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Cytokeratin 20 in Human Carcinomas

A New Histodiagnostic Marker Detected by Monoclonal Antibodies

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The authors have recently identified a new cytokeratin (CK) polypeptide, CK 20, whose expression is almost entirely confined to the gastric and intestinal epithelium, urothelium, and Merkel cells. Seven monoclonal antibodies (MAbs) specific for CK 20 were raised and characterized by applying immunoblotting and immunocytochemical screening. All of them reacted on frozen tissue sections. A further MAb, IT-K$_s$20.8, recognized CK 20 in sections of formalin-fixed, paraffin-embedded tissue samples. A total of 711 cases of primary and metastatic cancer, mostly carcinomas, were analyzed immunohistochemically for CK-20 expression, using CK-20 specific guinea-pig antibodies and MAbs. The expression spectrum of CK 20 in carcinomas resembled that seen in the corresponding normal epithelia of origin. CK-20 positivity was seen in the vast majority of adenocarcinomas of the colon (89/93 cases), mucinous ovarian tumors, transitional-cell and Merkel-cell carcinomas and frequently also in adenocarcinomas of the stomach, bile system, and pancreas. Most squamous cell carcinomas in general and most adenocarcinomas from other sites (breast, lung, endometrium), nonmucinous tumors of the ovary, and small-cell lung carcinomas were essentially or completely negative. The authors propose to use CK 20 as a diagnostic marker valuable in distinguishing different types of carcinomas, notably when presenting as metastases. (Am J Pathol 1992, 140:427–447)

In the fields of cell biology, embryology, and pathology, the importance of the multigene family of intermediate filament (IF) proteins is being increasingly recognized owing to the fact that the differential expression of these proteins is closely linked with specific programs of differentiation (for reviews,[1,2]). In pathology, e.g., IF proteins are used for the classification of both normal and malignant cells on a molecular basis.[1–3] Among these proteins, the epithelial subgroup, i.e., the cytokeratins (CKs), comprises a system of especially potent differentiation markers, since the highly diverse expression patterns of CK polypeptides are correlated with different pathways of epithelial differentiation, and thereby allow the accurate and sophisticated classification of epithelial cells into numerous subtypes.[4–13] In recent years, many investigators have outlined a variety of applications of CK polypeptides as diagnostic markers in tumor pathology, particularly for the differential diagnosis of carcinomas at the histologic level (for reviews,[1–3,6,11,13–16]). For example, procedures have been devised to identify poorly differentiated squamous cell carcinomas,[6,9,13,15,17,18] adenocarcinomas,[6,11,15,18–25] hepatocellular carcinomas,[6,22,26–28] and malignant mesotheliomas[29,30] on the basis of the expression or absence of particular individual or sets of CK polypeptides in such tumors.

CK typing is especially useful in cases in which accurate diagnosis may be difficult, e.g., poorly differentiated carcinomas, when neighboring structures have been invaded, and when the site of a primary tumor giving rise to metastases is unknown. In this last instance, the tumor in question is, in the majority of cases, an adenocarcinoma.[31] Metastases of adenocarcinomas with sites of origin as diverse as the breast, salivary glands, lung, stomach, pancreas, colon, ovary, and endometrium cannot be reliably distinguished using routine histologic methods. Furthermore, the value of established tumor markers such as carcinoembryonic antigen (CEA) and CA 19-9 for the differential diagnosis of such tumors is rather limited,[32] whereas few other markers, such as CA

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125[33] and villin,[34,35] are of some use in distinguishing certain adenocarcinomas. Unfortunately, for the subtyping of adenocarcinomas, currently available diagnostic procedures involving CKs also appear to be of restricted usefulness, being mainly based on the differential expression of CK 7 (particularly its consistent absence in adenocarcinomas of the colon[6,15,22,25,36]), one of the four CK polypeptides (CKs 7, 8, 18, 19) of the original CK catalog typically expressed in simple epithelia.[6] Furthermore, in some instances, minor CK components such as CK 5 (malignant mesotheliomas;[29,30] adenocarcinomas of endometrium and ovary[15,37]) or the coexpression of vimentin together with CKs[38] may yield some diagnostically helpful information.

We succeeded in identifying a fifth CK polypeptide of the simple-epithelium type of M_r 46,000 and less acidic than all other type-I CKs, referred to as CK 20,[39] which is of particular interest because of its restricted range of expression. CK 20 has mainly been found in intestinal epithelium, gastric foveolar epithelium, the urothelium, and in Merkel cells.[39] CK 20 is exceptional in its lack of immunologic crossreactivity with other type-I CKs as none of the dozens of monoclonal antibodies (MAbs) reactive with CKs, including some that react broadly with IF proteins in general,[40] did recognize this particular CK. This indicates that the relationship of CK 20 to the other CKs is more distant as is also indicated by the partial sequence data available.[39] Because of this notorious lack of immunologic crossreactivity it has taken almost a decade to ascertain the cytokeratinous nature of this cytoskeletal protein.[6,39] But the same fact also has allowed to generate several MAbs exclusive for this protein. In the present study, we report on a series of MAbs specific for CK 20 and their immunohistochemical value in the characterization of primary and metastatic human carcinomas. We particularly sought to determine whether CK 20 might be a suitable histodiagnostic tool for the subtyping of various carcinomas, including adenocarcinomas.

Materials and Methods

Tissues and Cultured Cells

Tissue from human primary and metastatic carcinomas of various origins and histologic types was obtained immediately after surgical removal, usually during the course of frozen section diagnosis. A few samples were obtained from autopsies. Various histologically normal human tissues were collected in a similar way, whereas further control tissues included pig duodenum obtained from a local slaughterhouse, dog small intestine obtained during an autopsy, and rat small intestine taken from healthy laboratory animals. Excised tissue specimens (max. diameter 1–1.5 cm) were immediately frozen in isopentane (precooled in liquid nitrogen to −140°C) or were placed directly in liquid nitrogen. The remaining tissue was fixed in formalin, and samples therefrom were routinely processed for paraffin embedding and subjected to pathohistologic evaluation.

In addition, paraffin blocks of several normal and malignant tissues samples were taken from the files of the Institute of Pathology of the University of Mainz and subjected to immunohistochemical staining using MAb IT-K_s20.8.

Human corneal epithelium was obtained during autopsies (up to 12 hr postmortem) by gently scraping the corneal surface with a scalpel and then immersing the material thus detached in phosphate-buffered saline (PBS) before centrifugation. Scalp hair follicles were isolated by plucking.

The colorectal-carcinoma–derived cell-culture lines HT-29 and DLD-1 were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland), and grown essentially as recommended by the producer.[39] Cells of the human mammary-carcinoma cell line, MCF-7, were grown as described elsewhere.[6]

Preparation of Cytoskeletal Fractions

From 20- to 30-μm–thick cryostat sections, regions containing normal epithelial (epidermis, tongue epithelium) or carcinoma tissue were prepared by performing microdissection under microscopic control, as previously described.[6,9] Cytoskeletal fractions were prepared from the microdissected tissues, as well as from cultured cells, by extraction in a buffer containing Triton X-100 and 1.5 M KCl followed by washing of the centrifuged pellet with a low salt buffer as described elsewhere.[6,41] Cytoskeletal material from the villi of duodenal mucosa, which contains a high concentration of CK 20, was prepared as described by Moll et al.[39]

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to the procedure of Laemmli.[42] Two-dimensional gel electrophoresis, using nonequilibrium pH gradient (NEPHG) electrophoresis in the first dimension and SDS-PAGE (with elevated ionic strength) in the second dimension, was performed as described previously.[41]

For immunoblotting,[41] proteins were transferred from gels onto nitrocellulose membranes in a semidry electrotransfer chamber (CTI, Idstein, FRG[43]) and further processed as described elsewhere,[41] except that the pro-

tein refolding buffer was omitted. Before the immunoreaction, the nitrocellulose membranes were stained for total proteins using Ponceau-S-red (Sigma, Munich, FRG) and then photographed. The bound primary antibodies were visualized by incubation with peroxidase-conjugated rabbit antibodies against guinea-pig or mouse immunoglobulins (Dakopatts, Hamburg, FRG) and after the appropriate washing steps,[41] subsequent staining with a 3,3′-diaminobenzidine (DAB) solution in PBS containing H_2O_2 and 0.015% nickel-II-sulfate.

Antibodies

The preparation, purification, and characterization of polyclonal guinea-pig antibodies specific for CK 20 have been described previously.[39]

In addition, a series of mouse monoclonal antibodies (MAbs) against CK 20 were raised; these were all derived from one fusion. Using preparative SDS-PAGE,[41] CK 20 was isolated from cytoskeletal material obtained from human duodenal mucosa. In brief, after exhibiting negative staining with 4 *M* sodium acetate, the bands containing CK 20 were excised, and the protein was first eluted by diffusion using 0.1% SDS and then concentrated by applying vacuum dialysis. After the addition of 3 mg mouse albumin (Sigma) per 100 μg CK 20, proteins were precipitated using acetone.[41] A 6-week-old female Balb/c mouse was immunized by a subcutaneous injection of an aliquot of the acetone-precipitated powder containing 20 μg CK 20 that had been mixed to form a fine suspension in 250 μl PBS, supplemented with 250 μl complete Freund's adjuvant (Sigma) and emulsified. Booster injections were given on day 22 (same dose, incomplete Freund's adjuvant, subcutaneous) and on day 54 (same dose, without adjuvant, intraperitoneal). On day 57, spleen cells were fused with cells of the mouse myeloma cell line X63-Ag8.653 at a ratio of 5:1 in the presence of 40% polyethyleneglycol 4000 (Roth, Karlsruhe, FRG[44]). After fusion, the cells were placed on ten Costar-3524 24-well microtiter plates (Costar, Cambridge, MA) and cultured in hypoxanthine-aminopterin-thymidine (HAT)-containing RPMI-1640 medium supplemented with 10% fetal calf serum. Hybridomas were screened by immunofluorescence microscopy using cryostat sections from human gastric mucosa, which is particularly suitable owing to its characteristic CK-20 pattern (Figure 5a). Positive clones were subcloned twice by limited dilution in 96-well microtiter plates. After subcloning, hybridomas were weaned off HAT-RPMI into RPMI medium alone and were grown in 25-cm^2 flasks or propagated as ascites in Pristane-treated Balb/c mice.[45] Immunoglobulin subclasses were determined by testing using an enzyme-linked immunosorbent assay (ELISA) with peroxidase-coupled secondary antibodies (Hybridoma subtyping kit; Boehringer).[45] The MAbs IT-K_s20.3, IT-K_s20.5 and IT-K_s20.8 are commercially available from Progen (Heidelberg, FRG).

For comparison, MAbs against other CKs, such as MAb K_s18.174 against CK 18 ([46]; available from Progen) and MAb K_s pan 1–8.136 against CKs 1–8([47]; Progen) were also used in immunohistochemical staining experiments.

Immunoelectron Microscopy

HT-29 colon carcinoma cells were processed for preembedding immunoelectron microscopy as described elsewhere.[48] Such cells were fixed in acetone. The primary antibodies were purified guinea-pig antibodies specific for CK 20,[39] whereas the secondary antibodies were coupled with 5-nm gold particles (Janssen, Beerse, Belgium).

Immunohistochemistry

From the frozen tissue blocks, 5-μm–thick cryostat sections were cut, air-dried, fixed in acetone (15 min at −20°C), and then air-dried again.

