Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: an overview

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Summary

Intermediate-sized filament proteins (IFP) are tissue specific in that antibodies to keratin, vimentin, desmin, glial fibrillary acidic protein (GFAP) and the neurofilament proteins can distinguish between cells of epithelial and mesenchymal origin as well as of myogenic and neural origin respectively. Malignant cells retain their tissue-specific IFP, which makes it possible to use these antibodies in tumour diagnosis. Carcinomas are exclusively detected by antibodies to keratin. Monoclonal antibodies to keratin have allowed the differentiation between subgroups of epithelial tumours until now between adenocarcinomas and squamous cell carcinomas. Lymphomas, melanomas and several soft tissue tumours are distinctly recognized by antibodies to vimentin. On the other hand, rhabdomyosarcomas and leiomyosarcomas are positive for desmin, while astrocytomas give a strong reaction with GFAP antibodies. Thus, antibodies to IFP are useful tools for differential diagnosis in surgical pathology.

Introduction

All mammalian cells contain intracellular networks of protein filaments that can be visualized in the electron microscope (Fig. 1a). These fibrillar components that make up the cytoskeleton can be divided into four types on the basis of their diameter (Schliwa and van Blerkom, 1981). In addition to microtubules (22–25 nm in diameter), microfilaments (5–7 nm) and microtubulare filaments (2–3 nm), another group of filaments with diameters between those of microfilaments and microtubules can be observed, the so-called intermediate-sized filaments (IF; 7–11 nm). The different filament types can also be visualized by the indirect immunofluorescence technique using specific antibodies directed against proteins that are part of these structures, that
Table 1. Tissue specificity of intermediate-sized filament proteins.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Protein constituent</th>
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<tbody>
<tr>
<td>Epithelial cells</td>
<td>Keratins (cytokeratins, prekeratins)</td>
</tr>
<tr>
<td>Mesenchymal cells</td>
<td>Vimentin</td>
</tr>
<tr>
<td>Muscle cells</td>
<td>Desmin</td>
</tr>
<tr>
<td>Neuronal cells</td>
<td>Neurofilament proteins</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Glial fibrillary acidic protein</td>
</tr>
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</table>

is, actin, tubulin, or the IF-proteins. Figs. 1b–f show some typical examples of cytoskeletal organizations present in cultured cells and visualized in the fluorescence microscope with this technique. Biochemical and immunochemical investigations have demonstrated a further subdivision of IF according to their protein subunits. Five different types of IF proteins (IFP) can be distinguished, each type specific for a special group of tissues (see Table 1; and also Franke et al., 1978; Anderton, 1981; Lazarides, 1980, 1981, 1982; Osborn et al., 1981; Franke et al., 1981a; Holtzer et al., 1981). Furthermore, recent investigations suggest that the tissue-specific IFP are retained during neoplastic transformation of these tissues and that no additional IF-cytoskeleton is expressed in solid tumours (see for example, Altmannsberger et al., 1981a,b, 1982a,b; Osborn et al., 1982; Caselitz et al., 1981; Gabbiani et al., 1981; Krepler et al., 1981, 1982; Ramaekers et al., 1981, 1982a,b; Schlegel et al., 1980a; Sieinski et al., 1981; Bannasch et al., 1980, 1981; Battifora et al., 1980; Löning et al., 1980; Madri & Barwick, 1982; Mettinen et al., 1982a,b,c; Summerhayes et al., 1981).

Therefore, antisera to these IF proteins may be used in neoplastic tissue recognition, especially since most of the antisera described so far have not shown cross-reactivity. In this paper, we describe the use of several IFP antisera (raised in rabbits) and monoclonal antibodies, obtained by the fusion technique described by Köhler & Milstein (1975), for the differential diagnosis of human malignant tumours and include a brief overview of the results described in the literature on this subject to date.

