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Immunohistochemical characterization of an anti-epithelial monoclonal antibody (mAB lu-5)

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Summary. A mouse monoclonal antibody (mAB lu-5) was prepared using a lung cancer cell line as an antigen. The selected clone produces an IgG with a gamma-1 heavy chain and a kappa-light-chain. Immunohistochemical testing of mAB lu-5 on 117 normal tissue biopsies and 474 tumours revealed reactivity with an intracytoplasmic, formaldehyde-resistant antigen present in most epithelial and mesothelial cells, but absent in mesenchymal cells. The antibody can therefore be used as a first order, pan-epithelial marker. It proved also useful for fast tumour diagnosis on frozen sections.

Key words: Monoclonal pan-epithelial antibody – Immunohistochemistry – Tumour diagnosis

Introduction

Monoclonal antibodies are able to characterize normal and malignant cells by recognizing tissue-specific, cell-line-specific or differentiation-specific antigens. Each type of reactivity has its merits in the differential diagnosis of tumours. A combined use of antibodies would eventually allow the specification of the tissue origin of a tumour and its grade of differentiation, which are important in prognosis and therapy.

We report here the preparation and testing of a mouse monoclonal antibody with characteristics of a pan-epithelial marker and a high immunohistochemical diagnostic efficiency on frozen and paraffin-embedded normal tissues and tumours.

Material and methods

Tissues. 474 tumours and 117 non-neoplastic tissue probes were investigated. For the study of frozen material 94 tumour specimens and 74 non-neoplastic tissue biopsies were collected

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from surgical material. Within minutes after excision multiple cubes of $1 \times 1 \times 0.5$ cm were snap-frozen and kept in air-tight plastic bags at $-70°$ C. In addition, autopsy material ($n = 18$) obtained within less than 24 h after death was processed identically. 405 biopsy and surgical specimens (380 neoplastic, 25 non-neoplastic) were fixed in 4% buffered formaldehyde or in SUSA's fixative (bone marrow biopsies, $n = 4$), dehydrated with alcohol or acetone and routinely embedded in Paraplast.

Antibody preparation. The antibody (mAB lu-5) was prepared by one of us (C.S.) essentially as follows: BALB c/j mice were immunized intraperitoneally with cells of a lung cancer cell line A549 and A2182 cells (Lieber et al. 1976; obtained from Dr. S. Aaronson, NCI, Bethesda, USA). 3. days after the second booster injection the fusion with mouse myeloma PAI cells (non-secreting myeloma derived from the line X63; Stocker et al. 1982) was carried out according to the procedure of Köhler and Milstein (1975). The cultures were screened by a solid phase antibody binding assay (SABA; Stähli et al. 1980; Stähli et al. 1983). The clone selected for this study produces an IgG with a gamma-1 heavy-chain and a kappa-light-chain. For this study supernatant was used up to a dilution of 1:4.

Immunofluorescence. Cryostat sections (4–6 μm) were collected on glass slides treated previously with chromic-acid and gelatine. The sections were air-dried, washed with phosphate-buffered saline (PBS) and incubated for 15 min with mAB lu-5. After two washes (5 min each) the sections were incubated with fluorescein-isothiocyanate (FITC)-conjugated sheep-anti-mouse IgG for 15 min. This antiserum was obtained after multiple booster injections with mouse gammaglobulin. A DEAE-cellulose IgG fraction was labeled with FITC according to Clark and Shepard (1963). The conjugate had a protein content of 8 μg/ml with a molar FITC ratio of 3.4, and was used at a working dilution of 1:10. On human tissues, this antiserum did not cross-react with tissue or serum components.

Controls included use of PBS as first layer and parallel incubation of known positive and negative test tissues.

Short incubation method (immunofluorescence). In order to make a fast immunodiagnosis on tumour material a short incubation method was developed. Unfixed sections (6 μm) obtained from biopsy specimens were frozen in a jet of CO_2. The sections were then air-dried (2–3) min with a fan, quickly immersed in PBS and immediately incubated with mAB lu-5 or PBS as control respectively (5 min, 37° C). Following a short wash in PBS (3–5 min) under gentle agitation, the sections were incubated with a commercial FITC labeled goat anti-mouse IgG (Nordic, lot Nr. 1–482, 10 min, 37° C), diluted 1:10. The sections were examined immediately with a fluorescence epiillumination microscope, usually not later than 30 min after receipt of the tissue sample. 88 biopsies were incubated under these conditions and the results were compared with those obtained with the conventional incubation.