Two immunohistochemical staining methods were employed:

1. Indirect immunoperoxidase microscopy was performed using a standard protocol.[49] The primary antibodies used have already been listed. The MAbs against CK 20 used here were in the form of either hybridoma supernatants or ascites fluid; the former were applied undiluted, while the latter were diluted 1:500 to 1:1000. As secondary antibodies, peroxidase-conjugated rabbit antibodies against mouse or guinea-pig immunoglobulins (Dakopatts) were applied. The staining reaction was performed using DAB and H_2O_2.
2. Indirect immunofluorescence microscopy was performed as described earlier.[46,49] As secondary antibodies, Texas-Red-coupled goat antibodies against mouse or guinea pig immunoglobulins (Dianova, Hamburg, FRG) were used.

Cultured cells grown as monolayers on coverslips were fixed in methanol (5 min at −20°C), briefly rinsed in acetone (−20°C), air dried, and subsequently immunostained using the immunofluorescence method described earlier.

To determine the reactivity of the CK-20 MAbs with protein A, immunohistochemistry was performed using cryostat sections of stomach mucosa. In place of the secondary antibodies, peroxidase-coupled protein A (Amersham, Braunschweig, FRG) was applied at a dilution of 1:200 in 1% bovine serum albumin (BSA) in PBS. Bound protein A was visualized using DAB and H_2O_2.

For the immunohistochemical staining of formalin-fixed, paraffin-embedded tissue, the avidin-biotin-peroxidase-complex (ABC) method[50] (ABC Kit; Vector, Burlingame, CA, USA) was applied. Four-micron–thick paraffin sections were dried overnight at 37°C, and after deparaffination and rehydration, endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2/40% methanol in PBS for 30 minutes. Subsequently, the sections were treated with 0.1% trypsin (Sigma) in 0.05 *M* Tris-HCl, pH 7.8, for 15 minutes at 37°C. After an avidin-biotin blocking step (Blocking Kit; Vector) and incubation with normal horse serum (diluted 1:10 in PBS), the sections were incubated overnight with the MAb, IT-K_s20.8, at 4°C. Bound antibody was detected using the ABC Kit. The staining reaction again involved the application of a DAB solution containing H_2O_2. Slides were weakly counterstained with Mayer's hematoxylin (Merck). In some instances, the indirect immunoperoxidase method was applied to trypsinized paraffin sections.

As negative controls, the specific primary antibody was replaced by PBS or by supernatant from a nonantibody producing hybridoma. These controls consistently yielded negative results.

Results

Characterization of Monoclonal Antibodies to Cytokeratin 20

CK 20 purified from human duodenal mucosa was used for the immunization of one mouse. After fusion, it was possible to isolate nine MAbs reacting with CK 20 (IT-K_s20.1 to IT-K_s20.9), all of which were immunoglobulin (Ig) G. Of these, MAbs IT-K_s20.1 to IT-K_s20.7 were extensively characterized in the present study (Table 1, Figures 1–4). One further MAb, IT-K_s20.8, recognized CK 20 in sections from routinely formalin-fixed, paraffin-embedded tissue as evident by the CK-20-typical staining pattern on various normal tissues (Figure 5b, c). Crossreactivity with some CK-20-negative epithelia (e.g., renal tubules) was observed for this MAb on cryostat sections but not on paraffin sections. The detailed characterization of this MAb will be presented elsewhere. The ninth MAb against CK 20 (IT-K_s20.9, IgG1) was not included in this study since immunohistochemically it showed crossreactivity with some component(s) of cell nuclei and muscle cells (cytoplasm).

In immunoblot experiments performed on SDS-PAGE-separated cytoskeletal proteins from human duodenal mucosa, MAbs IT-K_s20.1 to IT-K_s20.7 reacted specifically with CK 20 (Figure 1), and all failed to react with CKs 8, 18, and 19 also present in these preparations. Correspondingly, cytoskeletal proteins from MCF-7 cells, which contained CKs 8, 18, and 19, were negative for all of the antibodies (not shown). That the positive (M_r 46,000) band (Figure 1) genuinely represented CK 20 was confirmed by immunoblot experiments performed after duodenal cytoskeletal proteins had been separated by two-dimensional gel electrophoresis. As an example, Figure 2 shows the specific reactivity of antibody IT-K_s20.4 with the two major isoelectric variants of CK 20.

In further experiments, the CK-20 MAbs were tested

Table 1. *Characterization of Murine Monoclonal Antibodies against Cytokeratin 20*

Mab	Ig isotype	Reactivity with protein A*	Western-blot analysis (human CK 20)	Immunohistochemical detection of human CK 20 in tissue sections		Immunohisto-chemical crossreactivity	Immunohistochem. detection of CK 20 in intestinal mucosa of other species‡		
				Cryostat sections	Paraffin sections†		Pig	Dog	Rat
IT-K_s20.1	IgG1	–	+	+	–	–	–‖	–‖	–
IT-K_s20.2	IgG1	–	+	+	–	–	+	–	–
IT-K_s20.3	IgG1	–	+	+	–	–	+	–	–
IT-K_s20.4	IgG1	–	+	+	–	–	+	–	–
IT-K_s20.5	IgG2a	+	+	+	–	–	+	–	–
IT-K_s20.6	IgG1	–	+	+	–	Inner rooth sheath of hair follicles	–	–	–
IT-K_s20.7	IgG2b	+	+	+	–	Mesenchymal and smooth muscle cells	+	–	–
IT-K_s20.8	IgG2a	+	+	+	+¶	See text for cryostat sections	ND#	ND	ND

* As determined by the presence of the typical CK-20 staining pattern in stomach mucosa when protein A was used for detection of the primary antibody.

† Formalin-fixed tissue.

‡ Reaction of MAb with CK-20 ortholog of pig, dog or rat was considered positive when its staining pattern in intestinal epithelium was identical to that produced by guinea-pig antibodies to CK 20.[39]

‖ Weak reaction restricted to duodenal villi, probably not CK-20 specific.

¶ Trypsin treatment of sections was required prior to immunostaining.

ND, not determined.

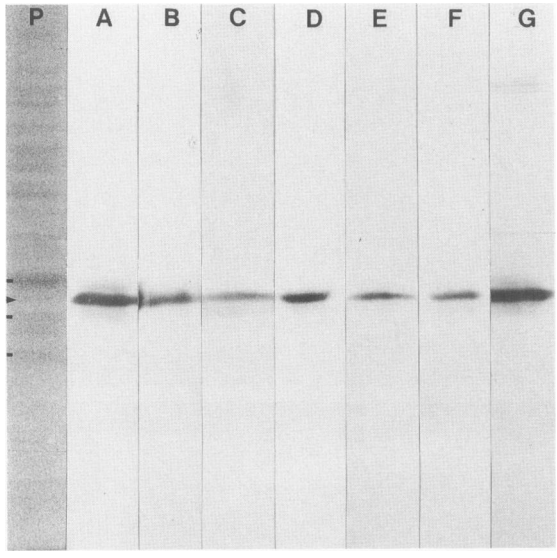

Figure 1. *Characterization of MAbs against CK 20 using immunoblotting. SDS-PAGE-separated cytoskeletal proteins of human duodenal mucosa were transferred to nitrocellulose membranes (shown in lane P as total protein staining using Ponceau-S-red) and immunostained using MAbs IT-K_s20.1* **(A)**, *IT-K_s20.2* **(B)**, *IT-K_s20.3* **(C)**, *IT-K_s20.4* **(D)**, *IT-K_s20.5* **(E)**, *IT-K_s20.6* **(F)**, *and IT-K_s20.7* **(G)**. *Note that each antibody specifically recognized CK 20 (arrowhead in lane P) but not CKs 8, 18 and 19 (dots in P; from top to bottom, respectively).*

for their immunoblot reactivity with proteins derived from various other human epithelial tissues, including 20 human epithelial CKs (Figure 3; for CK 7, Figure 10) as well as hair keratins. After immunoblotting with MAbs IT-K_s20.1 to IT-K_s20.7, the only immunoreactive band was that representing CK 20, which is present in the duodenal epithelium only (Figure 3, slots 1–3). No reaction with any other CK or non-CK protein was observed. Therefore, with regard to the immunoblot results, the CK-20 MAbs

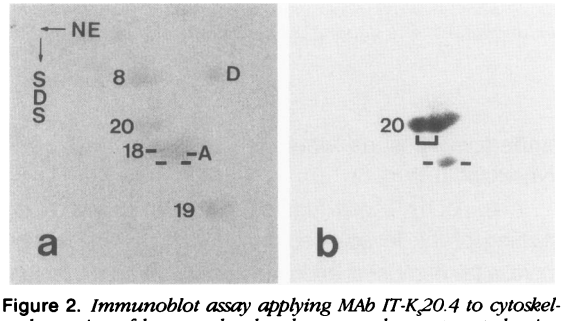

Figure 2. *Immunoblot assay applying MAb IT-K_s20.4 to cytoskeletal proteins of human duodenal mucosa, here separated using two-dimensional gel electrophoresis.* **a**: *Nitrocellulose membrane with total protein staining (Ponceau-S red).* **b**: *Corresponding immunoblot reaction, showing specific labeling of CK 20, including its two major isoelectric variants (bracket). The minor reactive spot between the horizontal bars represents a degradation product of CK 20 and is not visible in Ponceau-S-red staining* **(a)** *due to its minute amounts. NE, direction of first dimension using NEPHG electrophoresis: SDS, direction of second-dimension electrophoresis using SDS-PAGE; the numbers denote the CK polypeptides present.*[6] *A, endogenous actin; D, desmin derived from smooth muscle cells present in this preparation.*

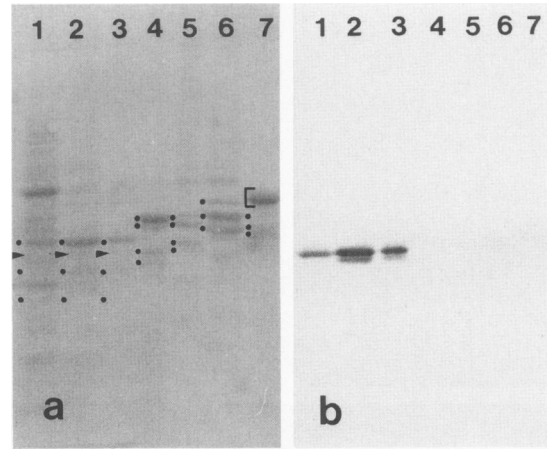

Figure 3. *Demonstration of the specificity of MAb IT-K_s20.6 by applying immunoblotting to various human tissues. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane.* **(a)** *shows total protein staining using Ponceau-S red. In each lane, the CKs present are indicated by dots, arrowheads (denoting CK 20) and brackets. Lanes 1–3, duodenal mucosa (lane 1, total proteins; lanes 2 and 3, cytoskeletal proteins; from top to bottom, CKs 8, 20, 18, 19); lane 4, plucked hair follicles (root sheaths, hair bulb, and hair shaft; total proteins; CKs 5, 6, 14/15, 16/17; hair cytokeratins are also present but not clearly resolved); lane 5, dorsal epithelium of the tongue (cytoskeletal proteins; CKs 4/5, 6, 13, 14); lane 6, corneal epithelium (total proteins; CKs 3, 5, 12); lane 7, foot-sole epidermis [cytoskeletal proteins; CKs 1/2/9 (bracket), 5, 10, 11].* **b**: *Corresponding immunoblot, showing the specific, exclusive reaction of MAb IT-K_s20.6 with CK 20 in duodenal epithelium (lanes 1 to 3; in lanes 2 and 3, a minor degradation product of CK 20 is also visible).*

were similar to the previously described guinea-pig antibodies.[39]

At immunofluorescence microscopy, all CK-20 MAbs were positive when applied to cell lines derived from human colonic carcinomas. In the case of DLD-1 cells, distinct fibrillar fluorescence was seen in the majority of cells (Figure 4a-d), but some were negative, resulting in a heterogeneous (mosaiclike) pattern of positive and negative cells as also noted for guinea-pig antibodies.[39] Essentially no differences in the immunostaining patterns were observed among the seven CK-20 MAbs. HT-29 cells were abundantly decorated by all of these CK-20 MAbs (Figure 4e), as well as by the guinea-pig antibodies described previously (Figure 4f, inset[39]). Figure 4f also shows that, in immunoelectron microscopy of HT-29 cells, the CK-20 antibodies specifically decorated IFs arranged in bundles, which is a typical feature of CK filament distribution. As expected, all of the antibodies against CK 20 yielded completely negative immunofluorescence results when applied to the mammary-carcinoma–derived MCF-7 cells (not shown).

