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The ultrastructural immunohistochemistry of oncofoetal antigens in large bowel carcinomas

W.D.G. Haynes, K.L. Shertock, J.M. Skinner and R. Whitehead
Department of Pathology, Flinders Medical Centre, Bedford Park, South Australia, 5042

Summary. Seven large bowel carcinomas were examined by light and electron microscopy for the presence of five oncofoetal antigens. Ultrastructural investigations involved a novel method whereby thick sections of gluteraldehyde-fixed material were cut on a vibratome and then labelled using slight modifications of a standard unlabelled antibody-enzyme (PAP) technique, before further processing.

Ultrastructural preservation, staining properties and the retention of antigen activity was seemingly better than that achieved by other investigators.

Specific, positive labelling for carcinoembryonic antigen (CEA), colon specific antigen (CSA) and pregnancy-specific β-1-glycoprotein (SP1) was seen in every case. Clear positive labelling for placental alkaline phosphatase (PLAP) and human chorionic gonadotropin (HCG) was seen in two cases.

Extracellular labelling was found in areas of cell debris, free lying or in phagocytic cells and on tumour cell brush borders. The pattern of intracellular labelling, however, was different for each antigen and reflected the probable sites of synthesis and release from the cells.

Thus CEA, a complex glycoprotein, was localised within the golgi apparatus, small apical cytoplasmic vesicles and mucous droplets in relatively well differentiated tumour cells. CSA, a chemically related glycoprotein, had a similar, but less dense distribution. SP1, by contrast, was localised within basally-located vesicles associated with the ribosomal endoplasmic reticulum and appeared to be released and persist as debris or taken up by phagocytic cells below the basal lamina. PLAP and HCG, both proteins, were found within simple single membrane-bound vesicles within relatively undifferentiated cells.

Key words: Immunohistochemistry – Ultrastructure – Oncofoetal – Antigens – Bowel – Carcinoma

Offprint requests to: W.D.G. Haynes at the above address

Introduction

The "oncofoetal antigens" are a heterogeneous group of proteins found in many human cancers and in the normal foetus, but usually absent, or present only in trace amounts, in normal adult tissues. Their appearance seems to be an accompaniment of the dedifferentiation that occurs during the evolution of neoplasms and their function, if any, is uncertain, although an immunosuppressive role has been suggested for pregnancy-specific β-1-glycoprotein (SP1) (Cerni et al. 1977).

Standard procedures of immunohistochemistry at the light microscope level can be used to demonstrate the presence of oncofoetal antigens within tumour cells, within tumour cell mucus droplets and in extracellular sites, but not to identify the specific organelle systems involved in antigen synthesis. Because the antigens are histochemically so dissimilar, varying from simple polypeptides to complex glycoproteins, a variation in the sites of synthesis and intracellular storage is to be expected.

Ultrastructural immunohistochemistry, which could provide more detailed information, is technically problematical because good fixation and ultrastructural appearance needs to be combined with retention of antigenic activity and satisfactory penetration of labelling molecules into the tissues. Two basic procedures have hitherto been applied to material obtained by resection of human tumours. In the first, blocks of tissue are frozen and sections are cut on a cryotome. These are then labelled with appropriate antibody before being processed for electron microscopy (Ahnen et al. 1982; Huitric et al. 1976; Peyrol et al. 1978). In the second, tissue is processed in the normal way for electron microscopy, but omitting postfixation in osmium tetroxide. Ultrathin sections are then treated to remove resin, labelled with antibody, and then osmicated and stained (Huitric et al. 1976; Isaacson and Judd 1978).

Neither of these methods has proven to be entirely satisfactory. Frozen tumour tissue has shown cell and organelle disruption or poor contrast, and there is difficulty in visualizing many organelles. Tissue embedded in resin before labelling often shows only weak antigenicity and very poor contrast. This paper describes our results using a method in which thick (about 20 mμ) sections are cut, from blocks of fixed and washed tissue, using a "vibratome" sectioning system (Oxford Laboratories Ltd.). These sections are then labelled with specific antibodies before postfixation and preparation for electron microscopy in the usual way.

Materials and methods

This investigation was concerned with the localisation of 5 antigens, namely, carcinoembryonic antigen (CEA), colon specific antigen (CSA), SPI, placental alkaline phosphatase (PLAP) and human chorionic gonadotrophin (HCG) in seven primary adenocarcinomas of the large bowel.

Adjacent tissue blocks from resected tumours were treated in three different ways. 1. Fixed in buffered formalin, embedded in wax and examined by light microscopy after staining with haematoxylin and eosin. 2. Snap-frozen in liquid nitrogen, then sectioned at 5 mμ using a Miles cryostat. Serial sections were then treated with 0.5% hydrogen peroxide to remove

endogenous peroxidase activity, incubated with appropriate antibodies, using the unlabelled antibody-enzyme (PAP) method of Sternberger (1974) and counterstained with Methyl Green. 3. Cut into 2 mm cubes, fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer for 30 min, then washed in several changes of buffer for at least 48 h. 20 mμ vibratome sections were then cut from each cube and stored until needed.

Examination of formalin-fixed, paraffin-embedded tissue was carried out first in order to confirm the diagnosis; the frozen sections were then examined in order to confirm positive antibody labelling.