Immunoperoxidase staining of paraffin embedded tissue. Sections (5 μm) were collected on glass slides pretreated with glue diluted in tap water (1:10 v/w; Cementit, Merz and Benteli SA, CH-3172 Niederwangen) and dried at 37° C for 24 h. After deparaffinization in xylene and rehydration, the specimens were brought into TRIS-buffer and treated with pronase (Sigma, Typ XIV, 0.1% in 0.5 M TRIS-buffer, pH 7.5, 37° C, 15 min), according to Huang (1975). The sections were then placed in ice cold TRIS-buffer for 30 min to stop the pronase activity. They were subsequently incubated with the primary antibody overnight at 4° C. The reaction was visualized by the avidin-biotin complex (ABC) method (Guesdon et al. 1979) using a commercial kit (Vector Laboratories, incubation schedule according to the instructions). The label was developed in 0.05% (w/v) diaminobenzidine-tetrahydrochloride under microscopic control, followed by counterstaining with hematoxylin, dehydration and mounting in Eukitt (Kindler, Freiburg i.Br., GFR). The specificity was controlled as described above.

To establish the reproducibility of the staining on formalin-fixed paraffin embedded material, we made a comparative study: frozen and fixed samples of the same specimen of 39 carcinomas (breast: 13, respiratory tract: 8, genital tract: 4, gastrointestinal tract: 5, skin: 2, endocrine glands: 5, thymomas: 2) and of 7 non-epithelial tissues (soft tissue sarcomas: 3, lymphomas: 2, normal spleen: 1, lymph node talcosis: 2) were incubated in parallel with an indirect immuno-

fluorescence technique and the ABC-method respectively. The results obtained on frozen and fixed tissue were identical. For this reason the extensive testing was subsequently carried out on formalin-fixed paraffin embedded material.

For both methods the reactivity was graded according to the proportion of reactive cells (estimated percentage) and staining intensity (+ to + + +).

Polyclonal anti-keratin serum. For a comparative study with polyclonal anti-keratin serum (Rabbit anti-human keratin, Ortho, Lot 024166) we applied the peroxidase antiperoxidase method (Sternberger et al. 1970). We incubated paraffin sections of 9 epidermoid carcinomas, 44 adenocarcinomas, 8 transitional cell carcinomas, 5 mesotheliomas, 8 oat-cell carcinomas and 10 carcinoids. The incubation was made according to the instructions of the kit.

Results

Comparison of the results obtained on frozen and paraffin sections

The results obtained on the 39 carcinomas were identical with respect to the number of stained cells and staining intensity. Non-epithelial tissues were invariably negative. It was concluded that the results obtained with unfixed frozen and fixed paraffin embedded sections could be combined in the subsequent evaluations.

Non-neoplastic tissue

The results of the staining with mAB lu-5 on a series of non-neoplastic tissues are summarized in Table 1. In 107 specimens of various organs the mAB lu-5 specifically stained all types of epithelia (Figs. 1a, 2a, 2c, 3), and mesothelial cells (Fig. 2b). The various types of epithelia showed a rather uniform fibrillar cytoplasmic staining pattern (Figs. 3 and 4) suggesting a reactivity of the antibody with cytoskeleton structures. The usual reactivity was of the all or none type. The results obtained on autopsy material were identical to those of surgical samples. The number of stained cells and the intensity of the reaction were high (60–100, and + + + respectively). For this reason the grading is not indicated in the tables. There were some exceptions to the rule: acinar and secretory cells were usually weakly stained, the extent of the reaction depending on vacuolization; umbrella cells of transitional epithelium showed a much more intense staining than the other cell layers (Fig. 2c); epithelium of bile duct and pancreatic ducts displayed a considerably more intense staining than the parenchymal cells. Normal epithelium adjacent to the tumour showed the same reactivity as epithelium distant from the tumour. Cell nuclei were not stained.

There was a focally positive reaction in some but not all specimens of the adrenal cortex and of synovial epithelium.

There was no immunoreaction in connective tissue, blood and lymphatic vessels, or smooth or striated muscle. The only exception was a weak cross reaction with smooth muscle cells of myometrium, prostate and gastric muscularis mucosa in both fixed and unfixed tissue probes. This non-specific reaction disappeared upon the use of the antibody at a dilution 1:4 which did not affect the intense reaction of epithelial cells.

