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Use of Monoclonal Antibodies to Keratin 7 in the Differential Diagnosis of Adenocarcinomas

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Monoclonal antibodies (MAbs) to specific keratin subtypes were prepared and characterized by immunoblotting and immunohistochemical assays on human cell cultures and normal and malignant human tissues. Chain-specific MAbs to keratin 7 (RCK 105, OV-TL 12/30) and keratin 18 (RGE 53, RCK 106, CK18-2), as well as broadly cross-reacting keratin MAbs (RCK 102, OV-TL 12/5) could be shown to react with different types of human epithelial tissues and were therefore tested for their usefulness in the differential diagnosis of carcinomas. The two broad-spectrum antibodies stained virtually all of the more than 350 carcinomas tested, especially when combined, and distinguished them from most nonepithelial tumors. The keratin 18 MAbs distinguished adenocarcinomas (which are keratin 18 positive) from most squamous cell carcinomas (which are generally keratin 18 negative). The MAbs to keratin 7 could be shown to recognize specific subtypes of adenocarcinoma and could, for example, distinguish between ovarian carcinomas (keratin 7 positive) and carcinomas of the gastrointestinal tract (keratin 7 negative), or between transitional cell carcinomas (keratin 7 positive) and prostate cancer (keratin 7 negative). In general, malignancies showed the expected keratin reactivity pattern as concluded from the keratin pattern of its cell of origin or its type of differentiation. The use of an extended series of malignancies did, however, also illustrate that exceptions to this rule exist. For example, certain antibodies to keratin 18 stained tumor areas in squamous cell carcinomas of the lung. Also a certain percentage of tumors, which generally showed no keratin 7 expression, were positive with RCK 105 or OV-TL 12/30. On the other hand, a certain percentage of tumors, which were generally positive for keratin 7, did not show a staining reaction with these MAbs. Furthermore subtle differences between reactivity patterns of different MAbs recognizing the same keratin protein were observed, both in the normal and malignant human tissues, indicating that specific keratin epitopes may be masked in certain tissues and that unmasking of such epitopes can occur with malignant progression. This phenomenon may be of some use in a further subtyping of carcinomas, especially those of the gastrointestinal tract. Despite these exceptional staining patterns, the keratin MAbs described above have proved to be useful tools in the characterization of epithelial tumors in routine histopathology and cytopathology, in which they add to a more refined diagnosis of (adeno)carcinomas. (Am J Pathol 1990, 136:641–655)

Keratins have been recognized to be epithelium-specific intermediate filament proteins and to comprise a family of at least 19 different polypeptides,[1,2] not including the hair keratins.[3] Also malignancies derived from such epithelial tissues have been shown to express specific keratins.[4–7] Combinations of the 19 different keratin proteins are distributed in a more or less tissue-specific fashion as initially detected by two-dimensional gel electrophoretic procedures.[1] The use of chain-specific monoclonal antibodies (MAbs) to individual keratins has confirmed these biochemical studies and allows the immunohistochemical distinction between different epithelia and carcinomas. For example, certain MAbs to keratin 18 separate most squamous cell carcinomas from adenocarcinomas,[6] while the degree of differentiation in a squamous epithelial tumor can be estimated by the use of keratin 10 or keratin 13 MAbs.[8]

In a recent study[9] we have described the preparation of a MAb specific for keratin 7 (RCK 105) and have shown

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Table 1. *Reactivity Patterns of Monoclonal Keratin Antibodies in Malignant Epithelial Human Tissues*

