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Expression of cytokeratin and neuron-specific enolase in small cell carcinomas of the lung

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Summary. Using a polyclonal antibody against human epidermal keratins and a monoclonal antibody against cytokeratins characteristic of simple epithelia, and the Avidin-Biotin system of immunohistochemistry, we have demonstrated cytokeratin expression in 46% and in 60% of small cell carcinomas of the lung at autopsy respectively. The latter gave a diffuse stronger reaction product than the polyclonal antibody. The results suggest that there is a cytokeratin rich and a cytokeratin poor type of small cell carcinoma. Neuron-specific enolase immunohistochemistry was positive in 60% of the cases. Coexpression with cytokeratin was seen in ten cases (30%). The expression of cytokeratin and neuron-specific enolase in small cell carcinomas strongly suggests that they are of an epithelial origin, but are capable of neuroendocrine differentiation.

Key words: Small Cell Carcinoma - Cytokeratin - Neuron specific Enolase - Immunohistochemistry

Introduction

Small cell carcinomas of the lung, which arise from the Kulchitzky cells of the bronchus, are separated from other lung carcinomas because of their epidemiology, poor response to therapy other than chemo- or radiotherapy, argyrophilic nature and because ultrastructurally they show poorly developed desmosomes and tonofibrils, and dense core neurosecretory type of granules in their cytoplasm (Fisher et al., 1978; Tateishi et al., 1978; Carter and Eggleston, 1980; Matthews and Gazdar, 1981; WHO, 1980; Yesner, 1983). The presence of neurosecretory granules is related to their production of a wide range of neuro-endocrine markers such as neuron-specific enolase (NSE), ACTH, calcitonin and others (Carney et al., 1982; Gould et al., 1983). Their nomenclature is still controversial as they are variously called small cell carcinoma, bronchopulmonary neuroendocrine carcinoma or Kulchitzky cell carcinoma (WHO, 1981; Gould et al., 1983; Paladugu et al., 1985).

The cytoskeleton of epithelial cells and tumors arising from them is characterised by a system of intermediate-sized filaments (7 to 11 nm) of the cytokeratin type in their cytoplasm (Franke et al., 1982; Moll et al., 1982; Osborn and Weber, 1982). They have been used in the differential diagnosis of tumors (Altmannsberger et al., 1981; Gabbian et al., 1981; Osborn et al., 1983). In small cell carcinomas, Bergh et al. (1984) and Lehto et al. (1983) have reported a lack of cytokeratin and the presence of neurofilaments in their cytoplasm in vitro and in vivo. On the other hand, cytokeratin expression and a lack of neurofilaments in these tumors has been reported (Saba et al., 1983; Van Muijen et al., 1984; Blobel et al., 1985; Broers et al., 1985).

We describe in this report, the expression of cytokeratin and neuron-specific enolase in thirty autopsy cases. We also show that small cell carcinomas may be separated into cytokeratin rich and cytokeratin poor subtypes.

Materials and methods

Study Population:

Between 1971 and 1980, 137 cases of lung cancer were autopsied at Kyushu University Hospital. The primary site and organ distribution of metastases when present were noted. The blocks of the primary site from the formalin fixed and paraffin embedded material were re-cut and stained with hematoxylin and eosin, alcian blue, periodic acid Schiff and Grimelius stains as necessary. The tumors were then classified according to the WHO (1981) criteria, and 30% (21.9%) of these were categorised as small cell carcinomas. Three to four