Materials and methods

Tissues

Normal and malignant tissues were obtained at surgery and small pieces were frozen in liquid nitrogen. The rest of the tissue was used for routine diagnosis and fixed in neutral formalin for

Fig. 1. Different types of cytoskeletal structures in cultured cells visualized by electron microscopy and in the indirect immunofluorescence technique. (a) Electron micrograph of a cultured bovine lens cell depicting microfilaments (MF), microtubules (MT) and intermediate-sized filaments (IF). (b–f) Immunofluorescence micrographs of cultured lens cells (b–d), and cultured hepatocellular carcinoma cells from guinea-pig (e) and man (f) incubated with antibodies to actin (b), tubulin (c), vimentin (d) and keratin (e, f). For details on lens cell fluorescence, see Ramaekers et al. (1980). (a) × 40 000; (b) × 600; (c) × 400; (d) × 800; (e) × 300; (f) × 500.
histology. Also tumours were cut immediately on a cryostat for diagnostic purposes. These sections (7 μm), and those cut from the material stored in liquid nitrogen, were kept frozen at -20°C for a few days before being stained by the indirect immunofluorescence and/or the immunoperoxidase technique.

**Antisera**

The following antisera were used in this study.

1. A rabbit antiserum directed against human epidermal keratins isolated from foot calluses essentially as described by Franke et al., (1978).
2. A rabbit antiserum raised against vimentin isolated from calf lens by preparative gel electrophoresis as described elsewhere (Ramaekers et al., 1981).
3. A rabbit antiserum directed against chicken gizzard muscle desmin isolated using modifications of the methods used by Geisler & Weber (1980) and Franke et al., (1980), briefly as follows. Chicken gizzard muscle tissue was minced in phosphate-buffered saline (PBS) at 4°C (all the subsequent isolation steps were performed at 4°C), stirred for 10 min and homogenized using an Ystral X10/20 homogenizer (Ystral GmbH, Ballrechten-Döttingen). After stirring for another 10 min and centrifugation at 6000 r.p.m. in a GSA rotor for 10 min, the pellet was homogenized in EDTA buffer (1 mM EDTA, 0.01% β-mercaptoethanol, 0.5% Triton X-100 and 10 mM Tris–HCl, pH 7.4) and extracted for 3 h.

After centrifugation as described, the resulting pellet was homogenized in potassium iodide buffer (0.6 M KI, 0.01% β-mercaptoethanol, 0.5% Triton X-100 and 10 mM Tris–HCl, pH 7.4), extracted for 3 h, repelleted and washed with PBS. Occasionally, this final pellet was treated with DNAase I (Sigma) for 20 min at 37°C. Desmin was purified from this crude preparation by preparative gel electrophoresis as described in the preparation method of vimentin (Ramaekers et al., 1981). Purity of the final desmin preparation was checked by one- and two-dimensional gel electrophoresis.

4. A rabbit antiserum directed against GFAP from human spinal cord was prepared essentially as described by Dahl & Bignami (1976). From 4.9 g spinal cord tissue approximately 750 μg degraded GFAP was isolated. On SDS–polyacrylamide gel electrophoresis, this preparation showed a multiple band pattern with polypeptides in the molecular weight region from 25 000 to 45 000. New Zealand white rabbits were immunized intracutaneously at 20 sites in the back with approximately 0.4 mg of the keratin preparation, 0.2 mg vimentin, 0.25 mg desmin or 0.2 mg GFAP. All preparations were dissolved in a buffer containing 2.3% sodium dodecylsulphate, 5% β-mercaptoethanol, 10% glycerol and 40 mM Tris–HCl, pH 6.8, as final concentrations. An equal volume complete Freund’s adjuvant was added before the injection. Rabbits were boosted subcutaneously at four sites repeatedly (up to three times with three-week intervals) with equal amounts of protein in incomplete Freund adjuvant and the sera collected three weeks after the last booster. Before immunization, the sera of the rabbits were tested for autoimmune antibodies on frozen section from human skin using the indirect immunofluorescence test (see below). Only those rabbits which had negative pre-immune sera were used for immunization.

Pre-immune sera were used as controls in parallel with the antisera described above. These sera were tested for specificity on frozen sections of human tissues. In addition, tissues from hamster and rat, as well as cultured cells, were used for quality screening as described earlier (Ramaekers et al., 1981, 1982a,b). For reactions of the vimentin antisemur, see also Klymkowsky (1982).

5. Anti-neurofilament antibodies were directed against the 68K protein from calf brain neurofilament preparations and were a gift from Dr Denise Paulin (Paris). Another neurofilament antibody preparation, also raised in rabbits, was also directed against bovine
Intermediate filaments in human tumours

brain 68K polypeptide isolated by preparative SDS–gel electrophoresis and absorbed with glial filament protein from bovine brain. This latter serum was kindly provided by Dr Chris Pool and Dr Willem van Raamsdonk (Amsterdam).