Diagnosis		Broad		Keratin 7		Keratin 18		
		RCK 102	OV-TL 12/5	RCK 105	OV-TL 12/30	RGE 53	RCK 106	CK 18-2
Squamous cell carcinomas								
Squamous cell ca. vulva		+		−		−	−	
Squamous cell ca. vulva		+	+	−	−*	−		
Keratinizing squamous cell ca. penis				−				
Lymph node metastasis poorly diff. squamous cell ca. penis		+		−	−	−*	+	
Moderately diff. squamous cell ca. cervix		+		−		−	−*	
Keratinizing squamous cell ca. tongue		+		−		−		
Keratinizing squamous cell ca. parotis		+		−		−		
Well diff. squamous cell ca. oesophagus		+		−		−		
Lymph node metastasis poorly diff. squamous cell ca.		+		−	−	−*	−*	
Lymph node met. squamous cell ca.		+		−	+†	−	−	
Lung tumors								
Squamous cell carcinoma	10×	+	+	−‡	+§	−‡	+&−‡	
Adenocarcinoma	10×	+	+	+	+	+	+	
Small cell anaplastic ca.	6×	+	+§	−‡	+&−‡	+§	+§	
Carcinoid	10×	+		−‡		+	+	
Adenocarcinomas of the GI tract								
Moderately diff. mucinous adenoca. stomach		+	+	−	−	+	+	+
Moderately diff. adenoca. stomach		+	+	−	−	+	+	+
Moderately/poorly diff. adenoca. stomach		+	+	+/−†	+†	+	+	+
Poorly diff. adenoca. stomach		+		−	−	+	+	
Poorly diff. adenoca. stomach		+	+†	−*	+†	+	+	
Poorly diff. anaplastic ca. stomach		+		−	−*	+	+	
Anaplastic ca. stomach		+		−*		+†		
Metastasis of a stomach adenocarcinoma in the ovaries		+		−			+	
Signet ring cell ca. stomach		+	+		+	+		
Mucinous adenoca. stomach		+	+		−	+		
Adenoca. stomach		+		−		+		
Anaplastic adenoca. stomach		+	+†	−*	+†	+	+	
Malignant degenerated adenovillous polypous stomach		+		−	−	+	+	
Adenocarcinoma jejunum		+	+		+	+		
Well/moderately diff. mucinous adenoca. colon	3×	+	+	−	−*	+	+	
Well diff. adenoca. colon		+	+	−*	−*	+	+	
Moderately diff. adenoca. colon		+		−	−	+	+	
Moderately diff. adenoca. colon	2×	+	+	−*	−*	+	+	
Moderately diff. partly papillary adenoca. colon		+	+		−	+		
Metastasis of this tumor in the ovary		+	+		−	+		
Moderately diff. multifocal adenoca. colon		+	+	−	−	+	+	
Metastasis of this tumor in the ovary		+	+	−	−	+	+	
Metastasis of a moderately diff. adenoca. of the colon	2×	+		−	−	+	+	
Poorly diff. adenoca. colon		+	+	−	−	+	+	
Poorly diff. adenoca. colon		+		−		+	+	
Metastasis of a mucinous colon adenoca. in the ovary	2×	+	+	−*	−*	+	+	
Intestinal carcinoids	2×	+		−*	+†	+	+	
Carcinomas of the pancreas								
Carcinoma in pancreas (autopsy material of patient with lung metastases)		+	+	+	+	+	+	
Mucinous adenocarcinoma		+		+†				
Adenocarcinoma		+		+		+	+	
Papillary adenocarcinoma		+	+	+		+		
Liver tumors								
Hepatoblastoma		+	+/−	−	+†	+	+†	
Hepatocellular carcinoma	5×	+		−	−*	+	+	+
Hepatocellular carcinoma		+		−*	+	+	+	+
Hepatocellular carcinoma	2×	+		+	+	+	+	+
Breast carcinoma								
	24×	+§	+§	+§	+§			
	80×	+‖		+§		+§	+	

Table 1 (*continued*)