The immunohistochemical results obtained for the intestinal mucosa of several mammalian species are listed in Table 1. For the pig, positive reaction was seen for five of the seven MAbs whereas the corresponding orthologous antigen of dog and rat was not detected (for guinea-pig antibodies[39]).

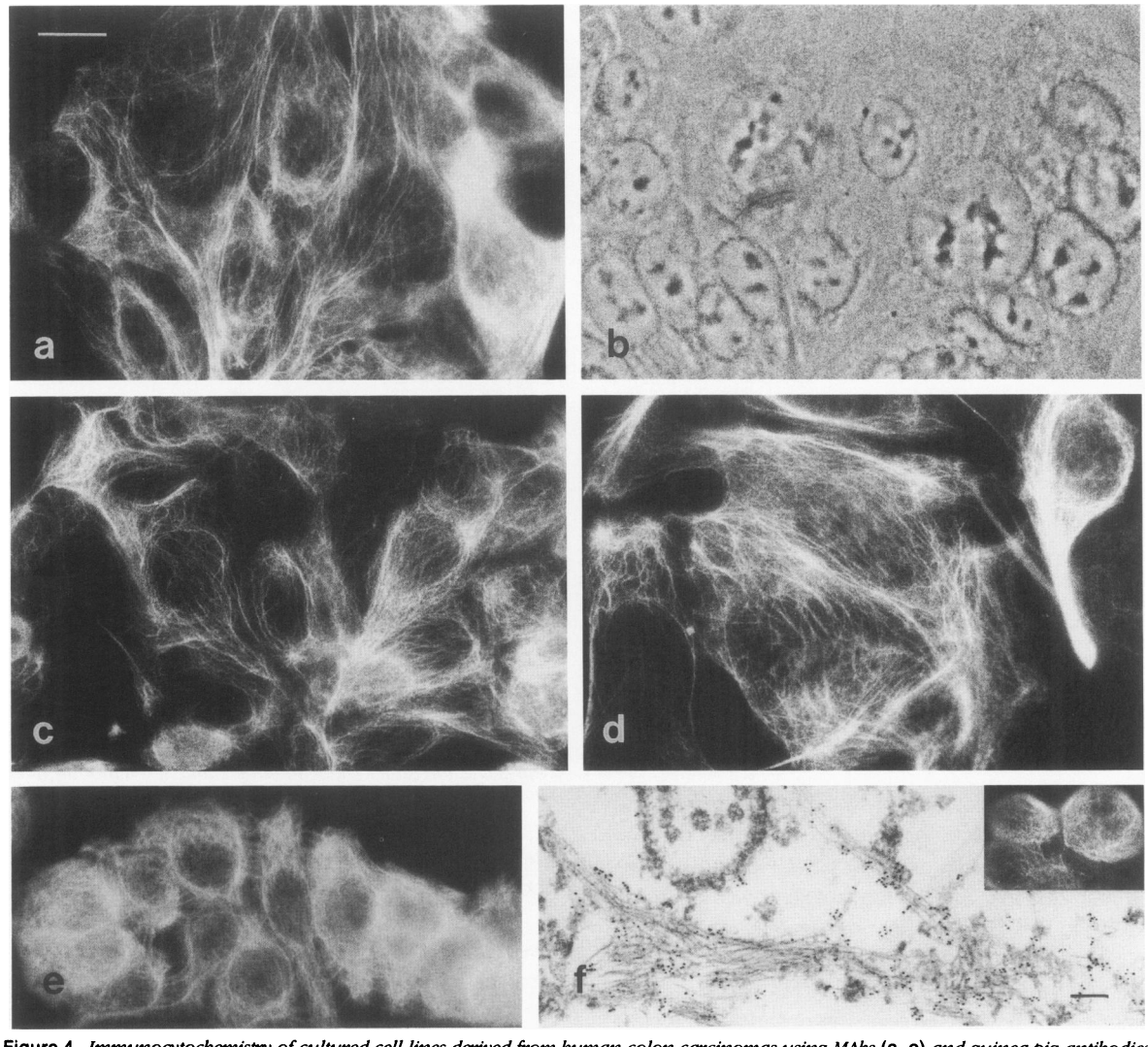

Figure 4. *Immunocytochemistry of cultured cell lines derived from human colon carcinomas using MAbs* **(a–e)** *and guinea-pig antibodies* **(f)** *specific for CK 20.* **a–d**: *Immunofluorescence microscopy of DLD-1 cells using MAbs IT-K_s20.2 (***a***;* **b**, *corresponding phase contrast micrograph), IT-K_s20.3* **(c)** *and IT-K_s20.5* **(d)**. *Note the distinctly fibrillar cytoplasmic staining of many (but not all) cells.* **e**: *Immunofluorescence microscopy of HT-29 cells using antibody IT-K_s20.4. Most cells exhibit cytoplasmic staining which partly appears fibrillar.* **f**: *Immunoelectron microscopy of HT-29 cells using guinea-pig antibodies to CK 20: note the specific decoration of bundled IFs by the antibodies as visualized by the 5-nm gold particles. The inset shows corresponding immunofluorescence staining using the same antibodies. Bars, 20 μm; in* **(f)** *(not inset), 0.1 μm.*

Distribution of Cytokeratin 20 in Normal Human Tissues as Determined by Immunohistochemistry

The immunohistochemical results obtained for normal human tissues using both MAbs and guinea-pig antibodies specific for CK 20 are summarized in Table 2.[39] A few specific examples, including the most important CK-20-expressing epithelial cell types, are shown in Figure 5. When applied to acetone-fixed cryostat sections (e.g., Figure 5a, d, e), similar staining results were produced by all of the CK-20 MAbs (IT-K_s20.1 to IT-K_s20.7) and the guinea-pig antibodies[39] (for the exceptional crossreactivities of two of these MAbs, see Table 1). MAb IT-K_s20.8 was observed to react with formalin-fixed paraffin-embedded tissues when the sections had been trypsinized (Figure 5b, c).

In stomach mucosa (Figure 5a), prominent and strong staining of the foveolar epithelium was noted; furthermore, a fair number of endocrine cells scattered throughout the upper portions of the pyloric glands were decorated (Figure 5a).[39] In cryostat and paraffin sections of duodenal mucosa, strong CK-20 positivity was present mainly in the epithelial cells lining the villi (Figure 5b). In the urothelium, specific albeit heterogeneous CK-20 immunostaining of superficial (umbrella) cells was noted (Figure 5c).

In the epidermis, the antibodies against CK 20 specifically decorated all Merkel cells (Figure 5d). Similar specific staining was noted for taste-bud cells in tongue

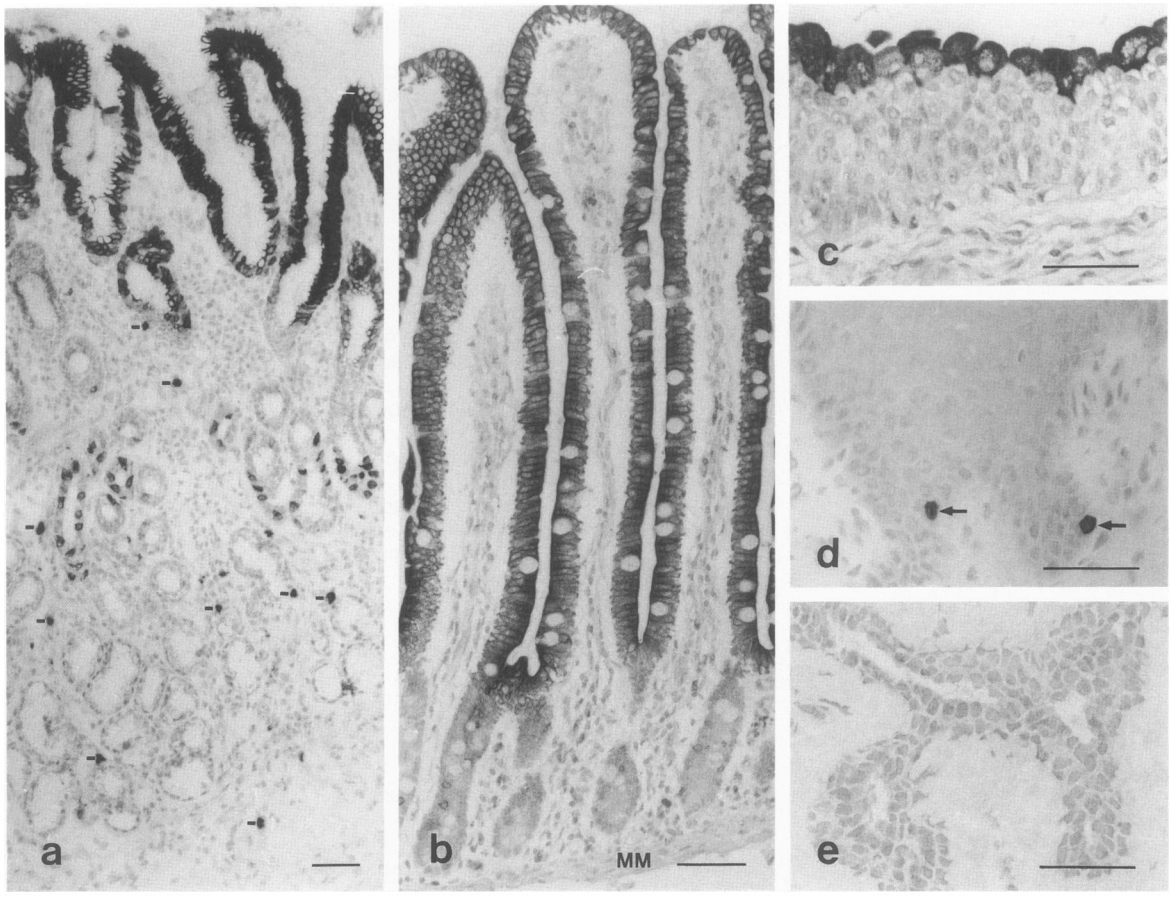

Figure 5. *Distribution of CK 20 in various normal human tissues as determined by immunohistochemistry.* **a**: *Stomach mucosa (pyloric region) showing strong staining of both the foveolar epithelium (top) and disseminated endocrine cells in the upper glandular zone (immunoperoxidase microscopy of a cryostat section; MAb IT-K$_s$20.5; short bars denote leukocytes stained nonspecifically due to the presence of endogenous peroxidase).* **b**: *Duodenal mucosa exhibiting strong staining of the mature epithelium lining the villi (paraffin section; ABC reaction; MAb IT-K$_s$20.8; MM, muscularis mucosae).* **c**: *Urothelium of the renal pelvis showing prominent staining of umbrella cells (paraffin section; ABC reaction; MAb IT-K$_s$20.8).* **d**: *In foot sole epidermis, CK 20 was specifically detected in basally situated Merkel cells (arrows; immunoperoxidase microscopy of a cryostat section; MAb IT-K$_s$20.5).* **e**: *Completely negative reaction of mammary-gland epithelium (terminal duct and acini of the resting gland; immunoperoxidase microscopy of a cryostat section; guinea-pig antibodies to CK 20). Bars, 50 μm.*

epithelium (not shown). Few other epithelial tissues contained sparsely distributed CK-20-positive cells (Table 2). Most other human tissues, including mammary-gland tissue (Figure 5e), were completely negative for the antibodies against CK 20 (Table 2).