(6) The monoclonal antibodies used in this study (RGE 53 and RKSE 60) were prepared essentially as follows: Balb/C mice were immunized intraperitoneally with approximately 50 μg HeLa-cell cytoskeleton, prepared by extracting a cell pellet with Triton X-100 and 1.5 M KCl and 100 μg human callus keratins, and after three weeks boosted twice (i.e., two and four days prior to the cell fusion). Splenic lymphocytes were fused with mouse myeloma Sp/0 Ag 14 cells in PEG-4000 and hybrids grown in 24-well clusters in RPMI 1640 (Dutch modification) containing 15% foetal calf serum. The cells were incubated for 24 h before adding HAT-medium and hybridoma cultures tested for antibody production two weeks later. Mixtures showing fibrillar staining when tested on HeLa-cell monolayers or a strong reaction on human epidermis in the indirect immunofluorescence technique were cloned, tested again for staining in HeLa-cells or epidermis, sub-cloned and grown according to standard techniques (Pazekas de St Groth & Scheidegger, 1980). Undiluted culture medium was used for tests on frozen sections of the human tissues in the indirect immunofluorescence technique (see below). As a control, growth medium from non-producing hybridomas was used.

Indirect immunofluorescence and immunoperoxidase staining
Air-dried cryostat sections prepared as described above were fixed in methanol for 5–10 min at −20°C and thereafter dipped in cold acetone. When the antisera to GFAP and neurofilament proteins were used, frozen sections were fixed with 2% paraformaldehyde, pH 7.4, for 5–10 min at 20°C instead of methanol-acetone fixation, followed by washes in water for 15–20 min and in PBS for 10 min.

After rehydration of the sections in PBS, the first antiserum was added (dilutions up to 1 : 100) and the sections incubated at room temperature in a moist chamber for 45 min. After washing with PBS (three washes of 10 min each), the fluorescein-labelled second antibody (goat-anti-rabbit IgG or rabbit-anti-mouse IgG, both conjugated with fluorescein isothiocyanate (FITC); Nordic, Tilburg, The Netherlands) was added and the sections incubated for another 45 min. After a second series of washes in PBS, the sections were mounted with 50% glycerol in PBS or in Gelvatol (Monsanto, St Louis, Missouri, U.S.A.) and viewed with a Leitz Dialux 20 EB microscope equipped with epifluorescent illumination using appropriate filters for fluorescein fluorescence. Pictures were taken on Tri-X film (Kodak) with an automatic camera using an ASA-setting of 400 or 800. For the immunoperoxidase technique, parafilm sections were deparaffinized using xylene (3 × 10 min) and ethanol (3 × 5 min), treated with 1% hydrogen peroxide in methanol for 30 min, and brought to PBS using a descending ethanol series.

The sections were incubated with 1% normal swine serum (obtained from Dako) for 30 min at room temperature followed by the first antiserum in 1% normal swine serum-PBS. Serum dilutions used in the peroxidase technique were 1 : 20 to 1 : 40 for the keratin antiserum, 1 : 100 to 1 : 400 for the GFAP antiserum, 1 : 40 to 1 : 80 for the vimentin antiserum and 1 : 80 to 1 : 160 for the desmin antiserum. The sections were incubated overnight at 4°C, followed by 2 h at room temperature. After three washing steps in PBS of 10 min each, the sections were incubated 30 min at room temperature with swine-anti-rabbit Ig (Dako; dilution 1 : 25) in 1% normal swine serum-PBS. After another series of washing steps, the sections were incubated for 30 min with the rabbit peroxidase-antiperoxidase complex (Dako; dilution 1 : 100) in 1% normal swine serum-PBS. After washing, the antigens were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB, obtained from Sigma) for 2–3 min. Mayer's Haematoxylin (1–3 min) was used as a counterstain.
Results

Fixation of tissue sections and the use of routine paraffin sections

In order to obtain optimal results and to avoid false negative results, it has to be realized that IFP detection is strongly influenced by the fixation method used. For example, fixation of tissues or tissue sections in formalin and subsequent paraffin embedding diminishes immunocytochemical detectability of keratin and vimentin (Osborn et al., 1977; Ramaekers et al., 1983a). When comparing results obtained with routine paraffin sections and frozen sections, it has become clear that adenocarcinomas, although positive for keratin in methanol-fixed frozen sections, very often appear unstained by the latter antibodies when formalin-fixed paraffin sections are used.