Diagnosis		Broad		Keratin 7		Keratin 18		
		RCK 102	OV-TL 12/5	RCK 105	OV-TL 12/30	RGE 53	RCK 106	CK 18-2
Female genital tract carcinomas								
Anaplastic carcinoma of the cervix		+		+†				
Adenosquamous carcinoma of the cervix		+		+†	+*	+	+	
Metastasis of a fallopian tube adenoca. in the cervix		+		+	+	+	+	
Serous cystadenocarcinoma	21×	+	+	+¶	+	+	+¶	+¶
Mucinous cystadenocarcinoma	6×	+	+		+	+		
Endometrioid carcinoma	8×	+	+	+¶	+	+¶	+¶	+
Metastasis endometrioid ca. on the mesocolon		+		+	+		+	
Nonclassified ovarian carcinomas	4×	+	+	+	+	+	+	+
Choriocarcinoma		+	+	+	+	+	+	+
Liver metastasis of a chorioca.		+		+		+†		
Brenner tumor		+	+	+	+	+	+	+
Male genital tract tumors								
Anaplastic seminoma		−	−	−	−	−	−	−
Embryonal cell carcinoma		+		−*		+†		
Prostate carcinoma	5×	+	+†	−*	−*	+	+	
Urinary tract carcinomas								
Renal cell carcinoma (Grawitz tumor)	11×	+		−		+		
Renal cell carcinoma	6×	+		−*		+§		
Renal cell carcinoma	2×	+		+		+		
Renal cell carcinoma	6×	+	+	−	−*			
Renal cell carcinoma	2×	+	−	−	−			
Wilm's tumor	3×	+		−		+§		
Wilm's tumor	1×	+	+		−	+		
Transitional cell carcinomas	59×	+		+§		+#	+	+
	4×	+	+		+			
Miscellaneous								
Mesothelioma	21×	+¶		+§		+¶	+¶	
Carcinoid	2×	+		−		+		
Adrenal pheochromocytoma	2×	−	−	−	−	−	−	

+/− Weakly positive.
* Some sporadic cells positive (in one or some specimens).
† Partly positive.
‡ Some positive cases or cases with positive areas.
§ Sometimes also tumors with negative areas or sporadically completely negative tumors were found.
‖ For RCK 102 40 cases were examined.
¶ Only few cases were examined with these antibodies.
# Variable staining pattern with 32 cases completely positive and 26 cases partly positive. One negative case showed squamoid differentiation.

that among normal human epithelia such a reagent can distinguish different types of columnar and glandular epithelia. Other authors have already pointed to the usefulness of keratin 7 MAbs in tumor diagnosis.[10–12]

In the underlying study the applicability of this MAb in the differential diagnosis of carcinomas was further examined. Because we were, however, aware of the fact that epitope masking may be a common feature when working with monoclonal (keratin) antibodies, we prepared a second MAb to keratin 7 (OV-TL 12/30) and used it in parallel with RCK 105. This antibody was obtained after immunization of mice with a human ovarian tumor cell line[13] and had been tested only on cultured cells.[14,15]

In this paper the antibody is characterized further by immunoblotting and applied to normal human epithelial tissues and to carcinomas. Next to these keratin 7 MAbs broadly cross-reacting monoclonal keratin antibodies (RCK 102 and OV-TL 12/5), as well as antibodies to keratin 18 (RGE 53, RCK 106, CK 18-2) were used in this study.

Materials and Methods

Tissues and Cell Cultures

Fresh normal and neoplastic human tissues (Tables 1 and 2) were obtained immediately after surgery or during autopsy, frozen in liquid nitrogen, and stored either in liquid nitrogen or at −80 C.

Frozen sections (4 to 7 μm thick) were cut on a cryostat, air dried, and in most cases also fixed in methanol (−20 C) for 5 minutes and dipped in acetone. All tissue specimens were diagnosed using hematoxylin and eosin (H&E)-stained paraffin sections. Ovarian carcinoma cell lines OTN11, OTN14, EFO-21, EFO-27, OAW-42, OVCA