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blocks of the primary site were cut at four microns, mounted on albumin-coated slides for immunohistochemistry. In addition, the following were also studied; two small cancer cell lines of human origin QG 90 and QG 96 (Kinjo et al., 1979), which were a generous gift from Dr. Hara, Kyushu Cancer Center Hospital. These were cultured in RPMI 1640 supplemented with 10% fetal calf serum. They were grown in flasks and on Lab-Tek tissue culture slides (Miles Laboratories, USA). When confluent the slides were used for immunohistochemistry, and the cells from the flasks were inoculated (20 million/ml) into the flanks of two nude mice of BALB/c origin obtained from Clea, Japan, which were kept in vinyl isolators. They were sacrificed when tumor diameter reached one centimeter (four weeks), and the tumor was divided into two portions, one was immediately frozen in dry ice acetone from which four micron sections were prepared for immunohistochemistry. The other portion was fixed in formalin and embedded in paraffin. Fresh tumor material was also obtained at autopsy from a 74-year-old, female, part of which was fixed in formalin in the usual manner, the other being immediately frozen in dry ice acetone from which cryostat sections were made. All cryostat sections were fixed in absolute ethanol at −20C for ten minutes.

Antibodies

The following antibodies were used; A rabbit polyclonal antibody against total keratin from human callus was purchased from Transformation Research Inc., USA. It was used at a dilution of 1:400. A mouse monoclonal human anti-cytokeratin antibody (Catalog. No. 7650) was purchased from Becton Dickson, California, USA, which has specificitiy for cytokeratins of molecullar weights 50, 43 and 39 kilodaltons (Moll's catalogue numbers 8, 18 and 19; Moll et al., 1982). It was diluted at 5 micrograms per milliliter. A mouse monoclonal antibody against human vimentin (Catalog No. MVi, molecular weight 57 kilodaltons) was purchased from Bio-Science Products AG, Switzerland, and was used at a dilution of 1:10. A rabbit anti-bovine gamma enolase antibody (anti-neuron specific enolase, NSE), a generous gift from Dr. Kuramitsu (Institute of Neuropathology, Kyushu University) was used at a dilution of 1:500.

Immunohistochemistry

The indirect immunoperoxidase method using an Avidin-Biotin system (ABC) (Hsu et al., 1981), was performed on the formalin-fixed and paraffin-embedded material and on the cryostat sections. The paraffin sections were deparaffinised, then for cytokeratin immunohistochemistry only, the sections were digested with 0.1% trypsin solution for 20 to 30 minutes at room temperature. All were washed, then incubated with 0.3% hydrogen peroxidase in 0.01 M phosphate buffered saline (0.15 M NaCl) pH 7.4 to consume endogenous peroxidase. They were incubated with the appropriate primary antibody overnight at room temperature. After washing, biotinylated goat anti-rabbit or horse anti-mouse immunoglobulin (Vector Laboratories, USA) was applied for 30 minutes, then incubated with a solution of avidin and biotinylated horseradish peroxidase (Vector Laboratories, USA) for another 30 minutes. The color was developed using a solution of 0.03% 3-3' diaminobenzidine. For the cytokeratins, human epidermis stained simultaneously with the tumor sections was used as a positive control. As a negative control, pre-immune rabbit or mouse serum instead of the primary antibody were used. For NSE, human colon incubated with the primary antibody or pre-immune rabbit serum was used as positive and negative controls respectively. For vimentin, normal fresh lung tissue was used as positive and negative controls.

Results

Morphology

13 cases were of oat cell subtype, and in another 8, areas showing an intermediate cell pattern were found. 8 were of intermediate cell subtype, of which two had a large cell pattern in part. One was a combined oat cell and adenosquamous carcinoma. Foci of squamous or adenoid differentiation were seen in two oat and two intermediate cell subtypes. Syncytial giant cells and tubules or rosettes were frequent in both varieties. Grimelius positivity was present in 21 cases (Table 1).