Specific CK-20 immunoreactivity was not detected in any of the nonepithelial cells or tissues tested (Table 2; for crossreactivity of MAb IT-K$_s$20.7, see Table 1), including smooth muscle, blood-vessel walls, lymph nodes, and tumor stroma, i.e., tissues in which other simple-epithelial CKs are occasionally expressed.[49,51]

Distribution of Cytokeratin 20 in Human Tumors

A broad spectrum of human malignant tumors (predominantly carcinomas) was immunohistochemically analyzed for the expression of CK 20. The series comprised 711 cases and included both primary tumors and metastases at various sites (lymph nodes and distant). Only metastatic carcinomas for which the site and type of the primary tumor had been unequivocally established were included in the series. The results obtained using cryostat sections are summarized in Table 3. For purposes of comparison, the CKs (other than CK 20) shown by previous studies to be typical for the various major carcinoma categories are also indicated.

Since, in CK-20 positive tumors, the proportion of stained tumor cells varied considerably, often with sharply delineated cell-to-cell heterogeneity, the immunohistochemical results were scored semiquantitatively according to the estimated percentage of positive tumor cells.

From Table 3, it is evident that:

1. There are pronounced systematic differences in the expression of CK 20 between the various types of carcinoma.
2. Most types of CK-20 positive carcinoma are de-

Table 2. *Distribution of Cytokeratin 20 in Normal Human Tissues as Determined by Immunohistochemistry**

Epithelia	
Skin	
Epidermis	−
Merkel cells	+ + +
Hair follicles	−
Sebaceous glands	−
Eccrine sweat glands	−
Apocrine glands	−
Mammary gland	−
Cornea	−
Oral cavity and salivary glands	
Gingiva (keratinocytes)	−
Merkel cells	+ + +
Buccal mucosa	−
Tongue mucosa (keratinocytes)	−
Taste buds	+ + +
Palatine tonsil	−
Small salivary glands	−
Parotid gland	−
Submandibular gland	−
Thymus (reticulum cells)	+
Respiratory tract	
Bronchial epithelium	(+)†
Bronchial glands	−
Lung alveoli	−
Gastrointestinal tract	
Stomach	
Corpus mucosa	
Foveolar epithelium	+ + +
Corpus glands	−
Pyloric mucosa	
Foveolar epithelium	+ + +
Pyloric glands	
Neck portion‡	+
Main portion	−
Duodenum	
Mucosal villi‖	+ + +
Mucosal crypts‖	+ +
Brunner's glands	−
Jejunum, ileum	
Mucosal villi‖	+ + +
Mucosal crypts‖	+ +
Colon, rectum	
Surface epithelium‖	+ + +
Mucosal crypts‖	+ +
Liver	
Hepatocytes	−
Bile ducts	−¶
Gall-bladder mucosa	(+)
Pancreas	
Ducts	−¶
Ductules	−#
Acini	−
Islets	−
Mesothelium (pleura, peritoneum)	−
Urinary tract	
Kidney	
Bowman's capsule	−
Tubules	−
Collecting ducts	−
Surface epithelium of renal papilla	−
Urothelium (renal pelvis, ureter, bladder)	
Superficial (umbrella) cells	+ +
Intermediate cells	(+)
Basal cells	−
Male genital tract	
Prostate gland	(+)
Seminal vesicle	−
Ductus epididymidis	−
Ductuli efferentes	−
Rete testis	−
Seminiferous tubules	−
Female genital tract	
Uterus	
Ectocervical squamous epithelium	−
Endocervical epithelium**	−
Fallopian tube mucosa	−
Ovary††	−
Endocrine glands	
Pituitary gland	−
Thyroid gland‡‡	−
Parathyroid gland	−
Adrenal gland	−
Placenta	
Amnion epithelium	−
Trophoblast	−
Nonepithelial cells and tissues	
Connective tissue	−
Blood-vessel walls	−
Lymph nodes‖‖	−
Synovium (chronic synovitis)	−
Smooth-muscle tissue (gastrointestinal tract, myometrium)	−
Skeletal-muscle tissue	−
Brain tissue (medulla oblongata)	−
Ependyme	−
Nerves (e.g., nervus hypoglossus)	−

* Data are based on findings for guinea-pig antibodies to CK 20 (all tissues tested) and for MAbs IT-K_s20.1–7 (at least the stomach, duodenum, liver, pancreas, kidney, renal pelvis, skin containing Merkel cells, and brain were tested). + + +, uniformly positive (>80% of epithelial cells); + +, heterogeneously positive (10–80% of epithelial cells); +, few cells positive (1–9% of epithelial cells); (+), very few cells positive (<1% of epithelial cells); −, negative reaction.

† A few very scarce columnar cells were positive.

‡ Scattered cells in the upper glandular portion were positive; many of them (if not most or all) represent endocrine cells, as identified by their staining for chromogranin.

‖ Enterocytes, goblet cells, and, in the crypts, some undifferentiated cells.

¶ Very few positive cells were observed in some intrahepatic bile ducts of one liver specimen (in the vicinity of a metastasis of a gastric adenocarcinoma) and in the relatively large ducts of two specimens of pancreas with chronic pancreatitis (in one case, in the vicinity of a pancreatic adenocarcinoma).

Terminal ductules exhibited a focal, relatively weak staining for MAb IT-K_s20.2 but were negative for all other MAbs as well as for guinea pig antibodies against CK 20. In Western blot experiments using a cytoskeletal preparation of human pancreas and MAb IT-K_s20.2, no immunoreactive band was obtained (not shown).

** Including subcolumnar reserve cells.

†† Including the surface epithelium and granulosa cells of primary and cystic follicles.

‡‡ Follicular epithelium; C cells were not identified in the sections.

‖‖ Containing CK-8:18 positive reticulum cells.

rived from normal epithelia that also express CK 20 (Table 2).

3. The expression of CK 20 is similar in both the primary tumor and metastases of each carcinoma type.

Among the various types of adenocarcinomas, the most consistent and extensive CK-20 staining was observed in primary and metastatic colorectal carcinomas (Figure 6a–e). Strong immunostaining was seen not only in well-differentiated tumors (Figure 6a) but also in tumors and tumor regions exhibiting less extensive morphologic differentiation (Figure 6b, d, e), including tumors with trabecular (Figure 6d) or even dissociated, anaplastic growth patterns (Figure 6e). CK-20 staining frequently showed cell-to-cell heterogeneity (Figure 6b–d), this has also been the case in glandular tumor structures (Figure 6c), and immunofluorescence microscopy was often able to reveal the fibrillar staining pattern that is typical of CK filaments (Figure 6c). CK 20 was also detectable in routinely prepared paraffin sections of colon carcinomas (Figure 6b). Of the 93 cases of colorectal carcinomas tested, only three undifferentiated carcinomas (solid or signet-ring-cell carcinomas, G4) were completely negative for the CK-20 antibodies (Table 3).

Adenocarcinomas of the stomach were often also CK-20 positive, these including tubular adenocarcinomas (Figure 6f) as well as undifferentiated and signet-ring-cell carcinomas (Figure 6g). However, in comparison to colon carcinomas, the proportion of positive tumor cells tended to be lower, and there were rather more negative cases (Table 3). Nevertheless, half of these tumors exhibited significant levels of CK-20 expression (at least 5% of tumor cells positive).

Among adenocarcinomas of gall bladder and bile ducts, the majority of cases was (heterogeneously) positive for CK 20 (Table 3).

Duct cell adenocarcinomas of the pancreas were characterized by the considerable degree of variation in the patterns of CK-20 expression. Often, conspicuous strong staining of single cells and small cell groups was observed (Figure 6h), but the overall proportion of positive tumor cells rarely exceeded 20% (Table 3). More than one-half of these carcinomas, including some well-differentiated cases, were negative or contained only occasional CK-20 positive cells.

In the small series of hepatocellular carcinomas studied, all tumors contained a few single CK-20 positive cells (Figure 6i, Table 3).

Most of the adenocarcinomas from other sites were negative for CK 20 (Figure 7, Table 3). Thus, most of the large series of breast carcinomas studied, including invasive ductal carcinomas, completely lacked immunoreactivity for CK 20 (Figure 7a, Table 3), even though many of these tumors exhibited tubular structures or produced mucin (e.g., lobular and mucinous carcinomas), and could often not be distinguished with certainty from gastrointestinal adenocarcinomas on the basis of morphological criteria. In a small proportion of the breast carcinomas examined, sparsely distributed CK-20 positive cells were detected (Figure 7b, Table 3). One case (G3) was more extensively positive.

Adenocarcinomas of the endometrium were also negative for CK 20 or contained few positive cells. In addition, most types of ovarian carcinomas, including serous and endometrioid tumors, were negative for the antibodies against CK 20 (Figure 7c). The only exception was the group of mucinous ovarian tumors, which consistently expressed CK 20 (heterogeneously) irrespective of their degree of malignancy (Figure 7d). Almost all tumors of the various cytomorphologic types of renal cell carcinomas exhibited no CK-20 immunostaining at all (Figure 7e). However, in adenocarcinomas of the prostate gland, the presence of a few (< 5%) CK-20-positive tumor cells was the rule (Table 3). One case (G3) was more extensively positive.

No CK-20 expression was observed in malignant mesotheliomas of the pleura, including the epithelial, adenocarcinomalike type of these tumors (Figure 7f). Most cases of adenocarcinomas of the lung also lacked detectable CK-20 expression, even though they exhibited tubular structures (Figure 7g) and often produced mucin. In rare cases, a few scattered CK-20-positive tumor cells were detected (Figure 7h), and one exceptional case was extensively positive. In general, negative results were obtained for the other types of lung carcinoma, including squamous cell carcinomas; however, of this last type, two cases of grade 3 exhibited a minor but significant proportion of CK-20 positive tumor cells (Table 3).