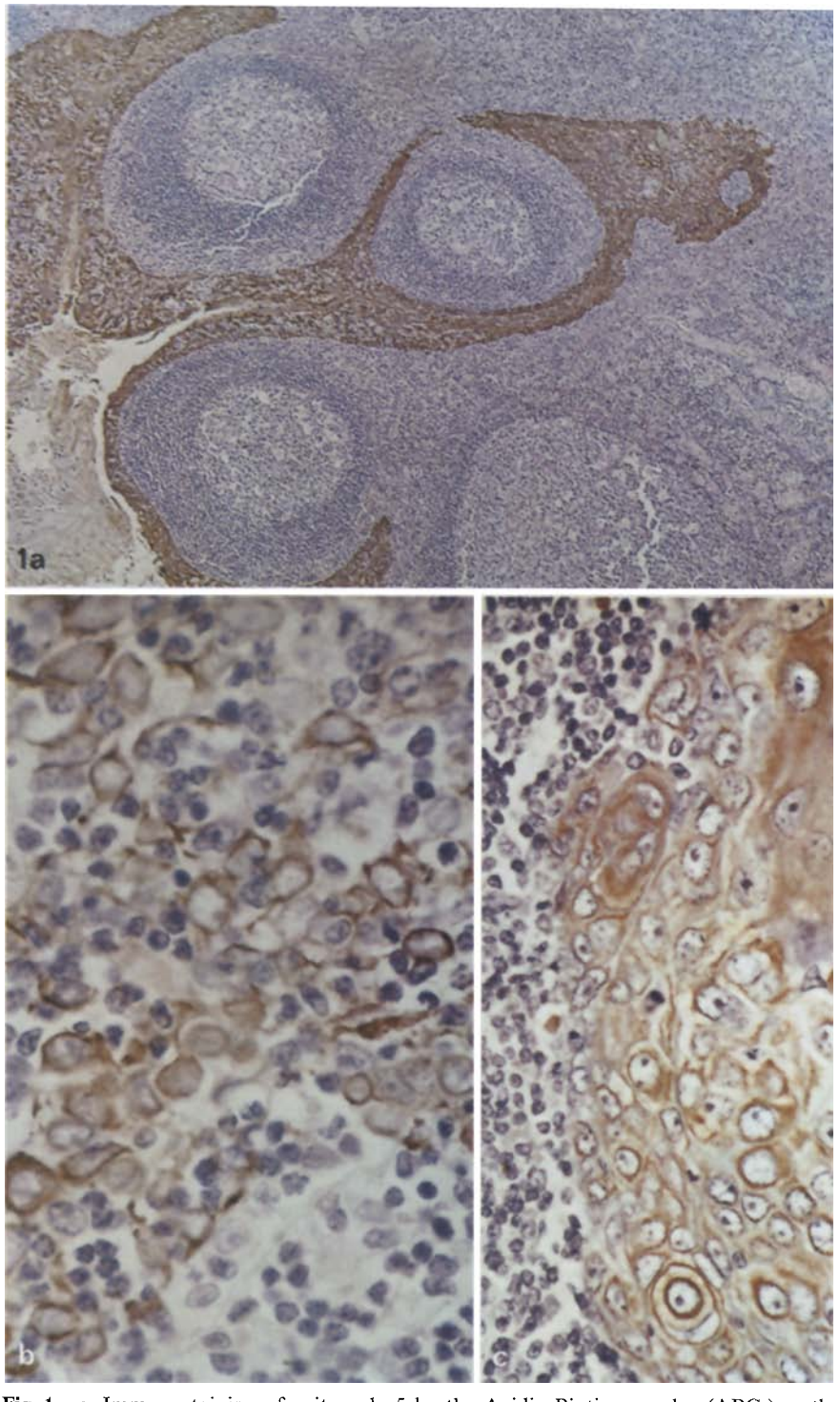


Fig. 1 a–c. Immunostaining of epitope lu-5 by the Avidin-Biotin complex (ABC-) method of normal and neoplastic epithelia in formalin-fixed, paraffin-embedded material, counterstained with haematoxylin: **a** Selective staining of squamous epithelium in chronic hyperplastic tonsillitis (100 ×). **b** Demonstration of epithelial component of thymoma (400 ×) **c** Identification of metastatic squamous cell carcinoma in a lymph node (252 ×)