Immunohistochemistry

a) Cytokeratin

In normal lung tissue, both the poly- and monoclonal antibodies stained the cytoplasm of all bronchial and bronchiolar cells (Fig. 1a), only some of the alveolar cells were stained by the monoclonal antibody. In the human epidermis used as positive control the polyclonal antibody showed a dense reaction product in the whole thickness of the epidermis and the ductal epithelium of seat glands were also positively stained, but non-epithelial tissues were not stained (Fig. 1b). The monoclonal antibody (Fig. 1c) showed reaction products only in the epithelium of sweat glands. Non-immune sera showed no reaction product. Both antibodies gave cytoplasmic reaction product in cryostat sections, and similar results in formalin fixed sections after trypsinisation. When trypsinisation was omitted, only very faint cytoplasmic reaction product was identified in a few cells (Fig. 2d). The formalin fixed- and paraffin-embedded tumor tissue revealed reaction products in the cytoplasm of 14 cases (46%) using the polyclonal antibody. This was spotty and was found only in a small proportion of cells (Fig. 3a). With the monoclonal antibody, 18 cases (60%) showed reaction products in the cytoplasm of a higher proportion of cells which was more diffuse compared to the reaction product with the polyclonal antibody (Fig. 3 and Table

2). Both oat and intermediate cells as well as tubules and rosettes were stained. Syncytial giant cells were rarely stained, but areas showing squamous or adenoid differentiation were always positively stained. Eleven of the oat cell tumors and two of eight intermediate cell tumors were cytokeratin positive (Table 3). Of the 14 cytokeratin positive cases, 11 were Grimelius positive and 10 of the cytokeratin negative cases were also Grimelius positive.

b) Vimentin

Vimentin positivity was observed in the cancer cell lines in culture and in the stroma of cryostat sections of fresh material (Fig. 2e). Formalin-fixed and paraffin-embedded tissues were negative.

c) Neuron-specific Enolase

In the human colon used as control, the ganglion cells in the intermuscular (Fig. 4a) and submucosal plexuses showed a dense reaction product. In addition, nerve fibres within the muscle layer were also positively stained. No reaction product was seen in the negative controls. The nerves within the tumor tissue were positively stained and were used as internal controls (Fig. 4b). In the tumor tissue 18 cases (60%) were positively stained and 14 of these and another 7 in the NSE negative group were Grimelius positive. Reaction product was scattered in the cytoplasm of cancer cells (Fig. 4c). Giant cells were frequently stained but areas showing squamous, large cell or adenoid differentiation were not stained. Oat cell tumors gave a higher proportion of positivity, 15 out of 21, than intermediate cell tumors, two out of eight (Table 3). Ten cases (nine with polyclonal antibody) were positive for both cytokeratin and NSE. In these cases, cytokeratin and NSE reaction products were seen in the same cells when serial sections were stained and compared (Fig. 5).

Table. 1. Histological characteristics of small cell carcinomas at autopsy.

Category	Oat cell subtype		Intermediate subtype	Combined subtype
	oat cell	oat and interm.		
Tubules or rosettes	2	5	6	1
Syncytial giant cells	5	5	3	–
Adenoid or squamous diff.	–	2	2	–
Large cell pattern	–	–	2	2
Adenosquam. carcinoma	–	–	–	1
Grimelius positive	11	8	2	–
Total Number	13	8	8	1

NB. 1. In the oat cell subtype, one case had tubules, syncytial giant cells and was Grimelius positive, and 4 had giant cells and were Grimelius positive.
2. In the oat and intermediate cell subtype, 2 had tubules, giant cells, and Grimelius positivity, 2 had giant cells and Grimelius positivity. One each had either tubules or giant cells and Grimelius positive. One each had tubules, giant cells, Grimelius reaction and either squamous or adenoid differentiation.
3. In the intermediate subtype, two had a Grimelius reaction and tubules, one had tubules and giant cells, two had a large cell pattern and had giant cells. One had giant cells and two had tubules.

Table 2. Summary of immunohistochemical findings in small cell carcinomas and in small cell carcinoma cell lines.

Case	Polyclonal anti-cytokeratin	Monoclonal anti-cytokeratin	Monoclonal anti-vimentin	Neuron-specific Enolase
In vitro				
QG 90	–	–	+	NT
QG 96	–	–	+	NT
In Vivo				
QG 90	–	–	+(stroma)	NT
QG 96	–	–	+(stroma)	NT
Alcohol fixed tumor tissue	+	+	+(stroma)	NT
Formalin fixed tumor tissue (30 cases)	+(14)	+(18)	–	+(18)

Key: + = positive, – = negative, NT = not tested.