Small cell lung carcinomas were CK-20 negative (Figure 7a), or at most, contained few positive cells (Table 3). This is particularly noteworthy, since Merkel cell carcinomas of the skin invariably exhibited the pronounced decoration of a large proportion or the majority of tumor cells, this appearing as paranuclear globular aggregates and/or delicate fibrillar (or diffuse) cytoplasmic staining (Figure 8b, c). Clearly, this represents an important finding for the differential diagnosis of such tumors. Intestinal and pancreatic neuroendocrine tumors were negative for CK 20 or exhibited only a sparsely distributed population of positive tumor cells (Table 3).

Interesting results were obtained for transitional-cell carcinomas of the urinary tract. In close correspondence with the expression of CK 20 in normal urothelium, most transitional cell carcinomas were heterogeneously positive or, in some cases, almost uniformly positive for the CK-20 antibodies (Figure 9a–d). In some well-differentiated papillary tumors, CK-20 staining was con-

Table 3. *Distribution of Cytokeratin 20 in Human Tumors as Determined by Immunohistochemistry*[a]

Site	Tumor type	Number of cases tested	CK-20 immunoreactivity[b]					Further CKs usually present[c]
			–	(+)	+	++	+++	
Colon, rectum	Adenocarc., G1, 2	68 (27)[d]	0 (0)[d]	1 (1)	5 (1)	37[e] (17)	25 (8)	8 18 19
	G3, 4	25 (12)	3 (0)	0 (0)	3 (2)	12 (8)	7 (2)	
Stomach	Adenocarc., G1, 2	11 (6)	1 (1)	6 (2)	3 (2)	1 (1)	0 (0)	(7) 8 (17) 18 19
	G3, 4[f]	21 (14)	6 (3)	3 (2)	2 (2)	9 (6)	1 (1)	
Gallbladder, bile ducts	Adenocarc., G1, 2	13 (4)	1 (0)	2 (0)	7 (3)	2 (1)	1 (0)	7 8 (17) 18 19
	G3, 4	6 (2)	2 (1)	0 (0)	2 (0)	2 (1)	0 (0)	
Pancreas	Adenocarc.[g], G1, 2	22 (5)	8 (2)	5[h] (0)	7 (3)	2 (2)	0 (0)	(4) 7 8 (17) 18 19
	G3, 4	23 (11)	11 (5)	3 (2)	8 (4)	1 (0)	0 (0)	
Liver	Hepatocellular carc.	4 (0)	0	4	0	0	0	8 18
Thyroid gland	Follicular (n = 2) and papillary (n = 4) carc.	6 (0)	6	0	0	0	0	7 8 18 (19)
	C-cell carc.	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	(7) 8 18 (19)
Salivary glands	Adenoid-cystic carc.	13 (4)	13 (4)	0 (0)	0 (0)	0 (0)	0 (0)	n.a.
	Mucoepidermoid tumor	3 (1)	3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	n.a.
	Adenocarc.	3 (0)	3 (0)	0 (0)	0 (0)	0 (0)	0 (0)	n.a.
Mammary gland	Invasive ductal carc., G1, 2	92[i] (7)	88 (7)	4 (0)	0 (0)	0 (0)	0 (0)	(7) 8 (14) (17) 18 19
	G3	19 (1)	15 (1)	3 (0)	0 (0)	1 (0)	0 (0)	
	Invasive lobular carc.	32 (2)	30 (2)	2 (0)	0 (0)	0 (0)	0 (0)	7 8 18 19
	Medullary carc.[j]	3 (0)	2	1	0	0	0	n.a.
	Mucinous carc.	4 (0)	3	1	0	0	0	n.a.
Endometrium	Adenocarc.[k], G1, 2	19 (2)	11 (1)	8 (1)	0 (0)	0 (0)	0 (0)	(4) (5) (7) 8 (13) (17)
	G3, 4	3 (1)	3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	18 19
Ovary	Serous, endometrioid, anaplastic and clear-cell tumors[l]	34 (7)	31 (7)	3 (0)	0 (0)	0 (0)	0 (0)	(4) (5) 7 8 18 19
	Mucinous tumors[m]	6 (0)	0	0	1	5	0	(7) 8 19 (19)
Kidney	Renal-cell carc.							
	Clear-cell type	25 (4)	24 (4)	1 (0)	0 (0)	0 (0)	0 (0)	8 18 (19)
	Chromophilic cell type	5 (0)	4	1	0	0	0	(7) 8 18 19
	Chromophobe cell type	9 (0)	8	1	0	0	0	7 8 18 (19)
Prostate gland	Adenocarc.	6 (1)	0 (0)	5 (1)	1 (0)	0 (0)	0 (0)	n.a.
Pleura	Malignant mesothelioma[n]	10 (0)	10	0	0	0	0	(4) 5 7 8 (14) (17) 18 19
Lung	Adenocarc.[o], G1, 2	23 (2)	19 (1)	3 (0)	0 (0)	0 (0)	1 (1)	(4) 7 8 18 19
	G3	10 (2)	9 (2)	1 (0)	0 (0)	0 (0)	0 (0)	
	Squamous cell carc., G1, 2	5 (1)	3 (0)	2 (1)	0 (0)	0 (0)	0 (0)	(4) 5 6 8 (10/11)
	G3	7 (0)	5	0	2	0	0	(13) 14 (15) 16 17 (18) 19
	Small-cell carc.							
	Intermediate-cell type	7 (3)	5 (3)	2 (0)	0 (0)	0 (0)	0 (0)	8 18 (19)
	Oat-cell type	8 (5)	7 (4)	1 (1)	0 (0)	0 (0)	0 (0)	
Skin	Merkel-cell carc.[p]	15 (4)	0 (0)	0 (0)	0 (0)	6 (2)	9 (2)	8 18 (19)
Small intestine, pancreas	Neuroendocrine tumors[q]	7 (3)	4 (2)	3 (1)	0 (0)	0 (0)	0 (0)	8 18 (19)
Urinary bladder, ureter, renal pelvis	Transitional-cell carc., G1, 2	7 (10)	0 (0)	2 (0)	1 (0)	2 (0)	2 (0)	(4) (5) 7 8 13
	G3, 4	17 (11)	1 (1)	2 (2)	0 (0)	10 (5)	4 (3)	(14) (17) 18 19
	Transitional-cell carc. with squamous metaplasia and squamous cell carc.	5 (2)	4 (2)	0 (0)	1 (0)	0 (0)	0 (0)	4 5 6 7 8 (10/11) 13 14 (16) 17 18 19
Cervix uteri	Squamous cell carc.	4 (0)	4	0	0	0	0	(4) 5 6 (8) (10/11) (13) 14–17 (18) 19
Oral cavity, pharynx, larynx	Squamous cell carc.[r]	67 (22)	61 (20)	5 (1)	1 (1)	0 (0)	0 (0)	
Esophagus	Squamous cell carc.	5 (4)	4 (3)	1 (1)	0 (0)	0 (0)	0 (0)	
Skin	Squamous cell carc.	9 (0)	9	0	0	0	0	5 6 (10/11) 14–17

Table 3. (*Continued*)

Site	Tumor type	Number of cases tested	CK-20 immunoreactivity[b]					Further CKs usually present[c]
			−	(+)	+	++	+++	
	Basal-cell carc.	3	3	0	0	0	0	5 (8) 14 (15) 17 (19)
	Malignant melanoma	5 (2)	5 (2)	0 (0)	0 (0)	0 (0)	0 (0)	None [rarely (8) (18)]
Testis, ovary[s]	Germ-cell tumors							
	Seminoma	4 (0)	4	0	0	0	0	None [rarely (8) (18)]
	Embryonal carc.	5 (1)	5 (1)	0 (0)	0 (0)	0 (0)	0 (0)	8 18 (19)
	Yolk-sac tumor	2 (0)	1	1	0	0	0	n.a.
Mesenchymal tumors[t]		10	10	0	0	0	0	None [rarely (8) (18)]

[a] Data are based on findings of immunofluorescence or immunoperoxidase microscopy performed on cryostat sections stained using guinea-pig antibodies and/or MAbs specific for CK 20.
[b] Scoring was performed according to the proportion of positive tumor cells: −, negative reaction; (+), <5% positive; +, 5%–20% positive; ++, 21%–80% positive; +++, >80% positive.
[c] Data compiled from previous studies based on gel-electrophoresis and/or immunohistochemistry findings (6, 9, 11, 13, 15, 20, 22, 24–30, 37, 46, 52–62). CKs present as minor components or in some but not all cases are indicated in parentheses. Rarely expressed CKs have not been included. n.a., no data available.
[d] First number = total number (primary tumors and metastases); number in parentheses = number of metastases (lymph node and distant).
[e] Including one case of adenocarcinoma of the small intestine.
[f] Including signet-ring-cell carcinomas [3 cases − or (+), 4 cases ++ or +++].
[g] Duct cell type.
[h] Including two cases of adenocarcinoma of the bile papilla.
[i] Including two cases of intraductal carcinoma [1 case −; 1 case (+)].
[j] Partly with portions of invasive ductal carcinoma.
[k] Including one clear-cell carcinoma (G2) and one papillary serous carcinoma (G2; both cases negative). Some adenocarcinomas exhibited focal squamous metaplasia (see ref. 37).
[l] Including borderline cystadenomas (4 cases, all negative).
[m] Including 1 cystadenoma and 2 borderline cystadenomas.
[n] 5 cases epithelial, 4 cases biphasic, 1 case sarcomatoid.
[o] Including 4 bronchioloalveolar carcinomas [2 cases −, 2 cases (+)] and 2 adenosquamous carcinomas (G3; both negative).
[p] In 8 cases, CK 20 was determined by two-dimensional gel electrophoresis only (++, relatively moderate amounts, 5 cases; +++, relatively large amounts, 3 cases).
[q] Comprising 2 intestinal carcinoids [both (+)] and 5 endocrine tumors of the pancreas.
[r] Various degrees of differentiation and keratinization; the cases scored "(+)" and "+" were predominantly poorly differentiated, nonkeratinizing specimens.
[s] One embryonal carcinomas (with teratoma) and one yolk-sac tumor were from the ovary (both negative).
[t] Including well-differentiated and myxoid chondrosarcoma, leiomyosarcoma, synovial sarcoma (biphasic), high-grade lymphoma, and malignant Schwannoma.

centrated in superficial tumor cells resembling umbrella cells. CK-20 staining could also be demonstrated in paraffin sections (Figure 9b). Notably, tumors of grades 3 and 4 were usually strongly positive (for lymph-node metastases of two such cases, Figure 9c, d). Only 1 of the 24 cases of pure transitional cell carcinoma studied was completely CK-20 negative. Squamous differentiation in these tumors resulted in a reduction in the level of CK-20 expression (Table 3).

Of our group of squamous cell carcinomas of the uterine cervix, oropharynx, larynx, esophagus, and skin, which included all grades of malignancy and degrees of keratinization, most cases were negative for CK 20 (Figure 9e), with the rest containing few or few positive cells (Figure 9f, Table 3).

All of the small series of nonepithelial tumors investigated (including primary and metastatic malignant melanomas, various types of sarcomas, and malignant lymphomas) were negative for CK 20 (Table 3).

In selected cases of carcinoma, gel electrophoresis and immunoblotting were performed to obtain direct evidence for the presence of CK 20. At two-dimensional gel electrophoresis, CK 20 could be identified, from its specific coordinates, in adenocarcinomas of the colon,[39] stomach (Figure 10a), and gall bladder (polypeptide "x" in[9]), transitional-cell carcinomas[46] as well as Merkel-cell carcinomas of the skin,[54,63] while no polypeptide corresponding to CK 20 could be recognized in other types of carcinomas and neuroendocrine tumors. The authenticity of the CK 20 resolved by gel electrophoresis was further confirmed by Western blotting. In such experiments using MAbs against CK 20, an immunoreactive protein band of M_r 46,000, definitely representing CK 20, was identified in adenocarcinomas of the colon but not in such tumors of the ovary and breast (Figure 10b, c).