Serial vibratome sections were then incubated with antibodies to the oncofoetal antigens and treated using a modified PAP method as follows:

1. Two washes in 0.1 M phosphate buffered saline, pH 7.2 (PBS), then incubation with 10% normal swine serum (NSS) in PBS, for 10 min at room temperature, in order to block nonspecific antibody binding sites.

2. Incubation with previously determined optimum dilutions of specific rabbit antibody, for one hour at room temperature or 24 h at 4° C. Antibody solutions contained NSS at 10X the nominal protein concentration of each antibody.

3. Two washes in PBS, followed by incubation in swine anti-rabbit antibody at a dilution of 1/20, for 20 min at room temperature.

4. Two washes in PBS, followed by incubation with a 1/50 dilution of rabbit antibody to horseradish peroxidase coupled to horse radish peroxidase (PAP) for 20 min at room temperature.

5. Two washes in PBS, followed by one in 0.05 M tris-HCl buffer, pH 7.6.

6. Localization of peroxidase activity with diaminobenzidine tetrahydrochloride, at 5 mg/10 ml in tris buffer containing 0.0002% fresh hydrogen peroxide for 3–5 min at room temperature.

7. Successive washes in PBS (twice) and distilled water, followed by post-fixation in 1% osmium tetroxide for 45 min.

Sections were incubated by floating on drops of incubation medium in wells pressed into parafilm. Medium could be changed either by removal with a syringe or by moving the section from well to well with a paint brush. Buffers were cleaned initially by membrane filtration (pore size 0.2 mμ) and all solutions (except for the peroxidase/antibody incubation medium) were spun at 300 rpm for 15 min in a GLC-2B centrifuge before use.

Antibody to CSA was raised in our laboratory; other antibodies were obtained from Behring or DAKO (Copenhagen, Denmark). Antibody production and purification and tests of specificity were performed as described by Skinner and Whitehead (1981). The specificity of each antigen was confirmed at the ultrastructural level by omitting steps 2 and/or 3 in the schedule above for some control sections with negative final results. It should be noted that, since no attempt was made to remove innate peroxidase activity from vibratome sections, red blood cells, and some neutrophil lysosomes gave positive results. However, no innate peroxidase activity was seen within tumour cells. Non-specific labelling of collagen in positive and control sections, was also seen.

One micron sections were cut initially from each labelled thick section and examined by light microscopy before and after staining with toluidine blue. Peroxidase-positive areas could usually be seen in unstained sections. Peroxidase positive zones containing representative areas of tumour cells were then cut for electron microscopy and examined before and after staining with uranyl acetate and/or lead citrate.

Results

Light microscopy

All the tumours showed a wide variety of appearances and most were moderately or well differentiated. Although two well differentiated tumours were negative for HCG, there did not appear to be a consistent relationship between the degree of tumour differentiation and the presence or degree

of antigen positivity. Most labelling appeared to be extracellular, on brush borders, within luminal or serosal debris, or within phagocytic cells (Fig. 1). Occasional tumour cells showed dense labelling, but most were antigen negative, or showed only equivocal labelling.

Electron microscopy

A total of approximately 470 ultrathin sections, from 58 labelled thick sections, were examined in detail. Ultrastructurally cell membranes and surface coats were intact, there was usually little or no swelling or disruption of organelles, and contrast, staining characteristics and resolution were good. A variable degree of mitochondrial swelling was seen in two tumours. Whatever the overall level of differentiation under the light microscope, at electron microscopy appearances varied from well differentiated cells that resembled normal colonic epithelial cells and contained a full complement of densely packed organelles, to small, undifferentiated cells, not arranged in recognizable acini and containing only a nucleus and occasional mitochondria and vesicles of endoplasmic reticulum.

Positive labelling of all five antigens within tumour cells was present to a depth of approximately 5 mμ within the thick sections. Examination of sections both before and after heavy metal staining proved particularly helpful when peroxidase labelling was light. Because staining enhanced the density of both the label and any underlying proteins, the procedure sometimes made the label more apparent, sometimes masked it.

Strong positive labelling for CEA, SP1 and CSA was seen in tissue from every tumour; unequivocal labelling for HCG and PLAP was present in only two tumours. The density and distribution of positive labelling was characteristic for each antigen.

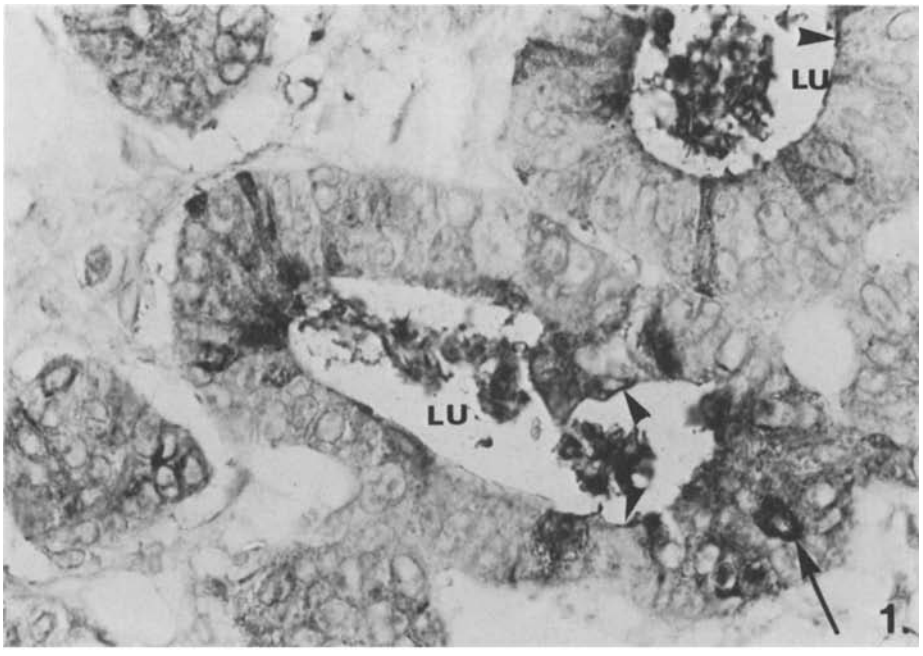
CEA (Figs. 2–3)