Table 3. Cytokeratin and neuron– specific enolase staining pattern by histologic subtype in small cell carcinomas.

Reaction	Oat cell subtype: oat cell	Oat cell subtype: oat and interm.	Intermediate cell subtype	Combined subtype
Cytokeratin positive	7	4	2	1
Cytokeratin negative	6	4[1]	6[1]	–
NSE positive	9	6	2	–
NSE negative	4	2	6	1
Total Number	13	8	8	1

[1]Two cases each were positive with the monoclonal anti-cytokeratin antibody.

Fig. 1a. A bronchiole from a 63-year-old male with an oat cell carcinoma stained with the polyclonal anti-cytokeratin antibody. Bronchiolar cells show cytoplasmic reaction product. At (a) is an area with desquamation of the epithelium. × 420

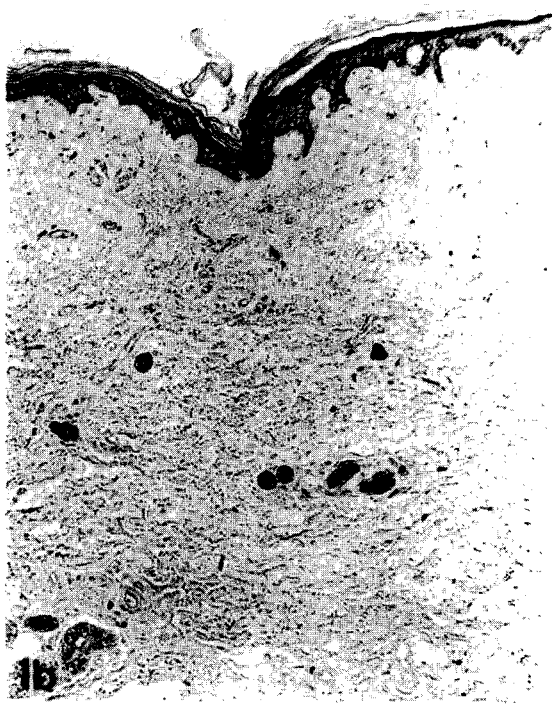

Fig. 1b. Normal human epidermis used as positive control stained with the polyclonal anti-cytokeratin antibody. Strong diffuse positivity in all layers of epidermis. Presence of melanin accentuates the positivity in basal cell layer. The sweat glands are also positively stained. × 150

Fig. 1c. Same specimen as 2b, stained with the monoclonal anticytokeratin antibody. The epidermis (top of figure) is unstained, the sweat glands are positively stained. × 60