This may explain some false negative results described in the literature (Schlegel et al., 1980a; Bejui-Thivolet et al., 1982; Gusterson et al., 1982). Altmanberger et al., (1981b) have, therefore, introduced a protocol involving ethanol fixation followed by embedding in paraffin wax. This method allows keratin to be demonstrated in glandular cells and adenocarcinomas. Squamous cell carcinomas and mesotheliomas can be detected in routine sections by the anti-keratin antibodies, probably because of their high tonofilament content (see also Warhol et al., 1982). Studies in progress will have to show whether or not proteolytic predigestion of the paraffin sections and/or increasing of the sensitivity of immunohistochemical techniques may result in detection of adenocarcinomas in routine sections. Desmin and GFAP can be detected easily and with high sensitivity in formalin-fixed paraffin sections (Ramaekers et al., 1983c; Miettinen et al., 1982a; Tascos et al., 1982; see also figs. 3a, b, 6a, b and 6e, f).

In this study, we have, therefore, used frozen sections for the detection of keratin, vimentin or neurofilaments in all epithelial tissues and tumours, and in most mesenchymal or neuronal tissues or tumours, respectively. For the detection of desmin or GFAP in most instances, routine paraffin sections were used.

Normal human tissues

Figs. 2 and 3 give a pictorial overview of the tissue specificity of the different types of IFP, while Table 1 summarizes these results. The typing described below refers to tissues in adults, since the staining reaction may be different in embryonal and neonatal tissues (see for example, Jackson et al., 1980, 1981; Dahl et al., 1981). Generally epithelial cells

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**Fig. 2.** Indirect immunofluorescence test on frozen sections from human tissues using antibodies directed against the different types of intermediate filament proteins. (a–c) Epidermis incubated with antibodies to keratin (a), vimentin (b) and desmin (c). (d–f) Small intestine incubated with antibodies to keratin (d), vimentin (e) and desmin (f). (g–h) Kidney frozen section incubated with anti-keratin (g) and anti-vimentin (h). (i) Reactivity of the anti-desmin antiserum on striated skeletal muscle. (j) Prostate and (k) thyroid tissue incubated with anti-keratin. (l) Nerve processes in the skin, positive for the anti-neurofilament antibodies. (a) × 63; (b) × 315; (c) × 184; (d) × 145; (e) × 193; (f) × 155; (g,h) × 193; (i) × 290; (j) × 145; (k,l) × 240.
contain proteins reactive exclusively with antibodies to keratin (also referred to as prekeratin or cytokeratin).

Using antisera to epidermal keratin in the indirect immunofluorescence technique, we – in agreement with other investigators (Franke et al., 1979a, b, 1980, 1981b, c; Sun et al., 1979; Asch et al., 1981; Denk et al., 1982; Schlegel et al., 1980b) – have observed strong staining in all stratified squamous epithelia, in dermal appendages such as hair follicles, sebaceous glands and sweat glands, myoepithelial cells as well as positive reactions in epithelial cells of the urinary tract, the gastrointestinal tract, the genital tracts, mesothelia, female breast lobuli and ducts, bile ducts and hepatocytes, pancreatic acini and prostate. Exceptions to this rule are formed by some epithelioid tissues of the eye (lens epithelium, lining cells covering the iris and ciliary body, retinal pigment epithelium; Ramaekers et al., 1980, 1982c) and Sertoli cells and germ cells in the testis, which all contain vimentin.

Vimentin antibodies stain fibroblasts in connective tissue, endothelial cells in blood vessels, macrophages, cells of cartilage and bone, some vascular smooth muscle cells and melanocytes.

The vimentin IFP has been referred to as non-specific since it occurs in nearly all cells in tissue culture and has also been observed by some investigators (Lazarides, 1980) in striated muscle and some types of glial cells (Dahl et al., 1981; Schnitzer et al., 1981). We have noted vimentin staining in certain types of glial cells in rat and human brain (Bergmann glia and some other astrocytes). Desmin antibodies react exclusively with striated skeletal muscle, cardiac muscle and smooth muscle (see Fig. 3a).
Intermediate filaments in human tumours

The neurofilament and GFAP antibodies were tested on rat brain sections, as well as sections from human brain. Antibodies to neurofilaments give a positive reaction in most neurons in rat and human brain. Furthermore, rat optical nerve axons and multi-polar ganglion cells in rat retina are positive.