Table 2. *Application of Keratin MAbs in Differential Tumor Diagnosis*

No.	Sex	Age	Localization	Histologic appearance	DD for localization/ classification primary malignancy	RCK 102	RCK 105	RCK 106	Final diagnosis	Remarks
1	F	53	Pelvic cavity	Moderately differentiated adenocarcinoma	Colon or ovary	nd	−	nd	Colon carcinoma	Elevated Ca 125 serum level but no immunohistologic OC-125 reactivity
2	F	41	Ovary	Moderately differentiated adenocarcinoma	Colon or ovary	nd	−	nd	Colon carcinoma metastasis	Colon carcinoma resected 8 months before detection of second tumor
3	F	65	Abdomen	Poorly differentiated adenocarcinoma	Colon or ovary	+	+	+	Ovarian carcinoma	
4	F	66	Inguinal lymph node	Poorly differentiated adenocarcinoma	GI tract, ovary or others	nd	+	+	Ovarian carcinoma	Immunohistologic reactivity with ovarian ca. markers OC-125 and OV-TL 3
5	F	65	Liver	Poorly differentiated adenocarcinoma	GI tract (liver, bile ducts, stomach or colon)	nd	+	+	Carcinoma of the extrahepatic bile ducts	Obstructive jaundice
6	M	74	Prostate	Poorly differentiated carcinoma	Urinary bladder or prostate	nd	+	+	Transitional cell carcinoma	Bladder tumor removed 7 years before detection of extension into prostate
7	F		Omentum	Undifferentiated large-cell carcinoma	Ovary or others	+	+	+	Ovarian carcinoma	
8	F	27	Ovary and kidney	Large-cell carcinoma	Kidney or ovary	+	−	+	Renal cell carcinoma metastasis	Renal cell carcinoma in left kidney
9	M	64	Cervical lymph node	Poorly differentiated adenocarcinoma	Lung, prostate, or others	+	+	+	Lung carcinoma	Lung biopsy positive for cancer; prostate biopsy negative for cancer. PSA negative.
10	M	47	Axillary lymph node	Undifferentiated large-cell carcinoma	Lung, urinary bladder, or others	nd	+	nd	DD lung carcinoma or transitional cell carcinoma	Cytology positive for urinary bladder carcinoma. Surgery revealed transitional cell carcinoma pT2; G3.
11	F	70	Lung	Adenocarcinoma	Lung, GI tract, or others	nd	+	nd	Bronchioalveolar carcinoma	Diagnosis supported by clinical data.
12	F	60	Small bowel	Poorly differentiated adenocarcinoma	Ovary or GI tract	nd	+	+	Endometrioid carcinoma	Immunohistochemical reactivity with ovarian tumor marker OV-TL 3.
13	F	56	Omentum	Poorly differentiated carcinoma	Ovary or GI tract	nd	−	±	Endometrioid carcinoma	Immunohistochemical reactivity with ovarian tumor markers OV-TL 3 and OC-125
14	F	56	Peritoneum	Undifferentiated carcinoma	Ovary or others	+	−	+	Mixed Müllerian tumor	No immunohistochemical reactivity with ovarian tumor markers OV-TL 3 or OC-125

nd, not determined.
DD, differential diagnosis.

433, and OVCAR 3 were grown as described,[13-15] while cell lines T24, RT4, HeLa, TR146 (provided by Dr. E. B. Lane, ICRF, London), KB, WiDr, LLCMK-2, and A431 were grown in Eagle's Minimum Essential Medium (EMEM) containing 10% newborn bovine serum.

Immuno(histo)chemical Assays

The indirect immunofluorescence technique on frozen sections (either air dried and/or fixed in methanol with or without an additional acetone fixation step) was performed essentially as described before.[16,17] Also the immunoperoxidase technique used on frozen sections in this study has been published.[8]

Cell cultures were washed with phosphate-buffered saline (PBS), fixed in methanol/acetone as described above, and rehydrated with PBS before the first antibody was added.

One- and two-dimensional SDS-polyacrylamide gel electrophoresis and immunoblotting of Triton X-100–extracted cytoskeleton preparations of cell cultures and solid tumor tissue were done as described by Ramaekers et al[9] and Broers et al.[8]

Antibodies

The following mouse monoclonal antibody preparations were used in this study.

1) Antibodies OV-TL 12/5 and OV-TL 12/30 were obtained from a fusion of Sp2/0-Ag14 myeloma cells with spleen cells from mice immunized with OTN 11 ovarian carcinoma cells.[13] These cells were grown in RPMI-1640 containing 15% fetal calf serum (FCS).[13] Cell suspensions were cryopreserved in medium containing 10% FCS and 10% dimethylsulfoxide and stored in liquid nitrogen.
   Eight-week-old male Balb/c mice were immunized by seven subsequent intraperitoneal injections, at 2-to-3-week intervals, with 2 to 3×10^6 cultured OTN 11 tumor cells.[13,15] Three days after the last injection the spleen was aseptically removed and fusion was performed by incubating 8×10^7 lymphocytes with 4×10^7 mouse SP2/0-Ag14 myeloma cells in the presence of 0.5 ml of a 50% (volume/volume) polyethylene glycol 4000 solution for 1 minute at 37 C according to the technique of Köhler and Milstein[18] and modified by Kennet et al.[19] Growth conditions, cloning, and testing procedures were essentially as described before for OV-TL 3.[16]
2) Antibody RCK 102, a mouse MAb, has been found to be specific for keratins 5 and 8 (for keratin nomenclature see Moll et al.[1]). A partial characterization of this antibody, recognizing many epithelial tissues and tumor types, has been described before.[9]
3) Antibodies RGE 53, RCK 106, and CK 18-2 have been shown to recognize specifically epitopes on keratin 18.[6,9] It should be noted that in certain tissues keratin 18 epitopes detected by the individual antibodies may be masked.[20]
4) Antibody RCK 105 has been shown to react specifically with keratin 7 and, as a result, to react with transitional epithelium and a subpopulation of columnar and glandular epithelia.[8,9,20]