Positive labelling for CEA was, overall, more conspicuous than that for any other antigen.

It was always observed within the mucous coat along the apical brush border and on associated luminal contents. A less intense labelling of the lateral and basal aspects of the cell membrane was seen occasionally.

Within malignant cells, CEA was present in some mucous droplets in goblet cells and in small or medium sized membrane-bound vesicles toward the apical region of well differentiated goblet cells, absorptive cells and intermediate cell types. The small vesicles were round or elongated and both the contents of the vesicles and the inner aspects of their surrounding membranes were labelled. Elongated vesicles often appeared to be aligned at right angles to the apical cell surface.

CEA was also found in the golgi apparatus in three tumours. Labelling was present both within the golgi lamellae and in small vesicles on the inner golgi membrane that resembled the CEA-positive apical cell vesicles.



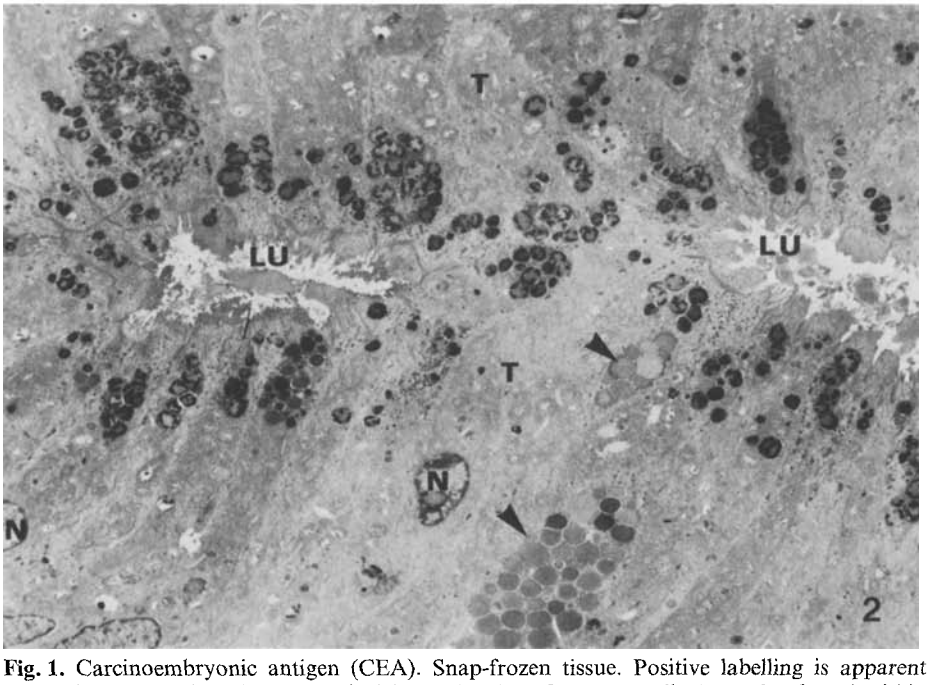


Fig. 1. Carcinoembryonic antigen (CEA). Snap-frozen tissue. Positive labelling is apparent within lumina (*LU*) and at the apical brush border of tumour cells (*arrowhead*) and within many tumour cells. Note the very densely labelled small tumour cell at lower right (*arrow*) × 600

Fig. 2. CEA. Vibratome-sectioned tissue. Positive labelling is seen on mucous droplets of well differentiated tumour cells. Occasional groups of droplets are unlabelled (*arrowheads*). *LU*, lumina; *N*, nuclei; *T*, tumour cells. × 3,500