Peripheral nerve processes can also be stained by the neurofilament antibodies (see Fig. 2c). Antibodies to GFAP react strongly positive in rat brain and human brain astrocytes, essentially as described by Shaw et al. (1981) and Yen & Fields (1981). GFAP-staining has also been observed in some peripheral nerve processes (see Jessen & Mirsky, 1980). Fig. 3b shows a staining pattern for astrocytes in human brain. For further details on immunohistochemical staining of different types of brain tissues for IFP, see Shaw et al. (1981).

Human tumours distinguished by polyclonal antisera to IFP
The results obtained in our laboratory with conventional rabbit antisera directed against the various IFP as well as data from the literature are summarized in Table 2 and illustrated in Figs. 4–6.

Carcinomas
Squamous cell carcinomas from skin, oesophagus and cervix as well as lymph node metastases of this type of epithelial tumour are strongly positive for keratin exclusively (see Fig. 4a). These tumours do not react with antibodies directed to any other type of IFP (Fig. 4b) (see Schlegel et al., 1980a; Gabbiani et al., 1981; Ramaekers et al., 1981, 1982a,b).

Table 2. Specificity of reactions of polyclonal antibodies to intermediate-sized filament proteins on human tumors (see also references).

<table>
<thead>
<tr>
<th></th>
<th>Keratin</th>
<th>Vimentin</th>
<th>Desmin</th>
<th>NF</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinomas</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Mesotheliomas</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Lymphomas</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Melanomas</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Rhabdomyosarcomas</td>
<td>-</td>
<td>(+)</td>
<td>++</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Leiomyosarcomas</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Other soft tissue tumours</td>
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<tr>
<td>(fibrosarcoma, liposarcoma, hemangiosarcoma, Schwannoma)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Osteosarcoma</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Neuroblastoma/ganglioneuroblastoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/−</td>
<td>-</td>
</tr>
<tr>
<td>Oat cell carcinoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Meningioma</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Astrocytoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ependymoblastoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Glioblastoma</td>
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<td>-</td>
<td>+</td>
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</table>
Adenocarcinomas in general are all positive for keratin but react less strongly than the squamous cell carcinomas. However, with respect to the presence of keratin in adenocarcinomas, results described in the literature are rather contradictory. Schlegel et al. (1980a), Gusterson et al. (1982) and Bejui-Thivolet et al. (1982) describe several types of adenocarcinomas, female breast carcinomas, and epithelial lung tumours to be negative for keratin. We have, however, tested 85 cases of human adenocarcinomas (Ramaekers et al., 1983a) from different tissues and adenocarcinomatous metastases in several organs.

In all cases, the tumour cells were positive for keratin only (see Fig. 4c–e). Furthermore, Almannsberger et al. (1981a,b, 1982a,b), Caselitz et al. (1981), Gabbiani et al., (1981), Krepler et al., (1981, 1982), Lehto et al. (1983) and Ramaekers et al. (1981, 1982a,b) have shown that most adenocarcinomas from lung, female breast, gastrointestinal tract and parotid gland are positive for keratin. Possible explanations for these discrepancies have already been described above.

Other pulmonary carcinomas tested, that is, epidermoid carcinomas, bronchioloalveolar carcinomas and large cell anaplastic carcinomas are positive for keratin (see Lehto et al., 1983). Oat cell carcinomas, however, do not react with these latter antibodies but are positive only for neurofilaments (Lehto et al., 1983). Mesotheliomas show a positive reaction with keratin (see Fig. 4f; see also Warhol et al., 1982). Further investigations are in progress to see what type of IFP is present in the mesotheliomas of predominantly fibrous (spindle-cell) appearance. Thymomas react exclusively with anti-keratin (Battifora et al., 1980). A rather striking exception in the list of keratin-positive adenocarcinomas is the renal cell adenocarcinoma (Grawitz tumour). In one case tested, we found tubular tumourous structures lacking positive staining for keratin but showing a positive staining exclusively with the anti-vimentin antibodies. In three other cases, some of the tumour cells were positive for keratin, while others were

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**Fig. 4.** Indirect immunofluorescence test on human carcinomas using antibodies to keratin (a, c–f) and vimentin (b). (a, b) Squamous cell carcinoma of the vulva. (c) Adenocarcinoma of the rectum. (d) Adenocarcinoma of the ovary (cystadenocarcinoma papilliferum serosum). (e) Metastasis of an invasive ductal breast carcinoma. (f) Malignant mesothelioma. Metastasis on the omentum. (a) × 77; (b) × 270; (c) × 640; (d,e) × 380; (f) × 290.