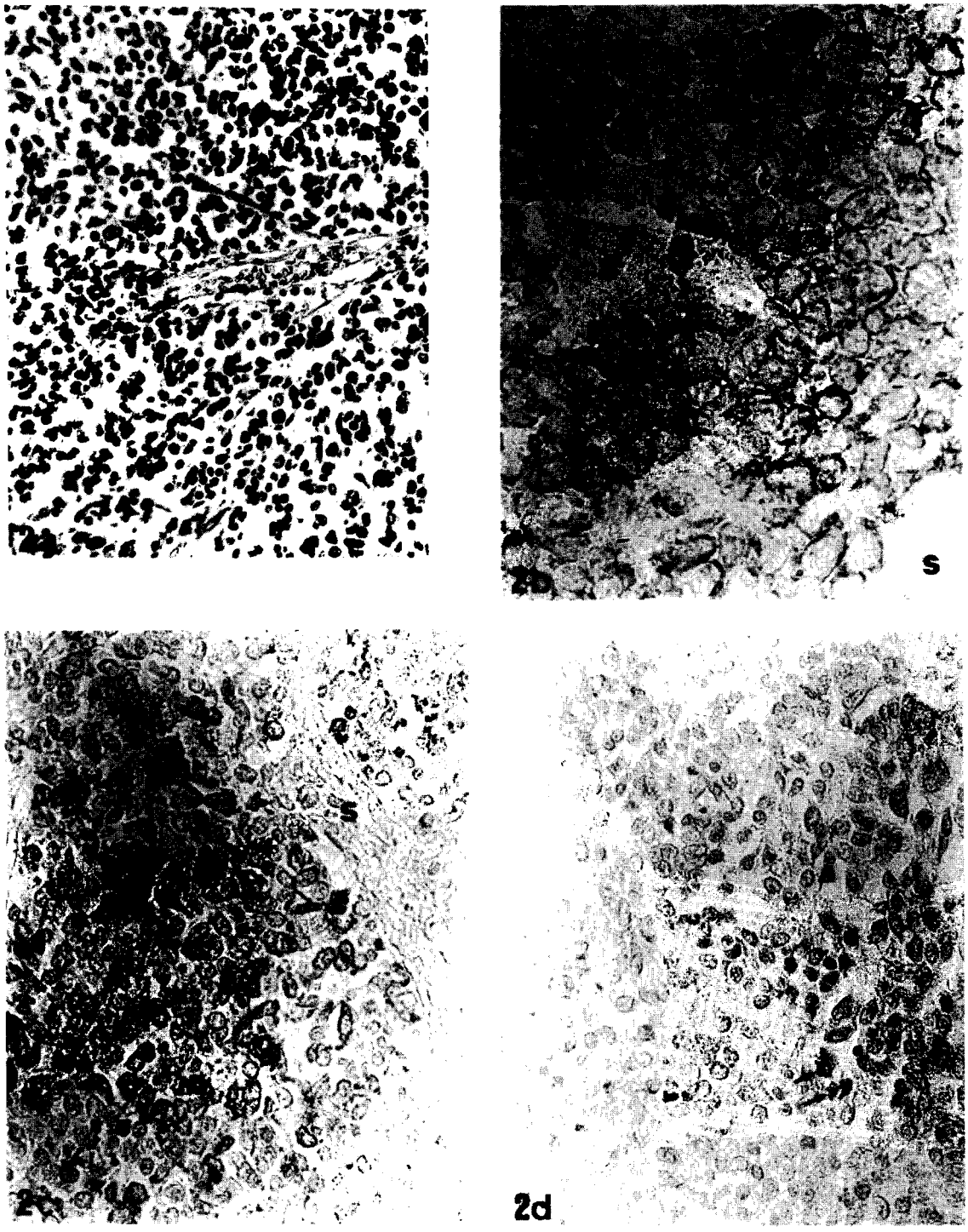

Fig. 2. Light microscopic and immunohistochemical findings in a 74-year-old female, with an oat cell carcinoma taken four hours post mortem. (a) Haematoxylin and eosin staining of formalin-fixed material showing small tumor cells with characteristic dense chromatin and scanty cytoplasm. × 220. (b) A cryostat section stained with monoclonal anti-cytokeratin antibody. Reaction product is seen diffusely in the cytoplasm of most cells, but the stroma (s), is unstained. × 800. (c) Formalin-fixed and paraffin-embedded section stained with the monoclonal antibody after preliminary trypsinisation. Reaction product is seen in most cells as in the cryostat section. Stroma (s), is unstained. × 450. (d) Section as in (c) above, but without prior trypsinisation. Reaction product is barely visible in a few cells (arrows). × 450. (e) Cryostat section stained with the monoclonal anti-vimentin antibody. Tumor cells (t), are unstained, but the endothelial cells of stroma (s), are strongly stained. × 220