Next to these, additional monoclonal keratin antibodies were used in the immunoblotting studies to determine the exact nature of the antibodies described above. They included antibodies M20 (anti-keratin 8), 6B10 (anti-keratin 4), AE3 (recognizing the basic keratins 1 to 8)[21], LP2K (anti-keratin 19; a gift from Dr. E. B. Lane, London), and E3 (anti-keratin 17, see Guelstein et al[22]; a gift from Dr. S. M. Troyanovsky, Moscow); and RKSE 60 (anti-keratin 10), see Broers et al,[8] and Guelstein et al.[20] Furthermore, the antibody RV202, specific for vimentin,[9,17] was applied in the immunoblotting assays.

Results

Monoclonal Antibody Characterization by Immunoblotting

Keratin antibodies RCK 106, RGE 53, and CK18-2 have been characterized extensively as described before.[6,9,20] For this study antibodies OV-TL 12/30, RCK 105, OV-TL 12/5, and RCK 102 were further characterized by one- and two-dimensional immunoblotting procedures.

The characterization of RCK 105 has been described[9] and was performed mainly by immunoblotting of cytoskeletal preparations of cell lines.

Figure 1a shows a comparative one-dimensional immunoblotting study of this antibody with antibody AE3[21] indicating that in those cell lines containing, among others, keratin 7, only this protein band is recognized by RCK 105 (Figure 1a, compare lanes 3 and 4, and lanes 5 and 6). Those cell lines known not to contain keratin 7 also do not show reactivity with RCK 105 in the blots (Figure 1a, compare lanes 7 and 8, and lanes 9 and 10). A human foreskin preparation, containing several of the high molecular weight keratins (Figure 1a, lane 1) is also negative for RCK 105 (Figure 1a, lane 2). The keratin 7 nature of the RCK 105 antigen was further confirmed by two-dimensional immunoblotting (Figure 1b and c). To extend the immunochemical characterization of RCK 105 to human malignant tissues, we used cytoskeletal preparations of