Discussion

General Considerations

Immunologically, the most remarkable result of our study is probably the generation of seven different MAbs spe-

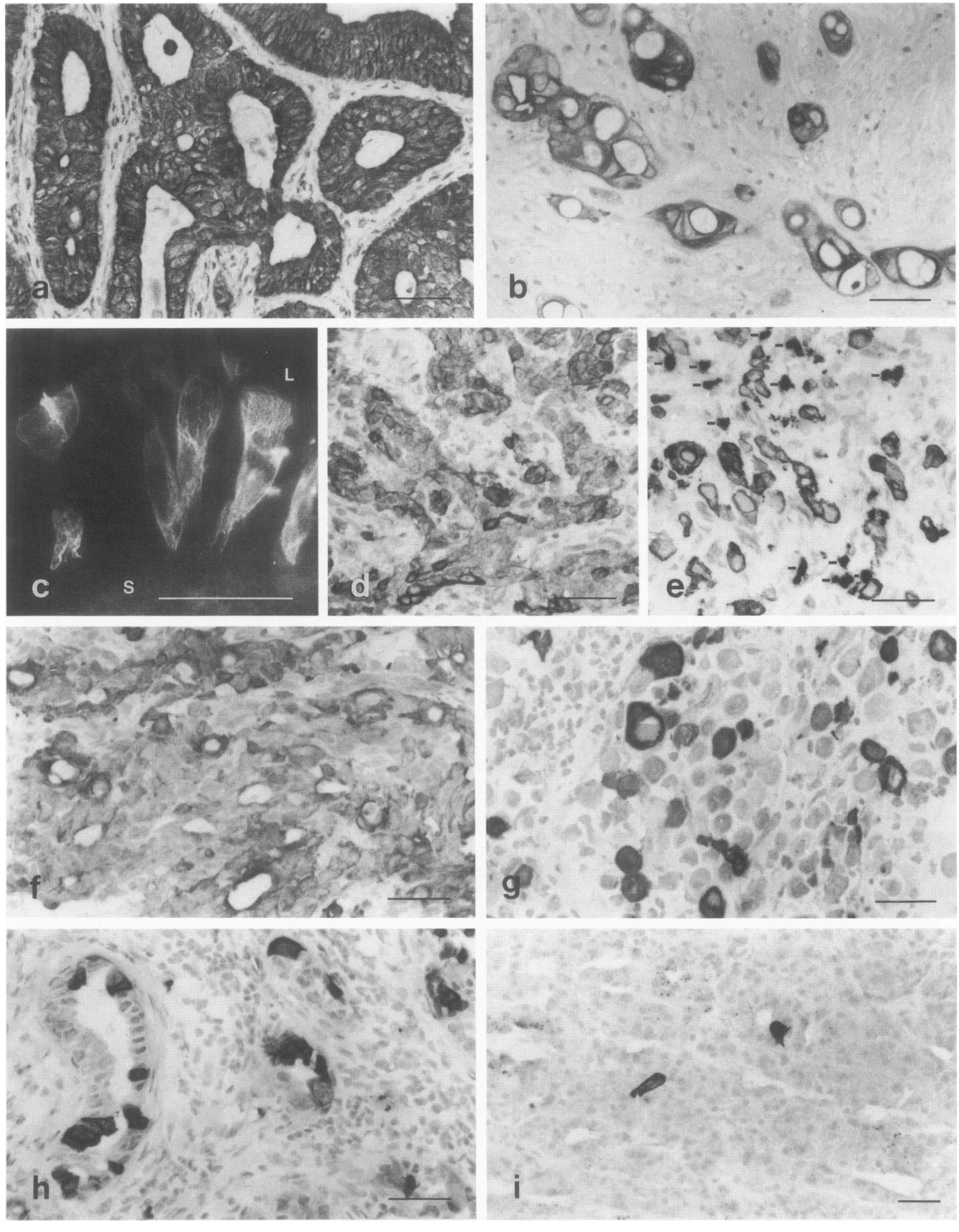

Figure 6. *Expression of CK 20 in adenocarcinomas of the colon* (**a–e**), *stomach* (**f, g**) *and pancreas* (**h**) *as well as in a hepatocellular carcinoma* (**i**), *as detected by applying immunoperoxidase* (**a, d–i**) *and immunofluorescence* (**c**) *microscopy to cryostat sections using guinea-pig antibodies and the ABC reaction to a paraffin section using MAb IT-K$_2$20.8* (**b**). **a**: *Well-differentiated adenocarcinoma of the colon (liver metastasis) showing almost uniform positivity for CK 20.* **b**: *Paraffin section of a moderately to poorly differentiated colon adenocarcinoma, showing positive staining of many tumor cells.* **c**: *Detail of moderately differentiated adenocarcinoma of the colon showing a fibrillar cytoplasmic immunofluorescence pattern; note that not all tumor cells are CK-20 positive (L, lumen; S, stroma).* **d**: *Significant, albeit heterogeneous, CK-20 expression has been maintained in this poorly differentiated adenocarcinoma of the colon sigmoideum with a predominantly trabecular growth pattern, which can here be seen infiltrating the periureteral tissue.* **e**: *Undifferentiated anaplastic portion of a colon carcinoma exhibiting the dissociation of tumor cells that still show strong CK-20 expression (short bars denote leukocytes stained nonspecifically due to the presence of endogenous peroxidase).* **f**: *Poorly differentiated tubular adenocarcinoma of the stomach exhibiting heterogeneous immunostaining of the tumor cells for CK 20 (L, lumen).* **g**: *Lymph-node metastasis of an undifferentiated carcinoma of the stomach containing signet-ring cells showing a small but significant proportion of CK-20-positive tumor cells (~30%).* **h**: *Moderately differentiated duct cell adenocarcinoma of the pancreas that was positive for CK 20 and exhibited distinct cell-to-cell heterogeneity.* **i**: *Only sparsely distributed single cells in this hepatocellular carcinoma reveal CK-20 expression. Bars, 50 μm.*

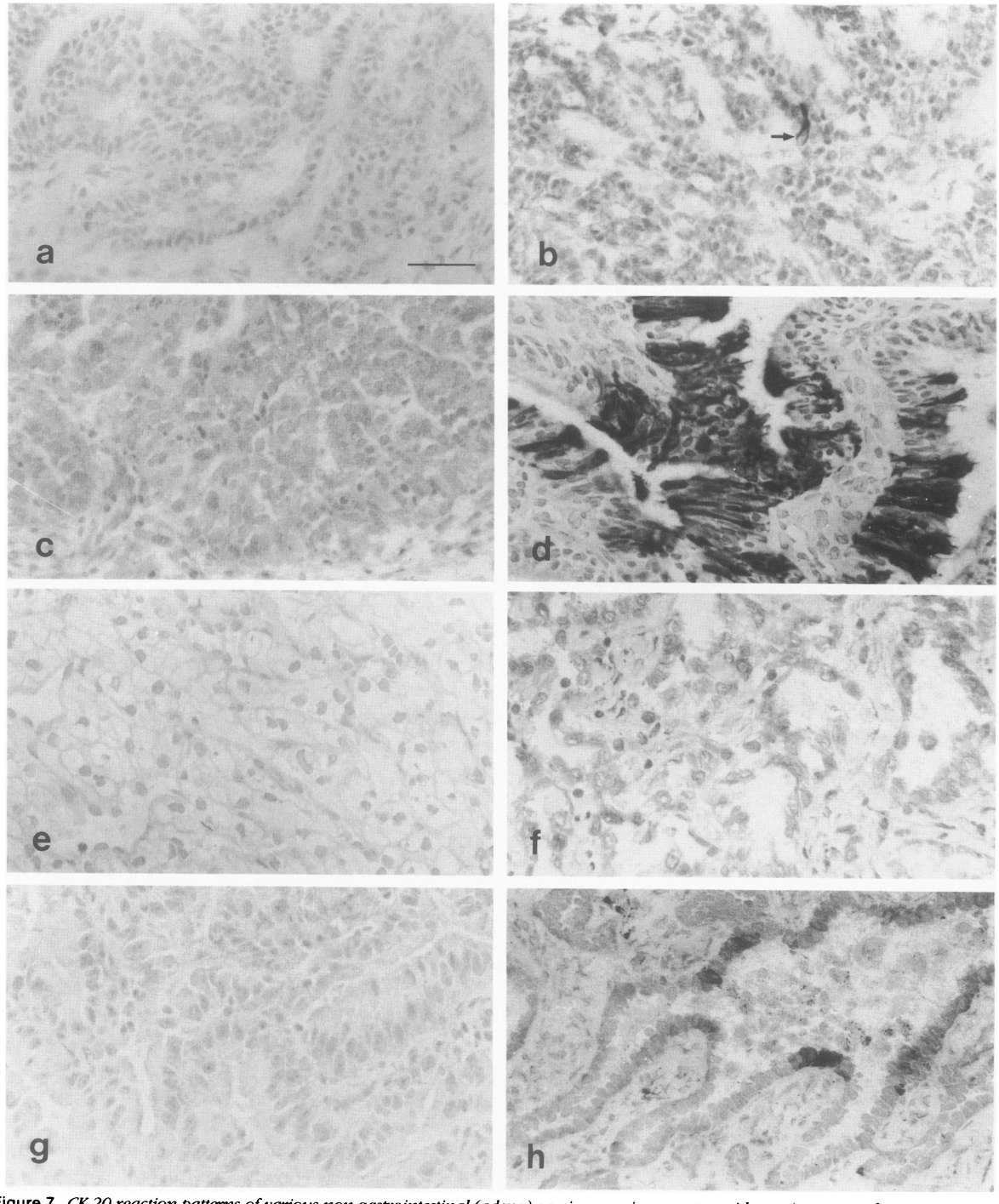

Figure 7. *CK-20 reaction patterns of various non-gastrointestinal (adeno)-carcinomas; immunoperoxidase microscopy of cryostat sections stained using guinea-pig antibodies.* **a, b:** *Invasive ductal carcinomas of the breast (***a***, G2;* **b***, G1) showing a completely negative reaction for CK 20* **(a)** *and, as a rare exception, sparsely occurring positive tumor cells (arrow in* **b***).* **c:** *Serous adenocarcinoma of the ovary (G3) that was negative for CK 20.* **d:** *In contrast, this mucinous cystadenocarcinoma of the ovary (low malignant potential) showed strong heterogeneous immunostaining for CK 20.* **e:** *Negative reaction of a renal cell carcinoma of the clear-cell type (G1).* **f:** *Malignant mesothelioma of the pleura (epithelial type) that was also negative for CK 20.* **g, h:** *Adenocarcinomas of the lung were usually CK-20 negative (***g***; G2) but the bronchioloalveolar carcinoma shown in* **(h)** *did contain a few positive tumor cells. Bar (in* **a***), 50 μm.*

cific for CK 20 none of which crossreacts with any of the other CKs, and a further MAb recognizing CK 20 in paraffin sections. Therefore, these MAbs provide specific tools in immunohistochemistry.