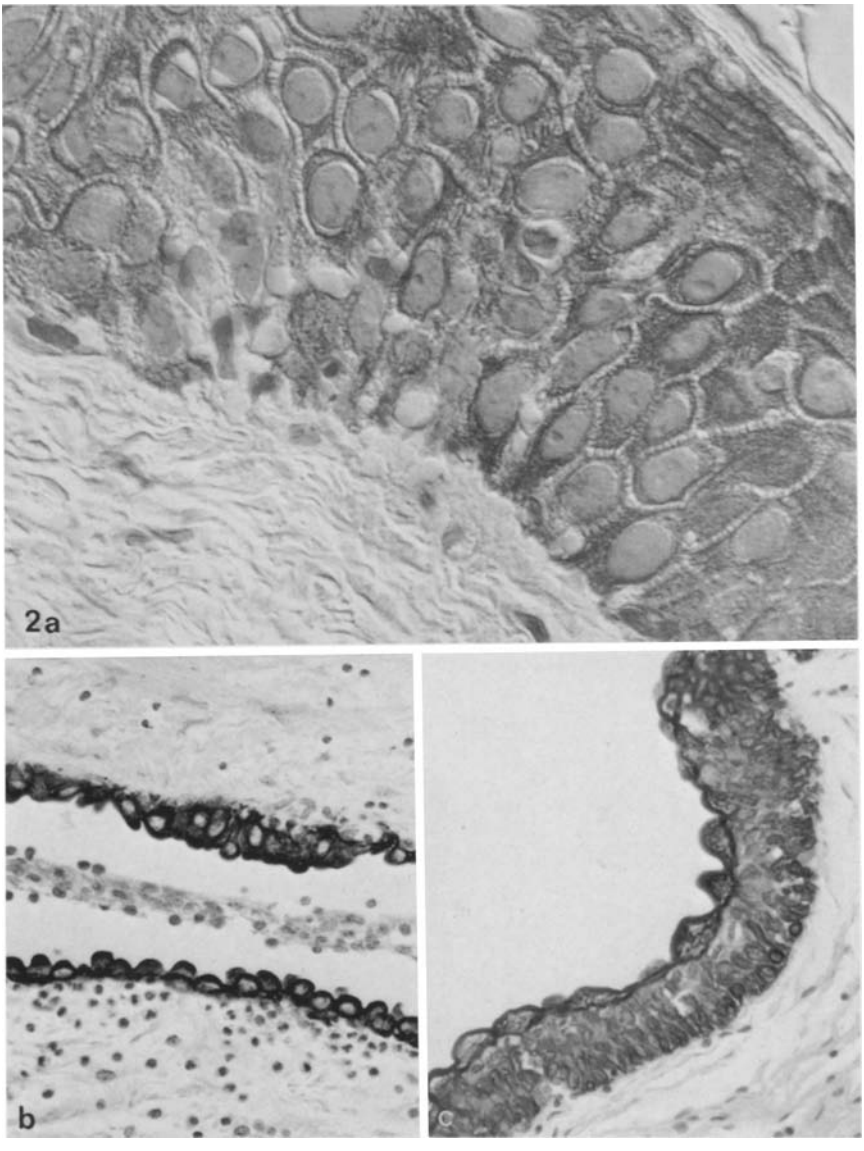
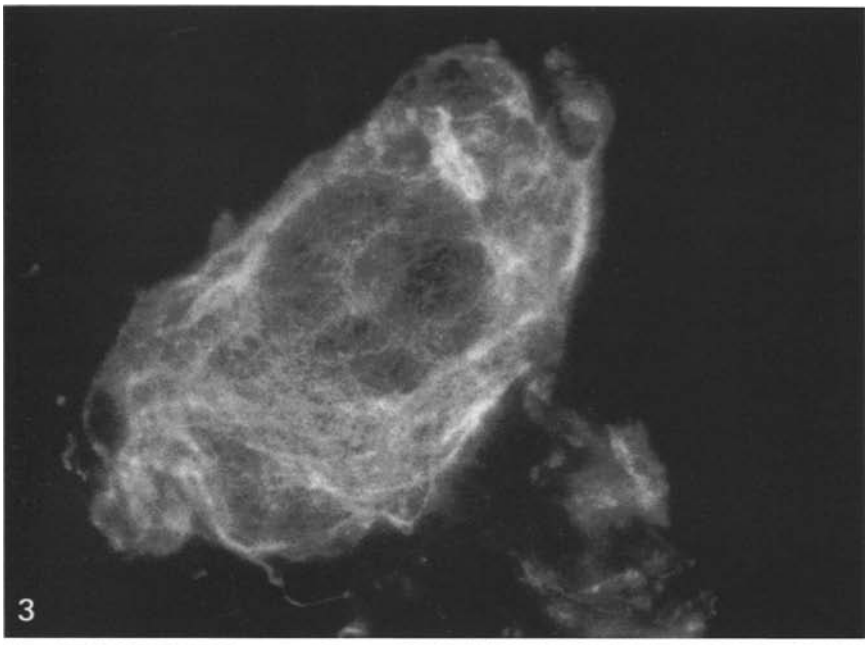