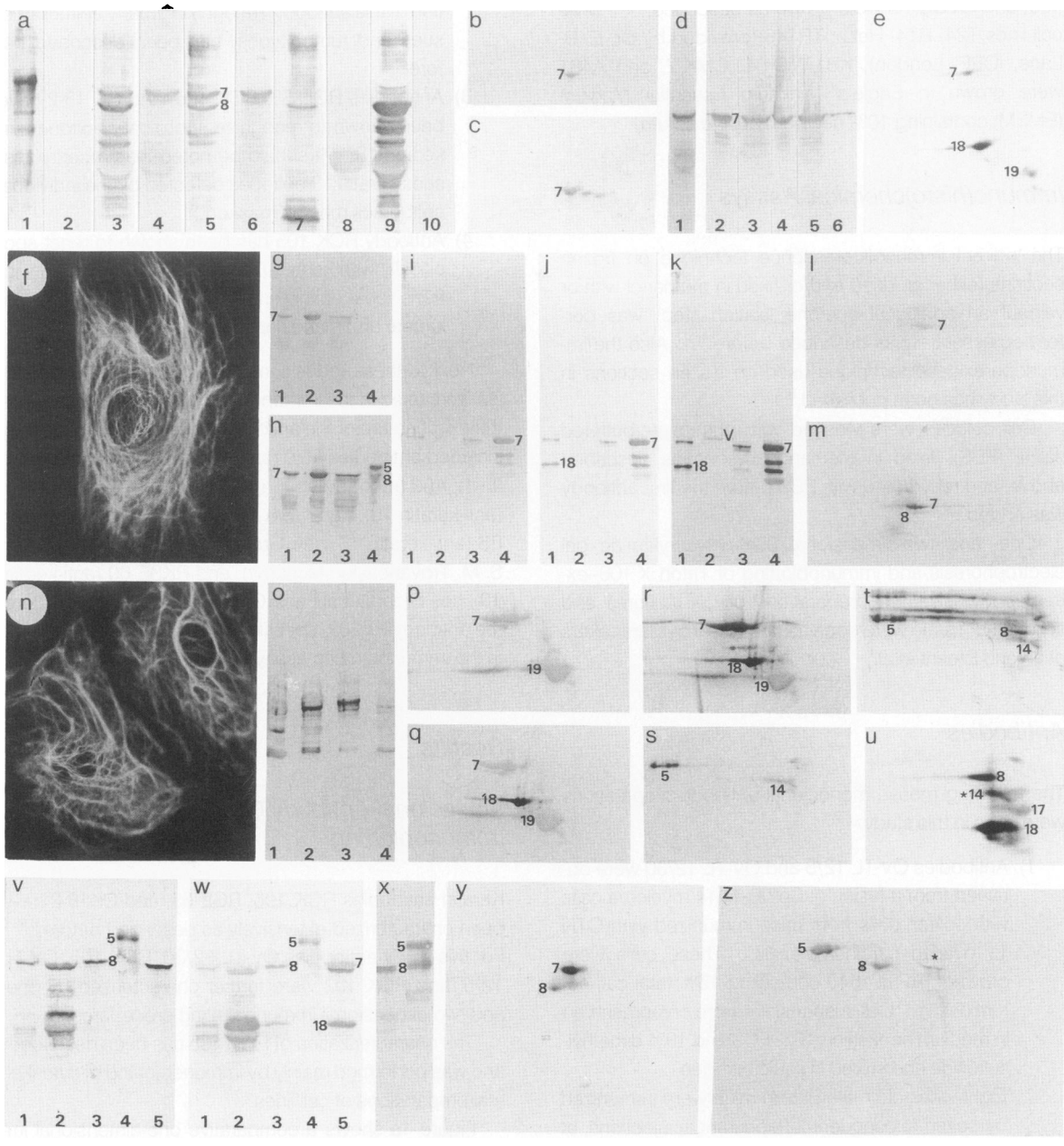

Figure 1. *Immunoblotting assays and indirect immunofluorescence micrographs (f, n) of antibodies RCK 105 (a–e), OV-TL 12/30 (f–m), OV-TL 12/5 (n–u) and RCK 102 (v–z).* **a:** *Cytoskeletal preparations from several tissues and cell lines stained with antibody RCK 105 (lanes 2, 4, 6, 8, 10) or with antibody AE3 (lanes 1, 3, 5, 7, 9). The cytoskeletal extracts were from the following tissues or cell cultures: lanes 1 and 2, human foreskin keratins; lanes 3 and 4, HeLa cells; lanes 5 and 6, KB cells; lanes 7 and 8, WiDr cells; lanes 9 and 10, LLCMK-2 cells. Note that the antibody AE3 recognizes several keratins (amongst which 7 and 8; indicated as such) and their breakdown products. The protein band recognized by RCK 105 comigrates with the keratin 7 band and is not visible in those preparations that have no detectable amounts of keratin 7 in the AE3 incubated blots (lanes 7–10).* **b:** *Two-dimensional immunoblotting of RCK 105 on KB cells.* **c:** *Two-dimensional immunoblotting of RCK 105 on A431 cells.* **d:** *One-dimensional immunoblotting of RCK 105 on cytoskeletal preparations from a lung adenocarcinoma (lane 1) and several transitional cell carcinomas of the bladder (lanes 2–6). Note reactivity with the cytokeratin 7 band and to a much lesser extent with breakdown products.* **e:** *Immunoblotting of RCK 105 on a two-dimensional blot of an adenocarcinoma of the lung revealed only one immunoreactive protein spot migrating in the position of keratin 7. The blot was reincubated with RCK 106 and LP2K, staining keratins 8 and 19, respectively, which were used as markers.* **f:** *Immunofluorescence micrograph of OV-TL 12/30 on a cultured human mesothelial cell.* **g, h:** *Cytoskeletal preparations from HeLa (lane 1), RT4 (lane 2) and A431 (lane 3) cells, as well as a keratin preparation from human foreskin (lane 4), showing that OV-TL 12/30 reacts only with (preparations containing) keratin 7. As a control, antibody RCK 102 was used to reincubate the blot shown in (g) resulting in blot (h), and indicating that the protein band recognized by OV-TL 12/30 is situated between keratins 5 and 8 on these one-dimensional blots.* **i–k:** *Immunoblots containing cytoskeletal preparations from HeLa cells (lane 1), bovine lens (lane 2), A431 cells (lane 3) and RT4 cells (lane 4), initially incubated with OV-TL 12/30 showing only keratin 7 and breakdown products (Figure 1i), and thereafter subsequently reincubated with RCK 106 (Figure 1j) indicating the position of keratin 18, and RV 202 (Figure 1k) to indicate the position of vimentin (v).* **l, m:** *Two-dimensional immunoblots of a cytoskeletal preparation of RT4 cells incubated with OV-TL 12/30 (Figure 1l) and thereafter with RCK 102 (Figure 1m).* **n:** *Immunofluorescence micrograph of OV-TL 12/5 on cultured human mesothelial cells.* **o:** *One-dimensional immunoblotting assay of OV-TL 12/5 on cytoskeletal extracts of RT4 cells (lane 1), a squamous cell carcinoma of the lung (lane 2), esophageal epithelium (lane 3) and a transitional carcinoma of the urinary bladder (lane 4).* **p–r:** *Two-dimensional immunoblotting study on a cytoskeletal preparation*