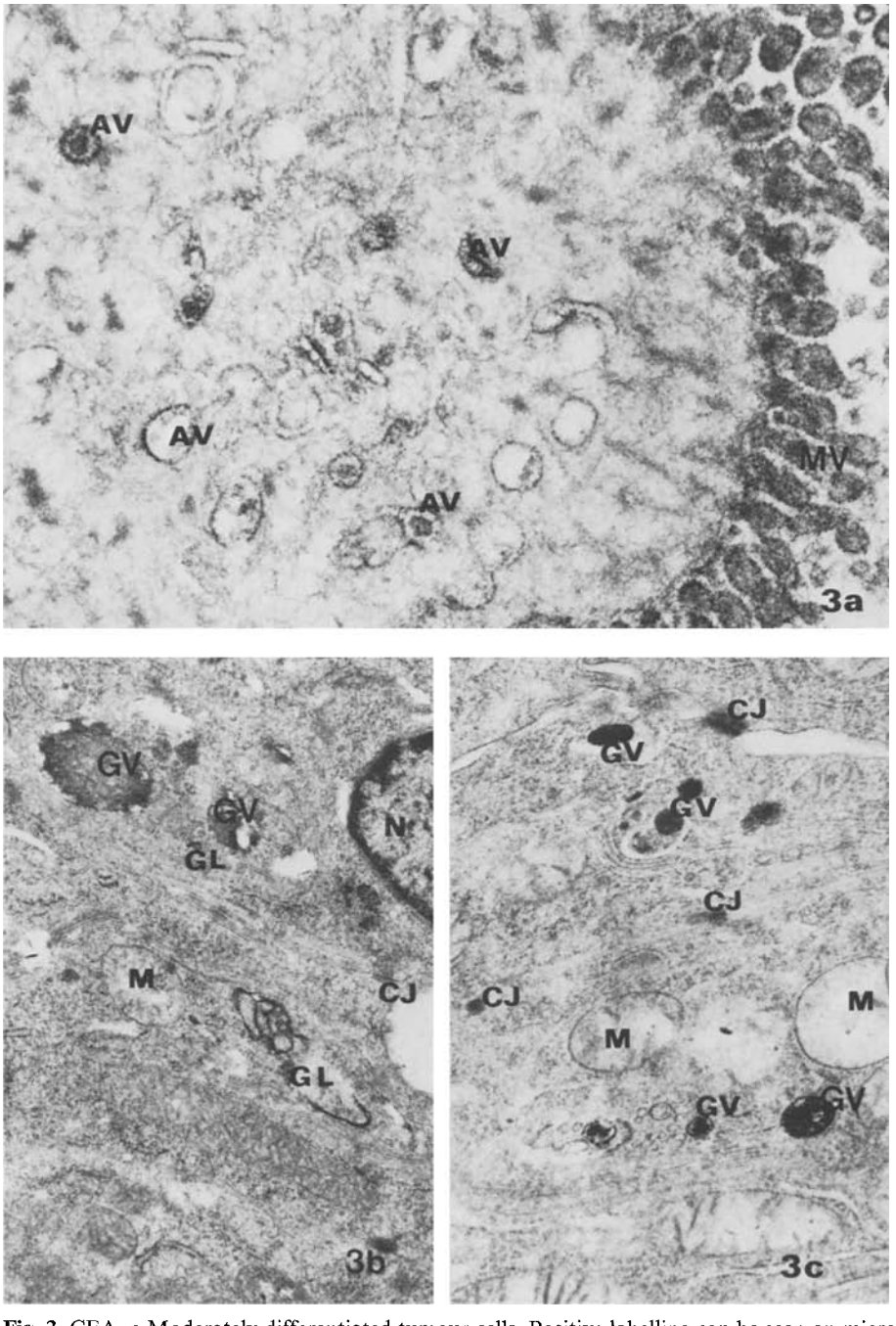


Fig. 3. CEA. **a** Moderately differentiated tumour cells. Positive labelling can be seen on microvilli. Small, apical vesicles show labelling on the inner aspect of vesicular membranes and their contents. ×33,000. **b** Positive labelling is present within elongated golgal lamellae and possibly in large vesicles on the golgal inner face. ×17,000. **c** Positive labelling of vesicles on the golgal inner face of two cells. ×24,000. *AV*, apical vesicles; *CJ*, cell junctions; *N*, nucleus; *M*, mitochondria; *MV*, microvilli; *GV*, golgal inner face; *GL*, golgal lamellae