Fig. 2a–c. Immunostaining of epitope lu-5 by the ABC-method of **a** normal skin (800×) (differential interference contrast optics) **b** normal peritoneal mesothelial cells (350×), and **c** normal transitional epithelium (350×). Formalin-fixed, paraffin-embedded material; counterstained with haematoxylin

Tumours

The results obtained with 369 epithelial and 105 non-epithelial tumours are listed in Tables 2 and 3. Virtually all epithelial tumours were reactive to mAB lu-5 (Fig. 5a, b). The following exceptions were noted: 5 theca cell and 1 granulosa cell tumours of the ovary, 1 bronchial carcinoid (probably due to bad tissue preservation) and 9 of 13 tumours of the adrenal cortex.



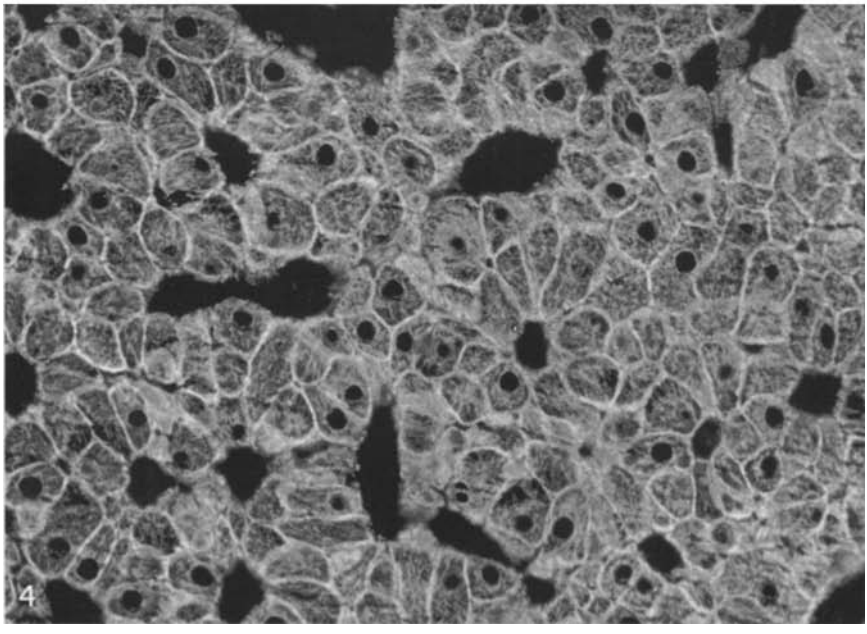


Fig. 3. Cytological smear of a breast carcinoma stained with mAB 1u-5. Fibrillar pattern of the reaction. Fixation in acetone, indirect immunofluorescence (840 ×)

Fig. 4. Visualization of epitope lu-5 in normal liver parenchyma. Frozen section, indirect immunofluorescence (220 ×)

