<td>Seminoma of testis</td>
<td>-</td>
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<tr>
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<td>Yolk sac tumour of testis</td>
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<td>Dermal cylindroma</td>
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<tr>
<td>Clear cell carcinoma of ovary</td>
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<tr>
<td>Synovial sarcoma</td>
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</table>

= negative reaction with antibody.
+ = positive reaction with antibody.

**Fig 1** SDS-polyacrylamide gel electrophoresis (four hours; 0.8 mV/cm gel) of raw lyophilised proteins from effusions of mesothelioma (lane A), transudate (lane B), and metastatic carcinoma (lane C). Mesothelioma and transudate protein patterns (lanes A and B) show fraction (thin line) corresponding to lowest cathodal band, which splits into two undifferentiated subunits. Metastatic carcinoma protein pattern (lane C) shows bands with different migration velocities, relative to two other patterns. (Comassie blue stain.)
Results

**ANTIGEN ISOLATION AND PURIFICATION**

SDS-polyacrylamide gel electrophoresis of 50 μg of lyophilised proteins from serous effusions of mesothelioma, transudate, and metastatic carcinoma showed differing migration patterns (fig 1). The mesothelioma protein pattern (lane A) showed three cathodal and two anodal bands. The lowest cathodal band probably consisted of two undifferentiated sub-

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**Fig 2** Polyacrylamide gel electrophoresis without SDS (eight hours; 0-8 mV/cm gel), of pure protein (lane P), raw fluids from mesothelioma (lane A), transudate (lane B), and metastatic carcinoma (lane C). Fifteen μg of the pure protein and 50 μg of total proteins from raw fluids were used; differing quantities of proteins were required for staining purposes. Band corresponding to native protein (arrow) is found in same position in both mesothelioma (lane A) and transudate (lane B) effusion protidograms, whereas it is absent in metastatic carcinoma effusion (lane C). (Comassie blue stain.)

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**Fig 3** Polyacrylamide gel electrophoresis (four hours; 0-8 mV/cm gel), without SDS for determining molecular weight of native protein. HMW: protein markers of known molecular weight; lane 1, first volume collected from elution of column; lane 2, second volume collected from elution of column; lane P, protein (large arrow) at end of purification procedure. As shown in lane P, molecular weight of protein is around 200 000 daltons. In lane 2 protein seems to be contaminated by several other proteins that have been removed during step two of purification procedure. At bottom of lanes (small arrow) are degraded peptides of low molecular weight and blue brominephenol band. (Comassie blue stain.)

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**Fig 4** Ouchterlony immunodiffusion. Antiserum (central well) precipitates pure antigen in well 1.
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units of the native protein of high molecular weight, which had not been shown by SDS. The two anodal bands were composed of peptides of low molecular weight. The transudate protein pattern (lane B) consisted of a broad cathodal band, a second intense band, and a slight protein trace that migrated in the same way as the subunits shown in the mesothelioma protidogram. Two anodal peptide bands were also evident. The metastatic carcinoma protein pattern (lane C) showed four cathodal protein bands, three intense and one slight, with different migration velocities, relative to the two other patterns (lanes A and B).

The entire electrophoresis technique, including SDS, was not used to show the purified protein as a single band nor to determine the molecular weight, as the cathodal native protein, IP 8:3, split into two undifferentiated subunits (fig 1). Without using SDS, electrophoresis provided a protein pattern that showed the native protein as a single band at IP 8:3 (fig 2). This band was found in the same position in mesothelioma (lane A) and transudate (lane B) effusion protidograms, but not in metastatic carcinoma effusion (lane C).

The molecular weight of the native protein IP 8:3 was determined by comparison with pure proteins of known molecular weight (fig 3). Electrophoresis was performed without SDS. Intermediate stages of the purification procedure were also shown—that is, the first (lane 1) and the second (lane 2) volumes collected from elution of the column. The protein impurities were completely removed (lane P) at the last step of the procedure.

**IMMUNODIFFUSION**

The reactivity of the antiserum against the mesothelial antigen was tested with the Ouchterlony's technique. fig 4 shows the results. The antiserum precipitated the pure antigen in well 1 (dilution 1/1), and the line of precipitation was seen after 48 hours of incubation. When the antiserum was allowed to migrate towards raw fluids from mesothelioma, transudate, and metastatic carcinoma, respectively, it failed to recognise the metastatic carcinoma.

**IMMUNOHISTOCHEMICAL STUDY**

A range of benign and malignant human tumours fixed in formalin and embedded in paraffin wax was selected from our files and tested immunohistochemically with the polyclonal antibody for the presence of the protein. The table shows the tissue examined and the reactions with the antibody.

When 1/80 antiserum was used only, reactive mesothelium, malignant mesothelioma of the pleura and the peritoneum, benign and malignant mesothelioma of the ovary of serous type (according to the classification of the ovarian tumours devised by the International Federation of Gynecology and Obstetrics), and adenomatoid tumours of the epithidymis readily bound the antibody (table) (fig 5a–f). The cytological distribution of the protein, as detected immunohistochemically, was cytoplasmic.