**Fig. 5.** Immunofluorescence micrographs of high-grade malignant lymphomas (a–d) and a melanoma in the skin (e–f) incubated with antibodies to vimentin (a,b,e), keratin (c,f) and desmin (d). (a,c) Immunoblastic sarcoma. (b,d) Lymphoblastic sarcoma in skeletal muscle. Courtesy Dr Th. Vroom. (a–c) × 340; (d) × 530; (e,f) × 365.

**Fig. 6.** Immunoperoxidase (a,b,e,f) and immunofluorescence (c,d) staining of human sarcomas (a–d) and brain tumours (e,f). (a,b) Rhabdomyosarcoma cells in a mesodermal mixed tumour from the ovary staining strongly positive with antibodies to desmin. (c) Seminoma positive for vimentin. (d) Mixed mesodermal sarcoma in the uterus with tumour cells strongly positive for vimentin. (e) Astrocytoma, reacting only with antibodies to GFAP. (f) Meningioma incubated with antibodies to vimentin. (a) × 290; (b) × 340; (c–f) × 240.
positive for vimentin. We suggest, therefore, that Grawitz-tumour should be renamed renal cell carcinosarcoma (Herman et al., 1983).

Tumour cells in pleomorphic adenomas from the parotid gland may contain both keratin and vimentin (see Caselitz et al., 1981; Krepler et al., 1982). Co-expression of vimentin and keratin is also observed in human epidermal tumour cells present in serous cavity fluids (Ramaekers et al., 1983c) in analogy with cells in tissue culture (Franke et al., 1979c).

When these cells resettle in the body and form solid metastatic tumours, they express exclusively keratin IF (see below).

Lymphomas
In collaboration with Dr Th. Vroom (Sloterwaart Hospital, Amsterdam), we have examined eighty cases of non-Hodgkin lymphoma (NHL), which included 14 different types. All NHL were positive for vimentin when tested in frozen sections (see Fig. 5a,b). No reaction was seen with the antibodies directed against keratin or desmin (Fig. 5c,d). These results are in agreement with those of Gabbiani et al. (1981) and are now being used in the differential diagnosis of carcinoma metastases and lymphomas in lymph nodes (see also Battifora et al., 1980). Tumorous leukaemia is positive for vimentin only.

Melanomas
Skin melanocytes and melanoma cells react exclusively with the anti-vimentin antibodies (Fig. 5e; see also Ramaekers et al., 1983b). No reactions were observed with antibodies to keratin (Fig. 5f), desmin, GFAP or neurofilament protein. These results are in accordance with findings of Sieinski et al. (1981) and may become useful in cases where differential diagnosis between amelanotic melanoma and carcinomas containing melanogenic inclusions is difficult or even impossible using conventional histological techniques. Vimentin and keratin staining may also help in cases of invasive amelanotic melanoma.

Rhabdomyosarcomas and leiomyosarcomas
These two types of myogenic tumours seem, in general, to contain both desmin and vimentin, although the presence of vimentin in rhabdomyosarcomas is still under discussion and a subject of contradiction in the literature (see, for example, Gabbiani et al., 1981; Altmannsberger et al., 1982a; Miettinen et al., 1982a,d).

In particular, undifferentiated small spindle shaped-rhabdomyosarcoma cells may be positive for vimentin (Gabbiani et al., 1981). Most important, however, is the fact that embryonal and alveolar as well as pleomorphic rhabdomyosarcomas react with the anti-desmin antiserum, while leiomyosarcoma has also been described to contain desmin (Gabbiani et al., 1981). It is, therefore, obvious that anti-desmin antibodies are a powerful tool in the differential diagnosis between myogenic tumours and other soft
tissue tumours. Furthermore, mixed tumours containing minor fractions of myogenic components may be diagnosed properly (Fig. 6a,b; see Ramaekers et al., 1981, 1982a,b, 1983d).