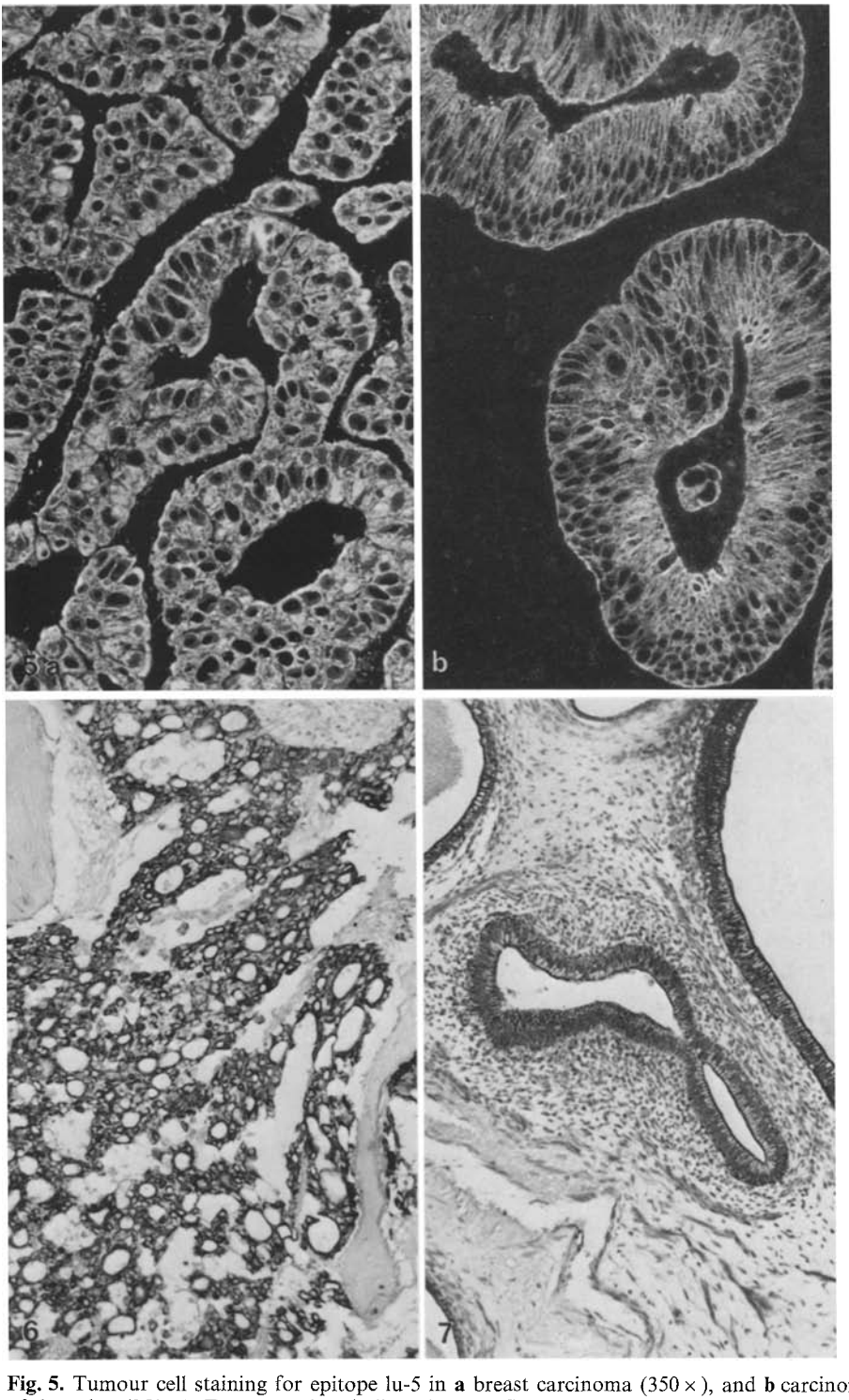


Fig. 5. Tumour cell staining for epitope lu-5 in **a** breast carcinoma (350 ×), and **b** carcinoma of the colon (350 ×). Frozen section, indirect immunofluorescence

Fig. 6. Presence of epitope lu-5 in a bone marrow metastasis of a prostate carcinoma. Susa's fixative, paraffin-embedded material, ABC-method. Counterstained with haematoxylin (88 ×)

Fig. 7. Positive reaction for epitope lu-5 in epithelial components of a teratocarcinoma of the testis. ABC-method. Formalin-fixed, paraffin-embedded material. Counterstained with haematoxylin (56 ×)

Table 1. Reaction of normal tissues with mAB lu-5 ($n=117$)

Positive reaction[a]	84/84	Focally positive reaction	5/10
Gastrointestinal tract		Adrenal cortex	3/7
Stomach	1/1	Synovial epithelium	2/3
Colon	6/6	Negative reaction	0/26
Liver	12/12	Spleen	0/4
Pancreas[b]	3/3	Muscle	0/2
Salivary gland[b]	1/1	Myocardium	0/2[c]
Respiratory tract		Myometrium	0/4
Tonsil	10/10	Brain (cortex)	0/3
Bronchial and alveolar epithelum	9/9	Nerves	0/2
Pleura	1/1	Lymph node	0/6
Urogenital tract			
Kidney	15/15		
Prostate	8/8		
Epididymis	4/4		
Ovary	2/2		
Vagina	1/1		
Fallopian tube	1/1		
Breast[b]	3/3		
Thyroid	4/4		
Epidermis (all layers)	3/3		