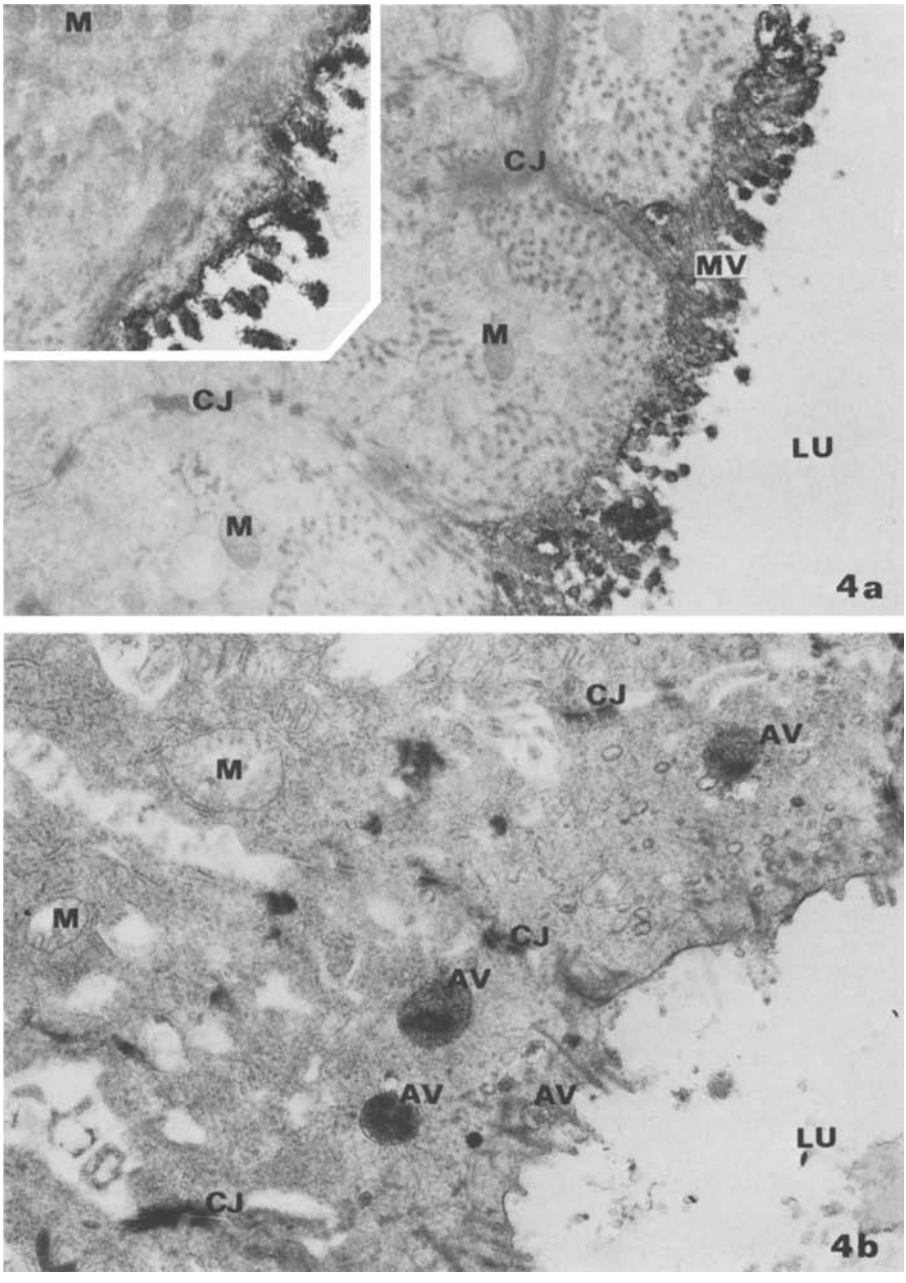


Fig. 4. CSA Moderately differentiated tumour cells. **a** Positive labelling of microvilli (MV) and apical cell membranes, but no intracellular labelling. Unstained section. *CJ*, cell junctions; *LU*, lumen; *M*, mitochondria. ×14,000 (INSET) More thickly labelled tumour cells from the same section. ×16,000. **b** Tumour cells showing weakly positive labelling of a few small apical vesicles (*AV*) but not of the apical cell surface. ×17,000. *AV*, apical vesicles

CSA (Fig. 4)

Dense CSA labelling was found along the apical brush border in every case; less conspicuous labelling was seen below the basal lamina, in areas of debris and within phagocytic cells.

Labelling within tumour cells was only seen in a single tumour and was restricted to frequent rather small, membrane-bound apical vesicles within moderately differentiated tumour cells. The intensity of labelling was less than for CEA positive vesicles of similar appearance.

SPI (Fig. 5)

Labelling of tumour cell apical microvilli and of luminal contents was seen in three tumours, but was always less intense than intracellular or basal labelling.

SP1 was always found within tumour cell vesicles of typical appearance, and within basal areas immediately adjacent to tumour cells. The vesicles were of the order of 1 mμ in diameter, round or oval and partially filled by densely-labelled amorphous contents; ribosomes were often attached to the outer aspect of the enclosing membrane. Several vesicles were often associated within membrane-enclosed areas of apparent focal cell necrosis and both vesicles and focal necrotic areas were always found towards the basal aspect of the cell. Similar areas of focal necrosis were present in adjacent phagocytic cells. Intense interstitial labelling was also seen and the underlying ultrastructure was thus masked. The great majority of labelling was seen within or associated with relatively undifferentiated tumour cells; occasional small positive vesicles were present in moderately differentiated cells.

PLAP (Fig. 6)

Labelling of PLAP was demonstrated in two tumours. Positive extracellular labelling was seen in basal regions, within lumina and on tumour cell apical microvilli, but was always most prominent in characteristic vesicles within the malignant cells.

PLAP-positive vesicles were rounded, of the order of 3–5 mμ in diameter and contained large masses of homogeneous, positive material. They were not restricted to any particular intracellular position. Neither the vesicles nor the positive material were surrounded by ribosome-linked membranes, although short lengths of ribosomal endoplasmic reticulum were very occasionally seen within the vesicles.

HCG (Fig. 7)

Positive labelling for HCG was restricted to occasional small groups of undifferentiated cells and associated areas of debris in two tumours.

Labelling within tumour cells was intense, but limited to a few smooth, membrane-bound rounded vesicles, about 0.5 mμ in diameter and filled with homogeneous material.