Our analysis of a large series of primary and metastatic carcinomas has shown that the characteristic, restricted expression of CK 20 observed in normal epithelia is maintained, to a large degree, during malignant trans-

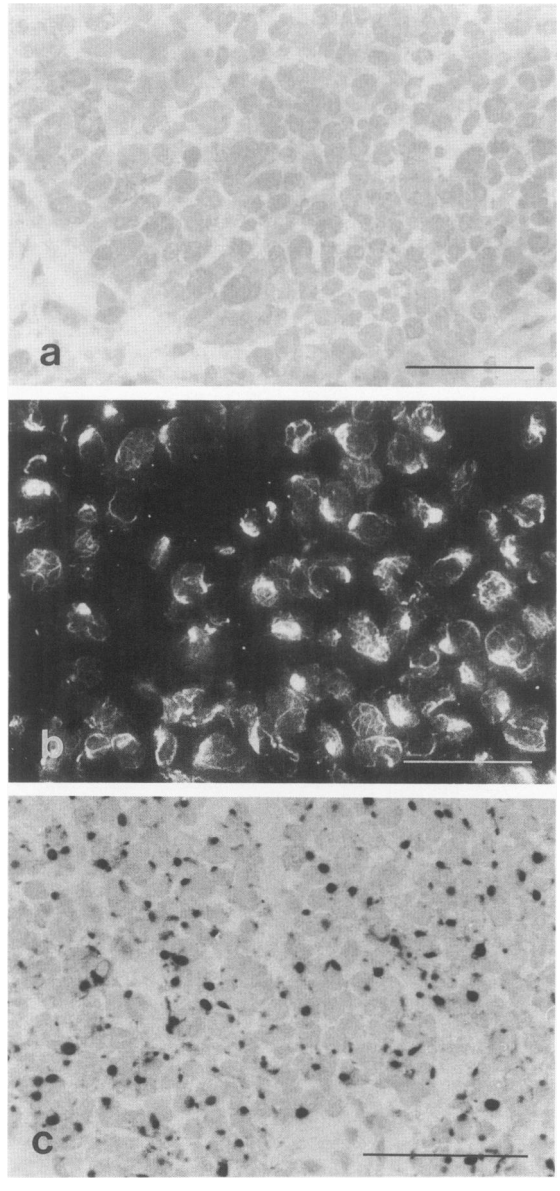

Figure 8. *Differential diagnosis of small-cell carcinoma of the lung* (**a**) *and Merkel-cell carcinoma of the skin* (**b, c**) *using immunohistochemistry to detect CK-20 staining (cryostat sections, guinea-pig antibodies).* **a**: *Negative reaction of a pulmonary small-cell carcinoma (immunoperoxidase microscopy); this particular case focally expressed neurofilaments exhibiting a plaque-like staining pattern (not shown).* **b**: *Merkel-cell carcinoma in which many tumor cells show positive staining, this mainly taking the form of coarse, plaque-like areas (immunoperoxidase).* **c**: *Another case of Merkel-cell carcinoma, again exhibiting the positive staining of most tumor cells for CK 20, but this time with a partially fibrillar pattern (immunofluorescence microscopy). Both cases of Merkel-cell carcinoma shown here also expressed neurofilaments in plaques (not shown). Bars, 50 μm.*

formation and tumor progression. Thus, the expression of this CK is a basic and virtually constitutive feature of a few specific programs of epithelial differentiation, so that despite the disturbances of gene expression occurring in tumor cells,[64] CK 20 continues to be synthesized in the corresponding carcinomas, even upon metastasis. For example, we have not found a single case of a Merkel-cell carcinoma in which the expression of CK 20 has been lost.[54,63] Remarkable stability of expression has also been noted for colorectal adenocarcinomas. For other carcinomas (stomach carcinomas, transitional-cell carcinomas), the degree of CK-20 preservation is not as high, so that a variable proportion of such tumors may lack CK-20. In stomach carcinomas, the expression of CK 20 may also be partially dependent on the involvement of intestinal metaplasia (which is CK-20 positive, R. Moll, M. Kasper, P. Stosiek, unpublished findings).

On the other hand, for a few epithelial tissues that normally appear to be CK-20 negative, the corresponding carcinomas may express this CK. However, this applies to a striking extent (number of positive cases and the proportion of positive tumor cells per section) only to carcinomas arising from the pancreas and the extrahepatic bile system, whose tissues of origin are ontogenically closely related to the CK-20-expressing gastrointestinal epithelia (occasionally, CK 20 is observed in individual cells of normal pancreatic ducts and biliary epithelium, more frequently so after non-neoplastic alterations[39,62]).

The infrequent finding of an ectopic CK-20 expression in sparse cells of carcinomas derived from CK-20-negative tissues, e.g., breast and endometrial carcinomas and squamous cell carcinomas, may indicate the loss of regulatory control mechanisms in individual cells similar to observations made for CKs 8 and 18.[65] In diagnostic tumor characterization, such rare and sporadic CK-20-positive cells are probably of little significance.

In most CK-20-expressing epithelia, the compartments containing the stem cells (intestinal crypt bases, gastric glandular necks, urothelial basal cell layer) mostly lack CK-20 at detectable levels, and this protein seems to be switched on during terminal differentiation and maturation. Therefore, in carcinomas arising from such stem cells, the expression of CK 20 may indicate local maturation of individual cells, resulting in cell-type heterogeneity in the same tumor.[56,57] Whether the specific heterogeneity of CK-20 positivity observed in a given tumor may reflect the spontaneous advent in some tumor cells (as a stochastic event) or local maturation remains to be examined, using other differentiation markers. With regard to squamous cell carcinomas, the focal expression of the keratinization-related CKs 1, 10, and 11[56] may be interpreted as reflecting such local maturation, since their presence is frequently closely correlated with horn-pearl formations. On the other hand, the almost uniform CK-20 staining pattern observed in some carcinomas, along with the fact that CK 20 may be present in mitotic cells both in solid tumors and in cell culture,[39] shows that, in

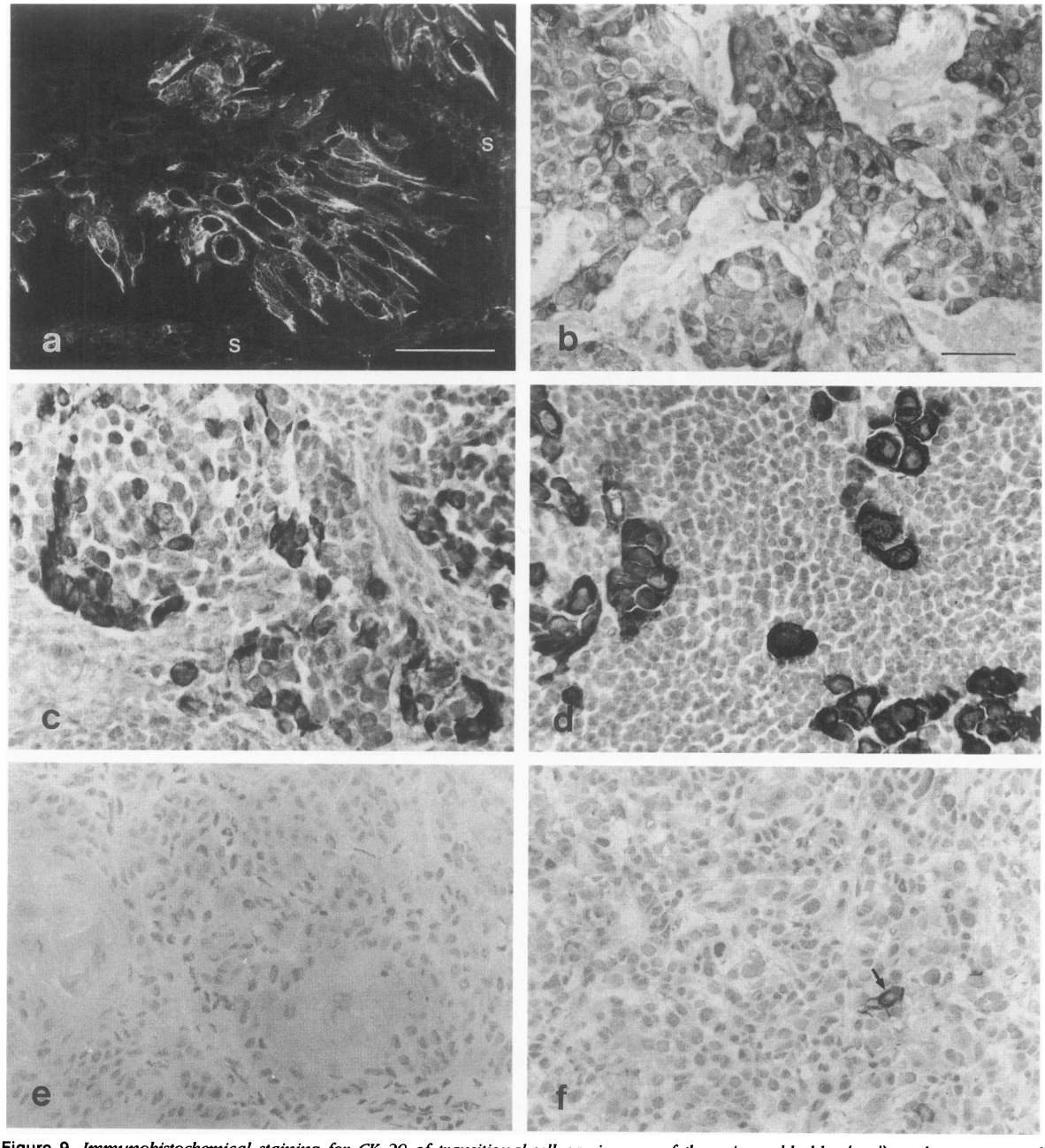

Figure 9. *Immunohistochemical staining for CK 20 of transitional-cell carcinomas of the urinary bladder* (**a–d**) *and squamous cell carcinomas* (**e, f**). *Immunofluorescence microscopy* (**a**) *and immunoperoxidase microscopy* (**c–f**) *of cryostat sections using guinea-pig antibodies;* **b**, *ABC reaction in paraffin section using MAb IT-K$_s$20.8.* **a**: *Papillary transitional-cell carcinoma of the bladder (G2; case no. 8 in ref.[46] showing mosaic-like immunofluorescence staining for CK 20 (S, stroma).* **b**: *Paraffin section of an invasive, nonpapillary transitional-cell carcinoma (G3) exhibiting some positive staining.* **c, d**: *Two cases of lymph-node metastases of nonpapillary transitional-cell carcinomas (*(**c**, *G3;* **d**, *G4); both show a positive reaction for CK 20.* **e**: *A moderately differentiated squamous cell carcinoma of the floor of the mouth that was completely negative for CK 20.* **f**: *A poorly differentiated, nonkeratinizing squamous cell carcinoma of the hypopharynx; exceptionally, a few sparsely distributed tumor cells are positive for CK 20 (arrow). Bars (in* **a** *and* **b**), *50 μm (*(**b–f**: *same magnification).*

certain carcinomas (e.g., colonic, urothelial), CK-20 expression occurs, and is continued, in actively proliferating cells.