Other soft tissue tumours
As briefly mentioned above, many soft tissue tumours other than myosarcomas contain vimentin IF. These include angiosarcomas, fibrosarcomas, liposarcomas, schwannomas (see Altmannsberger et al., 1982a), myxoma and malignant fibrous histiocytomas (see Gabbiani et al., 1981), epithelioid sarcomas (Miettinen et al., 1982b,d), seminomas and haemangiochromas (Ramaekers et al., 1981, 1982a,b). These tumours do not contain desmin, keratin, GFAP or neurofilaments (see Figs. 6c,d). In addition, bone tumours (osteosarcomas) seem to contain exclusively IF of the vimentin type. Interestingly, epithelial-like cells in biphasic synovial sarcoma contain keratin, suggesting that this type of sarcoma is of a unique type with true epithelial features (Miettinen et al., 1982c).

Nervous tissue tumours
Antibodies to GFAP show intense staining of tumour cells in astrocytic neoplasms. Fig. 6e shows an example of an astrocytoma stained by the immunoperoxidase technique with these antibodies. Furthermore, Tascos et al. (1982) have shown that anti-GFAP antibodies will also stain glioblastomas, subependymal giant cell astrocytomas, some ependymomas (2 out of 10), subependymoma and astrocytes in mixed neoplasms.

In addition, Osborn et al. (1982) have described a GFAP-positive ependymoblastoma. Brain tumours negative for GFAP included oligodendrogliomas, meningiomas, and most neurinomas (Tascos et al., 1982). Meningiomas stain strongly with antibodies to vimentin (Fig. 6b). Antibodies to neurofilaments have been shown to stain a ganglioneuroblastoma, a pheochromocytoma of the adrenal gland (see Osborn et al., 1982) as well as oat cell carcinomas (Lehto et al., 1983). Attempts to demonstrate neurofilaments in neuroblastomas or cultured neuroblastoma cells results in contradictory findings. Osborn et al. (1982) could not detect neurofilaments in human neuroblastomas with the immunofluorescence technique nor did they detect any other type of IFP in these tumours. Jørgensen et al. (1976), however, described the presence of neurofilaments in cultured neuroblastoma cells. The latter results should be interpreted carefully since their serum was not tested for the presence of anti-vimentin (auto)antibodies. Further studies are in progress to solve this problem.

The use of monoclonal anti-keratin antibodies to distinguish between different types of carcinomas
In order to develop antisera that can recognize and distinguish different types of carcinomas, we have prepared monoclonal antibodies to HeLa-keratins and human skin keratins. Two cell lines, producing specific antibodies, are now in use in our laboratory, that is lines RGE 53 and RKSE 60. Line RGE 53 produces antibodies that specifically recognize glandular epithelia as well as hepatocytes and tumours derived from these tissues. Mesothelioma is also stained by these antibodies (compare also Lane, 1982).
Table 3. Specificity of reactions of monoclonal antibodies to keratins on different types of human epithelial tissues and epithelial tumours.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RGE 53</th>
<th>RKSE 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glandular epithelia and adenocarcinomas</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Non-keratinizing stratified epithelia and squamous cell</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>carcinomas derived from these</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinizing stratified epithelia and squamous cell</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>carcinomas derived from these</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Line RKSE 60 produces antibodies which specifically react with keratinizing stratified epithelia, but not with non-keratinizing stratified epithelium or other epithelia.

The fact that both different types of monoclonal antibody preparations do not react at all with non-epithelial tissues, and keeping in mind that mice were immunized with a cytoskeletal preparation from HeLa cells and purified keratins from human skin calluses, make it very likely that both different antibody types, are directed against keratins or tonofilament-associated proteins. Moreover, RGE 53 has been tested for its reaction on cultured cells and in the immunoblotting assay. These experiments have shown that RGE 53 antibodies produce fibrillar staining patterns in cultured HeLa cells, in cultured human hepatoma cells, F9 mouse teratocarcinoma cells and in a human colon carcinoma cell line (WiDr-218). The fluorescence patterns obtained in these cells depict a typical intermediate filament cytoskeleton organization, (virtually) identical to the organizations visualized by the rabbit antiserum to human skin keratin. No reaction was seen in cultured bovine lens cells. Furthermore, immunoblotting has shown that RGE 53 antibodies interact with a cytoskeleton-associated polypeptide with a molecular weight of approximately 44 000 in both HeLa cells and cultured human hepatoma cells. This polypeptide corresponds to a keratin band typical for these cell lines. RGE 53 is an IgG 1 with a Kappa light chain. Table 3 gives a summary of the specific reactions of the two monoclonal antibody preparations, while Figs. 7 and 8 depict some typical examples of tissue sections visualized in the indirect immunofluorescence microscope with the two sera.