[a] Positive reaction = immunostaining of the epithelial part of the organ
[b] Including myoepithelial cells
[c] Weak reaction with undiluted antibody which disappears at a dilution of 1:4 without affecting the staining of epithelial cells in control sections

In 12 "mixed" tumours, i.e. carcinosarcoma, thymoma (Fig. 1b), pleomorphic adenoma and adenolymphoma, the antibody stained exclusively epithelial components. The epithelial structures of teratocarcinomas (Fig. 7) were always positive while mesenchymal parts were consistently negative. Occasionally areas of spindle cells (morphologically not classifiable as epithelial) were reactive with the antibody at a 1:4 dilution.

In 27 primary tumours and metastases thereof there were no apparent differences in the staining pattern, the percentage of positive cells and the intensity of staining (Figs. 1c and 6). 9 of 10 epithelial metastases of tumours of unknown localization were positive; one anaplastic metastasis, morphologically considered epithelial, was negative.

Table 4 shows that the mAB lu-5 stained different histological types of carcinomas ($n=251$). Polyclonal anti-keratin antibody stained all mesotheliomas (5/5) and all epidermoid (9/9) carcinomas, but only 2 of 8 transitional cell carcinomas and only 34 of 44 adenocarcinomas. All oat-cell carcinomas (0/8) and carcinoids (0/10) were negative.

105 non-epithelial tumours were invariably unreactive with mAB lu-5 (Table 3). A weak reactivity with the undiluted antibody was observed in 1 leiomyosarcoma of the uterus. This reaction was abolished by using a 1:4 dilution of the antibody.

Table 2. Reaction of mAB lu-5 with epithelial and mesothelial tumours ($n=369$)

Gastrointestinal tract	73/73
Tongue	1/1
Esophagus	1/1
Stomach	9/9
Small intestine	14/14
Colon	31/31
Salivary glands	2/2
Pancreas (exo)	1/1
Pancreas (endo)	11/11
Liver carcinomas:	
Hepatocellular	2/2
Cholangiocellular	1/1
Urogenital tract	72/78
Uterus (corpus)	9/9
Cervix	2/2
Ovary	9/15[a]
Vulva	1/1
Endometriosis	1/1
Testis:	
Teratocarcinoma	13/13[b]
Prostate	16/16
Kidney	7/7
Bladder	14/14
Respiratory tract	78/79
Epidermoid carcinoma	14/14
Adenocarcinoma	7/7
Oat-cell carcinoma	10/10
Carcinoid	47/48
Breast	40/40
Endocrine tumours	48/57
Thyroid	19/19
Parathyroid	3/3
Adrenal cortex	4/13
Pituitary	19/19
Paraganglioma	3/3
Skin	11/11
Epidermoid carcinoma	2/2
Merkel-cell tumor	9/9
Metastases of unknown primaries	9/10
Adenocarcinoma	3/3
Epidermoid carcinoma	3/3
Undifferentiated	3/4
Mixed-tumours[b]	12/12
Carcinosarcoma	5/5
Parotid gland:	
Adenolymphoma	2/2
Pleomorphic adenoma	5/5
Mesothelial tumours	9/9
Mesothelioma	8/8
Biphasic synovialoma	1/1[b]

[a] 5 theca cell and 1 granulosa cell tumors were negativ
[b] Reaction restricted to the epithelial part

Table 3. Reaction of mAB lu-5 with mesenchymal tumors ($n=101$) and seminomas ($n=4$)

Neural tumours	0/14
Astrocytoma	0/4
Medulloblastoma	0/3
Neurinoma	0/4
Meningioma	0/3
Melanoma	0/21
Seminoma	0/4
Lymphomas	0/25
Non-Hodgkin type	0/19
Hodgkin's disease	0/5
Plasmocytoma	0/1
Soft tissue tumours	0/41
Leiomyosarcoma	0/6
Leiomyoma	0/4
Liposarcoma	0/2
Fibrous histiocytoma	0/7
Hemangioendothelioma	0/6
Aggressive fibromatosis	0/2
Synovialoma (monophasic)	0/3
Others	0/11