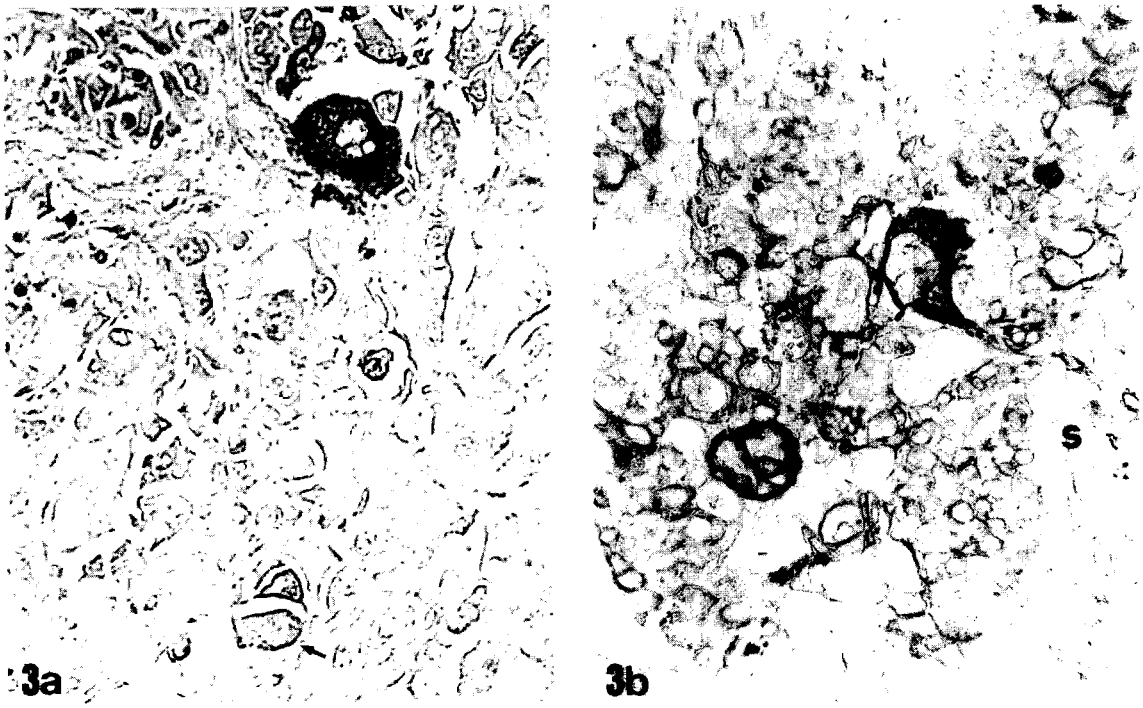

Fig. 3. Immunohistochemical findings in poly- and monoclonal anti-cytokeratin antibodies on formalin fixed and paraffin embedded tissue. (a) Fifty-nine-year-old male with an intermediate type of tumor stained with polyclonal antibody. Only a few cells of giant cell type have reaction product (arrows) in their cytoplasm. × 560. (b) Same cases as above stained with monoclonal antibody. Besides the giant cells, diffuse positivity is evident in most of the tumor cells. Stroma (s), is unstained. × 250. (c) A section taken from a 91-year-old female who had an intermediate type of tumor, stained with monoclonal antibody. Strong diffuse positivity is seen in most of tumor cells, but stroma (s), is unstained. × 270

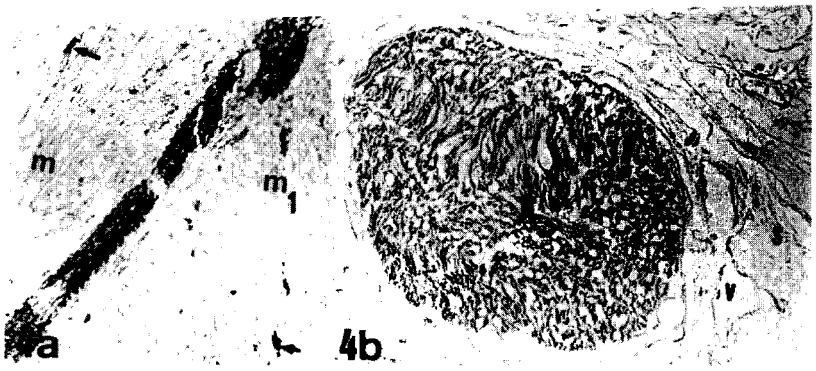

Fig. 4a. Human colon used as NSE positive control. A dense reaction product is seen in the ganglion cells and nerves between the longitudinal (m) and circular (m_1) muscle layers. Small nerve fibres within the muscle (arrows) are also positively stained. × 235