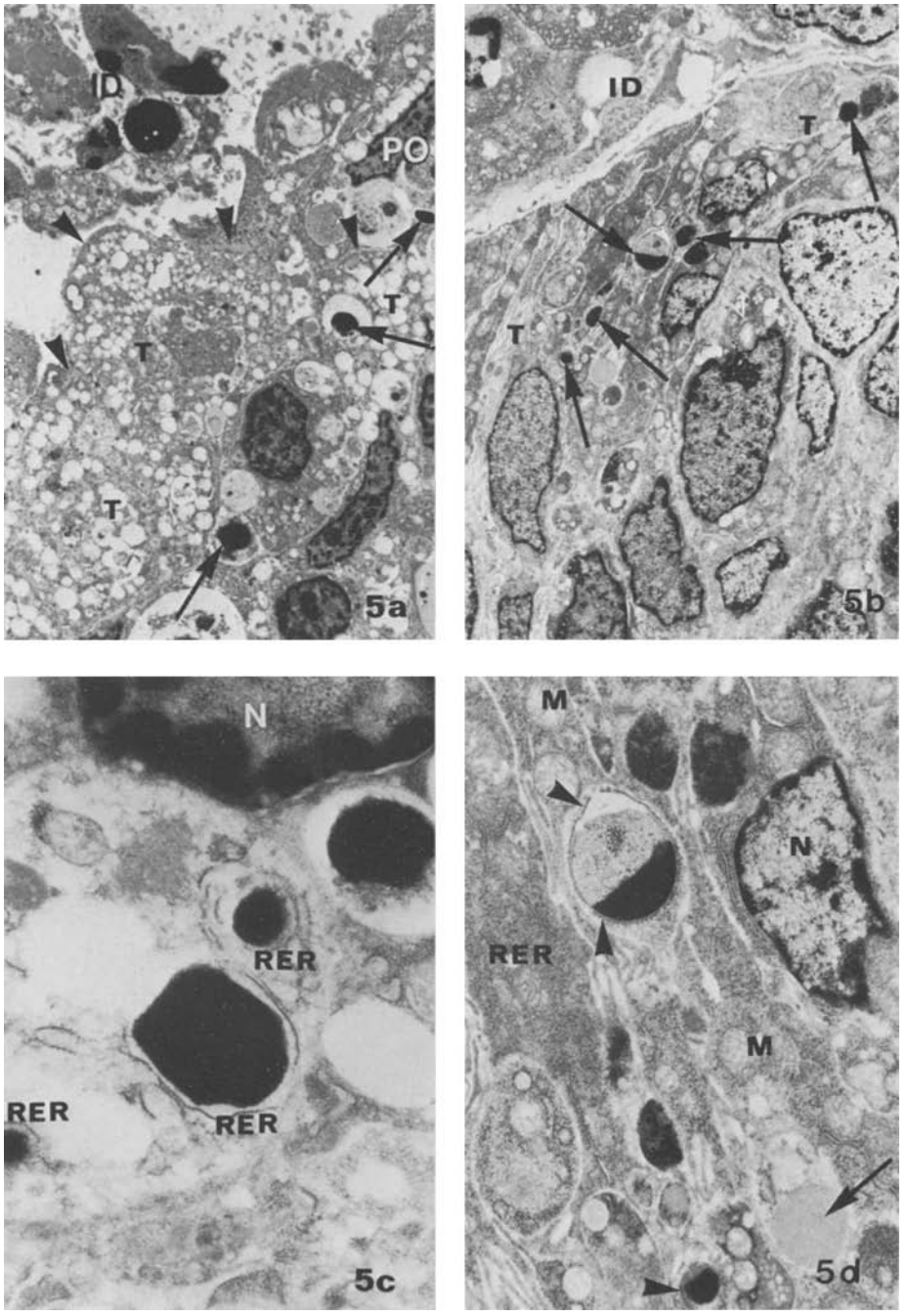
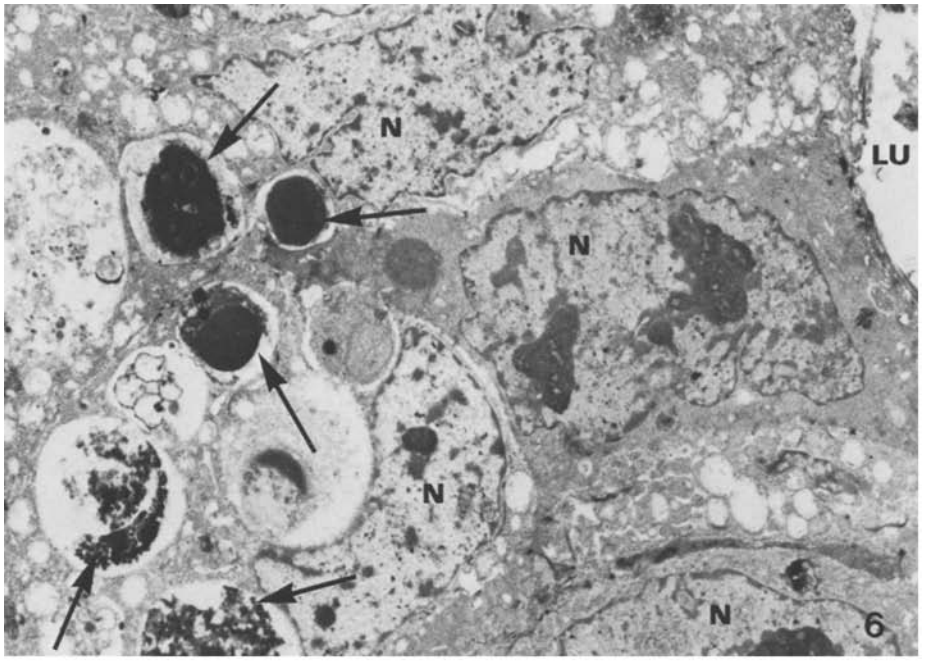