Table 4. Reaction of various types of epithelial tumours to mAB lu-5 ($n=251$) and to a polyclonal antikeratin serum ($n=84$)

	mAB lu-5	Anti-keratin (polyclonal
Epidermoid carcinomas	24/24	9/9
Adenocarcinomas	123/123	34/44
Transitional cell carcinomas	14/14	2/8
Oat-cell carcinomas	10/10	0/8
Carcinoids	71/72	0/10
Mesotheliomas	8/8	5/5

Short incubation method

13 of 88 short incubations could not be interpreted for technical reasons (high background). We found 58 positive and 17 negative tumours. The parallel incubation with the usual indirect fluorescence method revealed that the short procedure yielded false negative results in 6 epithelial tumours. We did not find false positive results.

Discussion

The monoclonal antibody lu-5 recognizes an epitope of an intracytoplasmic, formaldehyde-resistant antigen expressed in many epithelial and mesothelial cells, including alveolar epithelial and myoepithelial cells. It recognized 352 of 369 epithelial tumours (95%) regardless of their localization and grade of differentiation. An occasional weak reaction with mesenchymal cells or tumours disappeared in early dilution steps (1:2 or 4) of the primary antibody indicating a low affinity binding compatible with cross-reactivity. Despite dilution steps, rare foci of undefined cells in embryonic tumours were weakly stained.

The antibody is neither organ- nor tumour-specific. On immunohistochemical grounds it may be defined as a pan-epithelial marker reacting with an epitope present in normal and malignant epithelial and mesothelial cells. Nathrath et al. (1985) described an epithelial-specific polyclonal antiserum recognizing the Tissue Polypeptide Antigen (TPA). In comparison with the lu-5 reactivity, this antibody is not strictly pan-epithelial because it fails to stain hepatocytes, pancreatic acinar cells, kidney proximal tubulus cells, and all layers of the epidermis. Unlike most other groups who characterized "pan-epithelial" antibodies using essentially an immunoblot technique (Debus et al. 1982; Gigi et al. 1982; Tseng et al. 1982), we defined the specificity of mAB lu-5 by a broad immunohistochemical study. The pan-epithelial distribution of the antigen and the fibrillar staining pattern suggest a close association with the cytoskeleton. Our results raise two possibilities: (1) the epitope could be part of a molecule associated with a cytokeratin or (2) the epitope could be part of a cytokeratin molecule. In the latter case the epitope must be shared by almost all cytokeratins described by

Moll et al. (1982). Preliminary biochemical experiments using methods for the isolation of intermediate filaments and the immunoblotting technique point towards a cytoskeleton-associated antigen.

In surgical pathology the most relevant application for mAB lu-5 is differential diagnosis of epithelial versus mesenchymal tumours, large cell lymphomas and neural tumours. The antibody may be used most efficiently in combination with other tissue markers recognizing non-epithelial differentiation antigens, i.e. epitopes of intermediate filaments of the vimentin-, desmin- and glial fibrillary acidic protein type (Osborn et al. 1983; Schauer et al. 1984). Such a panel of antibodies of the first order, including mAB lu-5 would permit a first step in a diagnostic algorithm of tumour diagnosis. In this respect mAB lu-5 is different from most anti-cytokeratins, the reaction of which is not pan-epithelial, but more restricted (Moll et al. 1983; Raemaekers et al. 1983). Antibodies to various cytokeratins therefore can be used in a second step in the algorithm, in combination with antibodies directed against organ-specific (Stein et al. 1982) or differentiation-specific antigens, e.g. carcinoembryonic-antigen, alpha-foetoprotein, mesenchymal-specific epitopes (Donner et al. 1983; Brooks et al. 1982).

In a satellite study we also showed that mAB lu-5 may play a key role in the diagnosis of neuroendocrine tumours. Merkel cell carcinoma, malignant melanoma and malignant lymphoma can be differentiated using a combination of mAB lu-5, antibodies to "neuron-specific" enolase and the glial protein S-100 (Heitz et al. 1984).

In addition, we have shown that the mAB lu-5 may be successfully used for fast tumour diagnosis on frozen sections. At present approximately 30 min are necessary to carry out the two layer incubation. Using a labeled mAB lu-5 (one layer incubation) we hope to diminish the proportion of false negative results and, eventually, to reduce the time for fast immunological typing of tumours.

We suggest that mAB lu-5 may be used as mesothelial and pan-epithelial marker for immunocytochemical differentiation of epithelial from mesenchymal tumours.

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