Fig. 4b. A nerve in the tumor mass of a male, 72, with an oat cell carcinoma showing a positive reaction, but the stroma (s), and vein (v), are unstained. Anti-neuron specific enolase, × 500

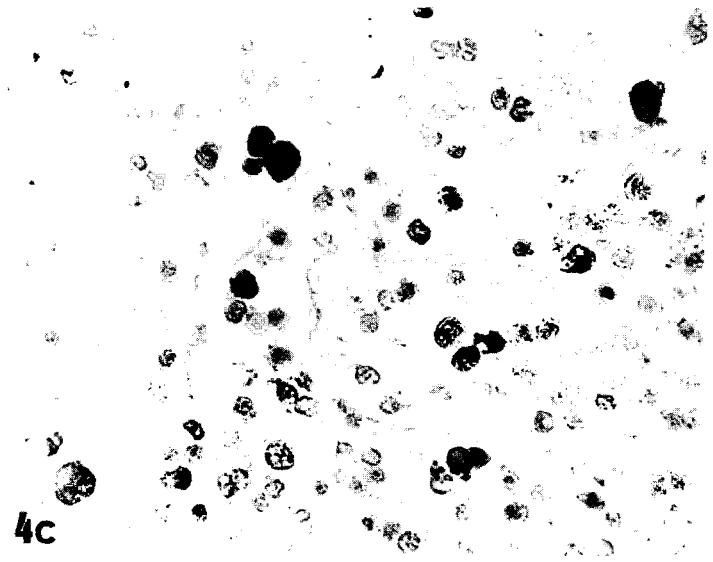

Fig. 4c. Same case as Fig. 4b, stained with anti-NSE. Reaction product is seen in several tumor cells, × 500

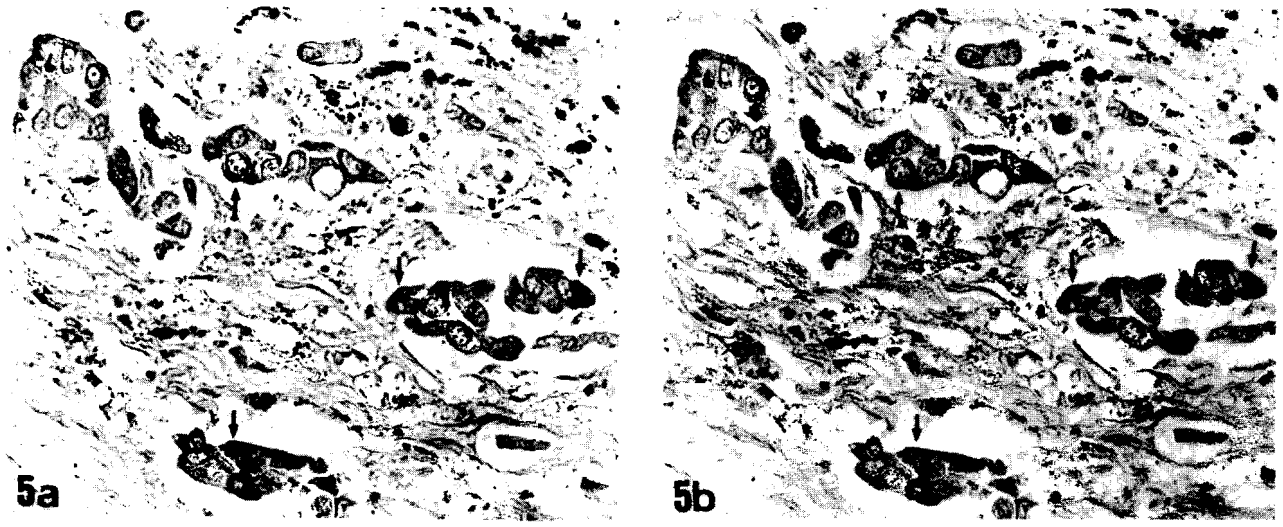

Fig. 5. Male, 84, with an oat cell subtype with intermediate cells. Two serial sections stained for Cytokeratin (a) and NSE (b). Arrows show reaction products in several tumor cells that are positive for both markers. × 410 respectively.