Fig. 7. Human carcinomas (a–e) and cultured colon carcinoma cells (f) incubated with the monoclonal antibody RGE 53 and viewed in the immunofluorescence microscope. (a) Moderately well-differentiated adenocarcinoma in the colon (lower left corner) and normal glandular tissue (upper right corner). (b) Well-differentiated invasive duct carcinoma from female breast. (c) Malignant mesothelioma metastasis on the omentum. (d) Scirrhus carcinoma of the stomach. (e) Oral squamous cell carcinoma. (f) WiDr-218 cells showing a fibrillar staining pattern. (a) × 86; (b) × 240; (c) × 315; (d) × 340; (e) × 290; (f) × 770.
Fig. 8. Double immunofluorescence labelling of a squamous cell carcinoma from penis incubated with the polyclonal antibody to keratin (a) and the monoclonal antibody RKSE 60 (b). (a,b) × 180.

Detection of intermediate filament proteins in metastatic tumour cells in serous cavity fluids

Using smears from human serous cavity effusions (ascites, pleural fluid) conventionally fixed in methanol–acetone, intermediate filament proteins can be detected in metastatic tumour cells (Ramaekers et al., 1983c).

In parallel with epithelial cells in tissue culture (Franke et al., 1979c), carcinoma cells and mesothelial cells in ascites or pleural fluid contain a vimentin IF-cytoskeleton in addition to their original keratin IF. The vimentin ‘transport’ cytoskeleton is lost when metastatic tumour cells resettle and form solid tumours.

In blood cells and in tumour cells of mesenchymal origin (melanoma cells, tumourous leukaemia cells), only vimentin IF can be detected. When using the monoclonal antibody RGE 53, only mesothelial cells and adenocarcinoma cells are detected. These two cell types cannot, however, be distinguished with this procedure. Future studies will, therefore, concentrate on the preparation of monoclonal antibodies specific for keratins in mesothelial cells.

Discussion

The present paper summarizes results obtained in our laboratory and data published by other investigators (see references) with respect to the use of antibodies to intermediate filament proteins in tumour diagnosis. From the foregoing it is obvious that, although several technical and cell biological problems will have to be solved, the method can be used successfully in many cases where differential diagnosis is difficult or even
impossible on the basis of routine histological procedures alone. Differential diagnosis of carcinomas and lymphomas, especially in lymph nodes, should be possible in nearly 100% of the cases by the use of antibodies to keratin and vimentin on frozen sections or ethanol-fixed, paraffin-embedded material. Moreover, histological differential diagnosis between sarcomas and carcinomas may be difficult, especially in cases of poorly differentiated neoplasms.

Problems in rhabdomyosarcoma diagnosis can be addressed by using desmin antiserum on routine paraffin sections, while melanomas can be distinguished from pigment-containing carcinomas since the former tumour type contains IF exclusively of the vimentin type. Furthermore, antibodies to vimentin, GFAP and neurofilament proteins may present new tools in brain tumour diagnosis. Meningioma can be distinguished from astrocytoma and ependymoblastoma in paraffin sections by the use of GFAP- and vimentin-antisera. Neuroblastomas and ganglionneuroblastomas should react positively with neurofilament antibodies although some negative results have been reported in these tumours (Osborn et al., 1982). The neurofilament antiserum should also allow the differentiation between oat cell carcinomas and other lung cancers (see Lehto et al., 1983). Another powerful technique for the preparation of antibodies that may, in the future, be of considerable help for differential diagnosis in surgical pathology is the fusion and cloning technique as described by Köhler & Milstein (1975). Monoclonal antibodies directed against keratins described so far can distinguish between groups of epithelial tissue types and the tumours derived from them. Future studies will show whether or not highly specific monoclonal anti-keratin antibodies can be prepared that recognize only one single type of epithelial tissue and its neoplastic derivatives. For example, Lane (1982) described an antibody apparently specific for kidney epithelium, a finding that may encourage further efforts in this direction. An antibody that is able to recognize only mesothelial cell keratin would be a great help in both histological and cytological diagnosis.

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Intermediate filaments in human tumours


Intermediate filaments in human tumours