Fig. 5. SP1. Relatively undifferentiated tumour cells; basal lamina of tumour cells (*arrowed-head*). Intense labelling is present in several medium-large vesicles (*long arrows*) within areas of focal cell necrosis in tumour cells (*T*) and in a probable phagocytic cell (*PO*) at top right. Labelling of interstitial debris (ID) is present at top left. ×3,000. *T*, tumour cells; *PC*, phagocytic cells; *ID*, interstitial debris. **b** Moderately differentiated tumour cells. Several positively labelled vesicles are present within tumour cells (long arrows), but areas of focal cell necrosis and labelling of extracellular material are absent. ×3,600. **c** An area of focal cell necrosis in a tumour cell immediately adjacent to **a**. Ribosomal endoplasmic reticulum (RER) can be seen around three of the four areas of positively labelled material present. *N*, nucleus. ×22,000. **d** Higher power of **b**. Ribosomes can again be seen to be attached to the limiting membranes of two areas of positively labelled material (*arrowheads*). Note the area of unlabelled proteinaceous material at lower right (*arrow*); this material has the same appearance in unlabelled sections. *M*, mitochondria. ×11,000



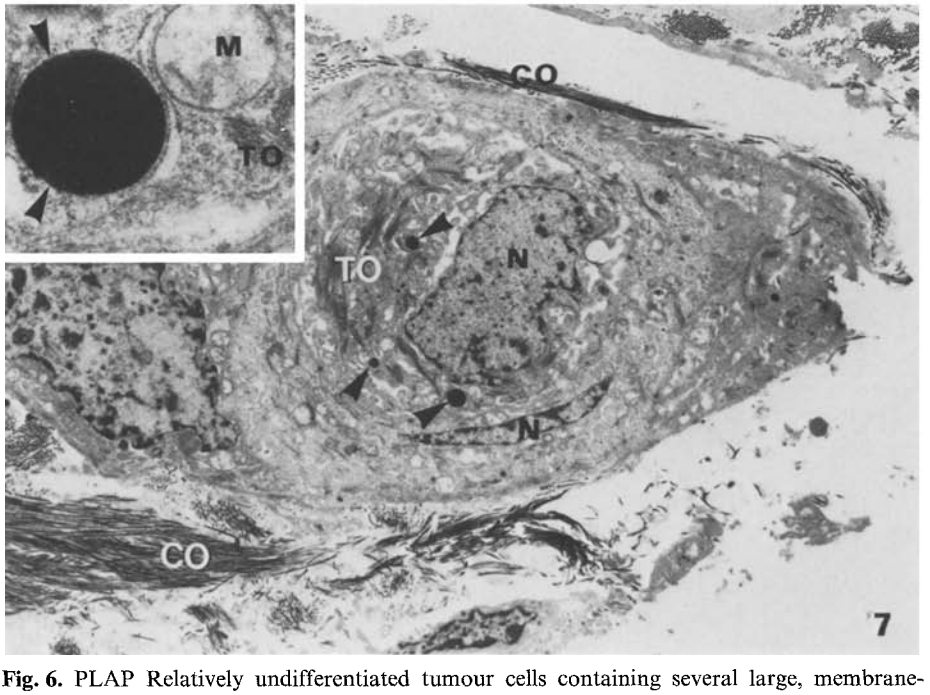


Fig. 6. PLAP Relatively undifferentiated tumour cells containing several large, membrane-bound vesicles containing positively labelled material (*arrows*). ×17,000. *LU*, lumen; *N*, nucleus

Fig. 7. HCG Small, undifferentiated tumour cells containing a few small, but intensely labelled vesicles (*arrowheads*). Cells contain numerous tonofilaments (*TO*). Note the intense (non-specific) labelling of interstitial collagen fibres (*CO*). ×4,100. (*INSET*) Higher power of largest vesicle. The single limiting membrane can be seen in places (*arrowheads*) ×37,000. *M*, mitochondria; *N*, nucleus

Discussion

The technique described has a number of practical advantages over those used by others working with oncofoetal antigens. Contamination of sections, a major hazard of the *en-grid* technique, is not a problem; any surface debris on vibratome sections is removed when 0.5 mμ sections are cut for light microscopy. Sections are easy to see and to handle and a single labelled vibratome section can, in theory, provide several hundred labelled ultrathin sections. The direct exposure of antibody solutions to cut cell surfaces before embedding means that antibody penetration is not a problem. Good ultrastructural preservation was obtained considering the problems involved in rapid fixation of tissue obtained at resection. Good staining characteristics and contrast in ultrathin sections are ensured by rapid initial fixation in gluteraldehyde and postfixation in osmium before embedding.

The major disadvantage of the technique is that serial ultrathin positive and control sections cannot be obtained; in practice, however, we were usually able to examine at least the same cells, if not the same organelles, in serial vibratome sections.

CEA was predominantly localized on the apical brush border, in some, but not all, mucus droplets, in small apical vesicles and within the golgi apparatus. These findings are similar to those described by Ahnen et al. (1982) in colon carcinomas, but we did not find the weak perinuclear staining that these earlier authors describe.