The biological significance of the peculiar distribution and range of CK-20 expression among normal epithelia and carcinomas remains difficult to understand, in particular as no function of this protein is known. Although endodermal derivation is a common denominator of most of the epithelia expressing CK 20, there are exceptions to this rule, e.g., Merkel cells. However, the unique spectrum of CK-20 epithelia is well conserved among mammals as far down the evolutionary scale as the rat,[39]

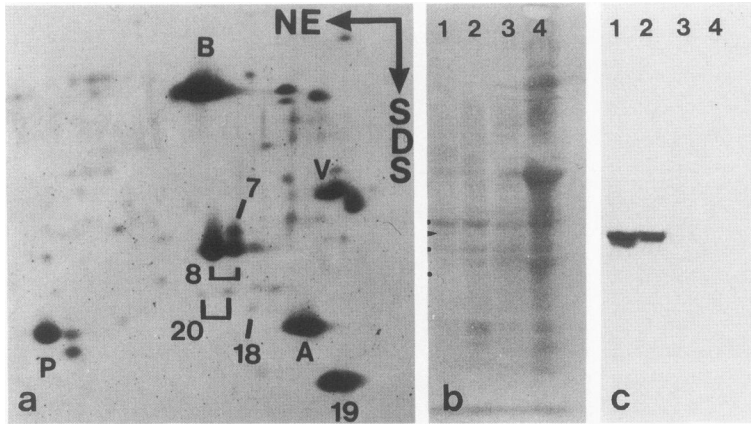

Figure 10. *Gel electrophoresis and immunoblot assays of cytoskeletal proteins of various carcinomas performed to investigate the presence of CK 20.* **a**: *Two-dimensional gel electrophoresis of cytoskeletal proteins from an adenocarcinoma of the stomach (G2), silver staining. Note the presence of small but significant amounts of CK 20. For symbols, see legend to Figure 2; P, 3-phosphoglycerokinase from yeast and B, BSA, both added as marker polypeptides.* **b, c**: *Immunoblot experiment using MAb IT-K$_s$20.6. Cytoskeletal proteins were separated by SDS-PAGE and transferred to nitrocellulose.* **b**: *Ponceau-S-red staining of total proteins;* **c**: *immunoblot. Lanes 1, duodenal mucosa (dots denote, from top to bottom, CKs 8, 18, and 19; the arrowhead designates CK 20); lanes 2, adenocarcinoma of the colon (liver metastasis); lanes 3, endometrioid adenocarcinoma of the ovary (also containing CK 7); lanes 4, invasive ductal carcinoma of breast. Note that an immunoreactive band corresponding to CK 20 is present only in normal intestinal mucosa and in the colon carcinoma (lanes 1 and 2 in* **c**).

which would seem to point to an important, as yet unknown biological role(s) for this CK.

Diagnostic Applications

It is evident from Table 3 that CK 20 exhibits different expression patterns in different types of carcinoma, even when these are morphologically similar, and that its expression is to a large extent conserved in metastases. It follows that this CK could well be a useful new histodiagnostic differentiation marker for problem cases, e.g., metastatic carcinomas whose primary tumor has not yet been detected.

Figure 11 illustrates the diagnostic relevance of the data obtained with carcinomas. It is probably appropriate to make a clear distinction between tumors in which 5% or more (up to 100%) of the tumor cells are CK-20 positive (CK-20 positive tumors, black segments in Figure 11) and tumors that are either completely CK-20 negative or contain less than 5% positive cells (white and dotted segments). Obviously, the different carcinoma types can be placed into one of the two categories, i.e., carcinomas that are commonly or frequently CK-20 positive and carcinomas that are mostly CK-20 negative (including cases with < 5% positive cells).

Consequently, CK 20 should be a suitable marker for several problems of differential diagnosis. One of these is the important and difficult diagnosis of specific tumor types among the large group of simple-epithelial tumors, including adenocarcinomas. The present data (Table 3, Figure 11) show that in tumors with a glandular morphology, CK-20 positivity is a strong argument either for an origin in the colon, stomach, bile system, or pancreas, or for a mucinous ovarian carcinoma. Such a finding excludes the possibility of an (adeno-) carcinoma of the endometrium, ovary (nonmucinous), kidney, thyroid, and salivary gland as well as a mesothelioma, whereas the likelihood of a lung or breast carcinoma would, at most, be low (the significance of the single cases in our series with considerable or strong CK-20 expression is, at present, difficult to assess). On the other hand, when an adenocarcinoma is CK-20 negative (or contains sparsely distributed positive tumor cells), various sites of origin need to be considered, e.g., the endometrium, ovary (nonmucinous), breast, lung, etc. whereas derivation from the stomach, bile ducts, or pancreas is less probable, while the presence of an adenocarcinoma of the colon is highly unlikely. Thus, CK 20 MAbs offer the marker desired to distinguish colon and breast adenocarcinomas (Yeger et al.[66] were unable to discriminate these tumors by their CK pattern).

Another field for the application of MAbs to CK 20 as a histodiagnostic marker is the differential diagnosis of Merkel-cell carcinomas (Merkel-cell tumors) of the skin as opposed to small-cell carcinomas of the lung (skin or lymph-node metastasis). Both tumors may be morphologically similar and have neuroendocrine characteristics in common,[67] and the criteria suggested for distinguishing them, e.g., the presence of neurofilaments and paranuclear IF aggregates in Merkel-cell carcinomas,[54,63,67,68] are not sufficiently specific (Figure 8a).[54,69,70] Conversely, these structures may be sparsely distributed or entirely absent in some Merkel-cell carcinomas.[63] In contrast, CK 20 is highly specific for Merkel-cell carcinomas, as previously suggested on the basis of gel-electrophoretic findings.[54,63] Thus, CK 20 is currently the best immunohistochemical marker for Merkel-cell carcinomas.

Moreover, this CK may provide a means of extending our understanding of the histogenesis of Merkel-cell car-

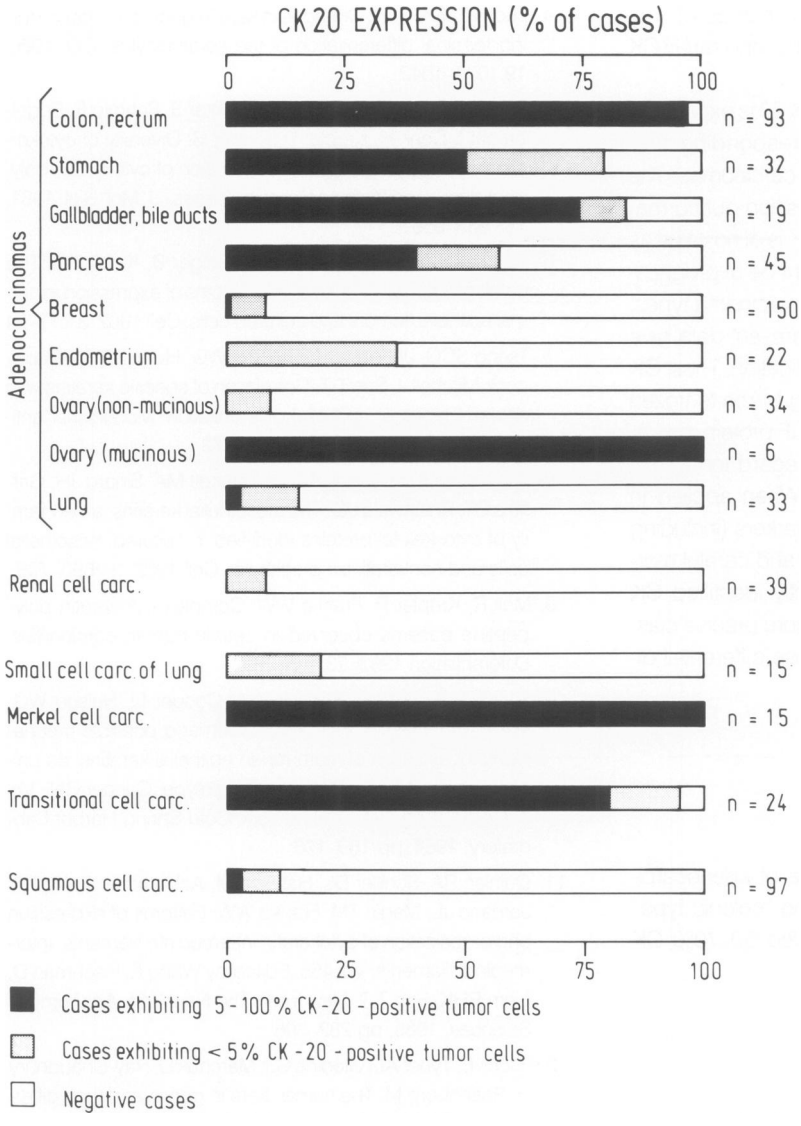

Figure 11. *Graphic representation of the expression of CK 20 in human carcinomas.*

cinomas. Although it has often been proposed that such tumors originate from cutaneous Merkel cells on the basis of common neuroendocrine features, direct evidence for this hypothesis is still lacking so that the question remains controversial.[67] As we have found that normal fetal and adult Merkel cells also express CK 20[39] (Figure 5d), this CK represents an additional (within the skin, highly specific) link between Merkel cells and these particular tumors, which would further support their histogenic relationship and the term "Merkel-cell carcinoma."

Finally, the importance of CK 20 as a marker of transitional-cell carcinomas of the urinary tract should be emphasized. In normal urothelium, CK 20 is mainly expressed in the upper, mature ("umbrella") cells ([39]; Fig. 5c). On the basis of their CK-20 expression patterns, there may be some form of relationship between normal urothelium and intestinal epithelium, as is also indicated by the occurrence of an intestinal-type glandular metaplasia in both normal urothelium ("cystitis glandularis") and transitional-cell carcinomas. During the development and progression of transitional-cell carcinomas, CK-20 expression is stably maintained, at least focally, as in poorly differentiated tumors and in lymph-node and distant metastases (Table 3; Figure 11). This is particularly important, since metastatic transitional-cell carcinomas lack distinguishing morphologic characteristics.

The overall CK profile of transitional-cell carcinomas is rather complex (Table 3). In addition to CK 20 and the other four simple-epithelial CKs (7, 8, 18, and 19), it frequently includes CK 13, whereas CKs 5, 14, and 17 occur less regularly.[46,71–73] Of these various CKs, both CK 13,[46,72,73] and CK 20 are useful markers; the latter appears to be especially suitable for the frequently relevant differential diagnosis of transitional-cell carcinomas and

poorly differentiated squamous cell carcinomas of various origins, which often express CK 13 but only rarely CK 20 (Table 3).

The present study has shown that CK 20 is expressed in both the primary tumors and corresponding metastases of a clearly defined subset of carcinomas. The high degree of stability of CK-20 expression during malignant transformation means that this CK is of no value as a marker of malignancy. Whether it might be of prognostic use, e.g., by subtyping of individual carcinoma types, will depend on future studies, but the present data give no grounds for postulating such an application. Thus, CK 20, as specifically detected by applying MAbs to frozen and paraffin sections, represents a new IF protein marker of promising diagnostic potential with regard to the histogenesis and typing of carcinomas. When applied in combination with other differentiation markers (including other CK polypeptides and IF proteins) and careful morphologic examination, this most recently identified CK should become a valuable aid for the more precise classification of many epithelial tumors whose differential diagnosis is otherwise difficult.

Note Added in Proof

The authors have recently seen a case of adenocarcinoma of the right ethmoidal sinus of the "colonic type" which by immunohistochemistry revealed 50–70% CK 20-positive tumor cells.

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