Discussion

The monoclonal antibody whose specificity is directed against cytokeratins characteristic of simple epithelia showed a stronger and a more diffuse positivity than the polyclonal antibody which showed only focal and rather weak positivity (Fig. 3). This may be due to the fact that the polyclonal antibody was prepared from the callus of stratified squamous epithelium. This has a characteristic cytokeratin pattern that is different from that of simple epithelia, like the respiratory epithelium, which the monoclonal antibody had specificity for (Moll et al., 1982; Blobel et al., 1984; Cooper et al., 1985). Formaldehyde fixation has been shown to adversely affect the stainability of cytokeratin compared to alcohol fixation or cryostat sections (Altmannsberger et al., 1981; Banks-Schlegel et al., 1984). As also reported by others (Pinkus and Corson, 1985), we found that preliminary trypsinisation restores optimal immunoreactivity of cytokeratin in formalin-fixed and paraffin-embedded tissues especially if used with an appropriate antibody (Fig. 2b,c,d). For small cell carcinomas of the lung, the antibody should have specificity directed against cytokeratins characteristic of simple epithelia.

A lack of cytokeratin expression in small cell carcinomas has been reported (Lehto et al., 1983; Bergh et al., 1984). Contrariwise, cytokeratin expression in small cell carcinoma tissue and in small cell carcinoma cell lines has been demonstrated (Saba et al., 1983; Van Muijen et al., 1984; Blobel et al., 1985; Broers et al., 1985). We found that the oat cell subtype of tumor has higher positivity for both cytokeratin and NSE than the intermediate cell subtype. We do not know whether this means that this subtype has better differentiation than the intermediate cell subtype. This data seems to indicate that there are two subtypes of small cell carcinoma, one is cytokeratin positive, and the other is cytokeratin negative in immunohistochemical studies. Carney et al. (1985) have shown that cancer cell lines of classical small cell carcinoma express cytokeratin, while those of variant small cell carcinoma lack cytokeratin, but express vimentin and neurofilament. Variant small cell carcinoma cell lines are chemo- and radioresistant compared to cell lines of classical small cell carcinoma. It may be, therefore, that these two subtypes of small cell carcinoma have different biologic behaviour. In in vitro studies, a failure of cytokeratin expression has been shown to be associated with a loss of cell to cell interaction and with dedifferentiation (Winter et al., 1980; Venetianer et al., 1983; Ben-ze'ev, 1984). In vivo studies showed a more invasive growth pattern and a shorter survival time in rats injected with a cytokeratin poor cell line compared to those injected with a cytokeratin rich cell line (Hirono et al., 1964; Kinjo et al., 1984).

Neuron-specific enolase, a neuroendocrine marker, could be demonstrated in 60% of our patients. Coexpression with cytokeratin was seen in ten cases. Similar findings have been reported recently (Sheppard et al., 1984; Blobel et al., 1985). This would indicate that neuroendocrine differentiation may occur in cells showing a typical epithelial phenotype, ie., cytokeratin positivity.

Because the expression of cytokeratin is a characteristic of epithelia and epithelium-derived tumors (Gabbian et al., 1981; Franke et al., 1982; Osborn and Weber, 1982, 1983), the widespread cytokeratin positivity we found in this study indicates that all the major types of lung carcinoma, including small cell carcinomas, have a common epithelial origin. Such a common cell of origin would explain the frequent occurrence of heterogeneity in lung carcinomas that is found by both light (Hirsch et al., 1983; Roggli et al.,

1985) and electron microscopy (Mennemeyer et al., 1979; Horie and Ohta, 1981; Saba et al., 1983; Banks-Schlegel et al., 1984).

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