It seems likely that CEA is manufactured within the golgi apparatus and transported from there to the apical cell surface in small vesicles or via mucus droplets. The small apical vesicles, especially the more elongated ones, resemble elements of the endoplasmic reticulum, but if they contain material from the golgi apparatus they should more properly be called secretory vesicles.

Ahnen et al.'s description of CEA antigenicity within the perinuclear space, however, suggests that a protein precursor of the complete CEA molecule can be detected by commercially prepared antiserum to CEA. However Ahnen used periodate-lysine-formaldehyde (PLP) rather than gluteraldehyde as initial fixative. A number of light microscope studies have shown that gluteraldehyde diminishes CEA antigenicity more than PLP and the difference between Ahnen's results and our own may therefore reflect an alteration of the precursor molecule by gluteraldehyde fixation. After glycosylation in the golgi apparatus, this alteration is no longer possible.

Ahnen et al. also suggest two possible mechanisms for the presence of CEA along the basolateral surfaces of less well-differentiated tumour cells: leakage of antigen between the intercellular junctions or a loss of cell-surface polarity that might result in exocytosis of antigen through the basolateral cell membranes. We would support the first suggestion. There was no sign at all of small positive vesicles in lateral or basal intracellular positions in our study.

The ultrastructural distribution of CSA is less well shown than that of CEA. Labelling of the brush border is again conspicuous and there

is occasional labelling of small apical vesicles that resemble CEA-positive secretory vesicles. But intracellular labelling is very weak overall and the sites of CSA manufacture or storage are not apparent. Neither mucus droplets nor golgi apparatus are labelled.

Quite large accumulations of densely labelled homogeneous SPI-positive material can be seen within basal vesicles and the association of these vesicles with swollen lamellae of the ribosomal endoplasmic reticulum is often clear. This distribution is quite different from that of CEA and CSA but the intracellular SP1 positive vesicles are similar in size and appearance to the SP1 positive vesicles described by Azer et al. (1983), in cultured cells from an ovarian cystademona. The latter vesicles were also thought to originate in the ribosomal endoplasmic reticulum. SP1 positive vesicles appear to accumulate in areas of focal cell necrosis. The latter are then presumably released into the extracellular space or taken up by phagocytic cells.

Once outside the tumour cells SP1-positive material remains as large accumulations of debris on their basal aspect. None of the other oncofoetal antigens accumulate so conspicuously in such sites. This is an interesting finding in view of the suggestion by Cerni et al. (1977) that SP1 may have immunosuppressive properties. It seems possible that the presence of relatively large amounts of an immunogenic protein along the basal aspect of tumour cells might indeed reduce effective anti-tumour macrophage activity.

The presence of small amounts of SP1 on the apical surface of tumour cells may represent leakage between the intercellular junctions of relatively undifferentiated cells.

The origins and precise nature of the large PLAP-positive vesicles are unclear and no evidence of the release of labelled material was seen, although positive labelling of cell apices and luminal and basal debris was present. However, our findings are similar to those of Wilson et al. (1981) working with neutrophils from healthy and leukaemic patients. They found that while alkaline phosphatase activity was confined to the plasma membrane of normal cells in malignant cells, it was found only within large, abnormal rounded "lysosomes" which they termed phosphasomes and which resemble the positive vesicles in our study. Again, no structural counterpart of a release mechanism from the cells was seen.

HCG proved to be the most difficult antigen to localise ultrastructurally, as at the light microscope level. It was only found within a few cells in two tumours. Only a few labelled vesicles were found per cell and a mechanism of release of positive material from the cells was not seen. The vesicles resembled, but were smaller than, the large, membrane bound HCG positive vesicles demonstrated in the normal human placental syncytiotrophoblast by Dreskin et al. (1970). These were later assigned to the ribosomal endoplasmic reticulum, but this suggestion is probably in error as they do not appear to have associated ribosomes. Our results would suggest that HCG is formed in the smooth endoplasmic reticulum.

A degree of correlation between the level of differentiation of tumour cells in this study and the specific oncofoetal antigens produced by those cells is more apparent ultrastructurally than at the light microscope level.

CEA, for example, is produced predominantly by well differentiated cells and is the only antigen studied to be produced by well differentiated goblet cells. CSA is produced principally by moderately differentiated cells. SP1 is produced by relatively undifferential and moderately differentiated cells, PLAP by relatively undifferentiated cells and HCG by undifferentiated cells. Antigens produced only by relatively undifferentiated cells cannot require complex organ systems for their synthesis. Thus PLAP and HCG (respectively an enzyme and a simple polypeptide hormone) appear to be formed in simple, smooth membrane bound vesicles. Synthesis of SP1 appears to require the presence of properly organised ribosomal endoplasmic reticulum, while synthesis of CEA, a complex glycoprotein, additionally involves the golgi apparatus.

We found a consistent, non-specific labelling of collagen fibres (Fig. 7). While this did not interfere with ultrastructural visualisation of specific antigen positivity, it did result in a relatively high level of background staining in control tissues at the light microscope level. It seems most likely to be caused by the non-specific binding by collagen aldehyde groups of the antibodies used, because the effect is not present in sections merely stained with DAB.

This study describes a new methodology for the ultrastructural localisation of intracellular antigens in tumours. It relates the approximate level of tumour cell differentiation in terms of its intracellular organelles to the cell's ability to synthesise specific antigens.

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