None of the other tumours, either benign or malignant, tested in this study reacted with the antibody (table) (fig 5g–h).

A non-specific binding of the antibody was seen in most of the neoplastic epithelial tissues when antiserum was used at lower dilutions—that is, from 1/10 to 1/40, whereas no binding was found at higher dilutions (1/80) in any tumour tissue.

The following observations should be emphasised. Firstly, in all mesotheliomas, the protein was detected in epithelial type cells (fig 5b), as well as in "transitional" cells—that is, intermediate between epithelial and mesenchymal cell type, and in cells similar to fibroblasts of the connective tissue type of mesothelioma (fig 5d). Secondly, as for the epithelial type pattern, the intensity of the staining varied among different mesotheliomas and also within the same tumour. Thirdly, in mesothelial tumours of the ovary that feature a mixture of serous and mucin filled cells only the serous cells gave a positive reaction with the antibody (fig 5e).

The antibody also detected both reactive (fig 6a) and malignant (fig 6b) mesothelial cells in cytological smears of serous effusions. In vitro cultured human mesothelial cells from a new born hernial sac also reacted with the antibody (fig 6c).

**Discussion**

Controversial results have been reported when immunological techniques have been used for the detection of markers that might be specific for mesotheliomas or adenocarcinomas. The reasons for this discrepancy stem mostly from methodological differences and variations in antiserum specificity.

Only one specific antimesothelial cell serum has been developed and found to react solely with benign and malignant mesothelioma tissues, initially, by using an indirect immunofluorescence procedure and, subsequently, by an immunoperoxidase method. Unfortunately, such antiserum is not readily available to the pathologist, and furthermore, its diagnostic usefulness has been evaluated only in a limited sample of adenomatoid tumours.

According to the results just reported, the antibody we developed can be used as a reliable diagnostic tool for distinguishing mesothelial and mesothelioma cells in both histological and cytological specimens. In fact, it has proved to be highly sensitive in the identification of all tumours of mesothelial origin.
Fig 5  Positive (a to f) and negative (g and h) staining reactions of various lesions with antibody (Immunoperoxidase method; Mayer's hematoxylin counterstain.) a) reactive hyperplasia of pleural mesothelium; b) pleural mesothelioma, epithelial type; c) pleural mesothelioma, transitional cell type; d) pleural mesothelioma, cells similar to fibroblasts; e) cystoadenoma of ovary, mixed type. Antibody reacts with serous cells (arrow). No staining of mucus filled cells; f) adenomatoid tumour of epididymis; g) adenocarcinoma of lung; h) adenocarcinoma of large bowel. All figs × 108.
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Fig 6  a) Smear from reactive pleural effusion. "Activated" benign mesothelial cells bind antibody. (Immunoperoxidase method; Mayer's hematoxylin counterstain.) × 400.  
b) Smear from malignant pleural effusion secondary to mesothelioma. Malignant mesothelial cells bind antibody. (Immunoperoxidase method; Mayer's hematoxylin counterstain.) × 250.  
c) Cell from in vitro culture of human mesothelial tissue. Fine granular staining of cytoplasm with the antibody. (Immunoperoxidase method; Mayer's hematoxylin counterstain.) × 540.

only (table). The protein can be regarded as a specific marker of mesothelial cells, as it has not been expressed in a representative sample of tumours from all the systems of the human body, apart from the original coelomic cavities, including their derivates and remnants.

The fact that the antigen was detected in serous ovarian tumours and adenomatoid tumours of epididymis supports the theory of a mesothelial derivation of these neoplasms, the histogenesis of which has been a source of controversy.19–22

It should be emphasised that the antibody is specific for mesothelial cells and is not exclusive for mesothelioma cells. If cells from a malignant tumour, however, can be shown to have the antigen characteristic of a mesothelial cell it would be reasonable to conclude that the tumour is a mesothelioma.

In ovarian mesothelial tumours only serous cells react with the antibody, whereas mucus filled cells do not. A possible explanation for this might be that the protein might be active in the cytoskeleton, which seems to organise the cytoplasm and hence can influence various types of cytoplasmic activity.

The expression of protein in mesothelioma cells is of particular importance in distinguishing malignant mesothelioma from adenocarcinoma, which affects pleural and peritoneal surfaces. This differential diagnosis is a well known area of difficulty in surgical pathology.

The use of the antibody in an easy and readily available immunohistochemical procedure means that the confidence in making the morphological diagnosis of mesothelioma, especially on limited biopsy material or serous effusions, or both, can be improved. Nowadays, this is of particular importance: to establish a correct therapeutic approach to patients with mesothelioma early diagnosis using minimal intervention, such as closed needle biopsy, is vital; and open chest surgery, which is hazardous in this disease,23 should be avoided.

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References


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