from RT4 cells. Incubations on the same blot were subsequently done with OV-TL 12/5 (p; reaction with keratins 7 and 19), RCK 106 (q; additional spot for keratin 18 occurs), and RCK 105 (r; the spot for keratin 7 becomes more intensely stained, while also some breakdown products become visible). **s, t:** *Two-dimensional immunoblotting study on a cytoskeletal preparation from TR 146 cells. Incubations on the same blot were subsequently done with OV-TL 12/5 (s; reaction with keratins 5 and 14), and AE3, 6B10 and M20 (t; additional reactivity with keratin 8 and several higher molecular weight basic (skin) keratins. These latter keratins, which are recognized by antibody AE3 are most probably minor contaminants in the TR 146 preparation).* **u:** *Detail of a similar blot as in s and t but reincubated with M20, E3, and RCK 16 recognizing keratins 8, 17 and 18, respectively, to further prove that OV-TL 12/5 does indeed react with keratin 14 (asterisk).* **v, w:** *One-dimensional immunoblotting study with RCK 102 on cytoskeletal preparations of cultured human hepatoma cells (lane 1), HeLa cells (lane 2), A431 cells (lane 3), RT4 cells (lane 5), as well as a human foreskin keratin preparation (lane 4). Note in (v) that RCK 102 reacts only with keratins 8 and 5 (in lane 4) and some keratin 8 breakdown products (lane 2). Reincubation of this blot with RCK 106 and RCK 105 (recognizing keratins 18 and 7, respectively; w) confirms this latter statement.* **x:** *One-dimensional immunoblots of RCK 102 on cytoskeletal extracts of a human mesothelioma cell line (lane 1; only recognizing keratin 8) and a squamous cell carcinoma of the lung (lane 2) in which both keratins 5 and 8 are recognized.* **y:** *Two-dimensional immunoblot of a cytoskeletal preparation of KB-cells incubated with RCK 102 (which only recognized keratin 8) and thereafter with RCK 105, which stained the additional keratin 7 band.* **z:** *Two-dimensional immunoblot of a human foreskin keratin preparation, showing very clearly the reactivity of RCK 102 with both keratins 5 and 8, but no other keratins. The asterisk indicates the position of keratin 10, which was reactive with antibody RKSE 60.*

solid bladder transitional cell carcinomas and lung carcinomas. Figures 1d and e show two examples of one- and two-dimensional immunoblots of these preparations, indicating that also in tumor tissues only keratin 7 is recognized by RCK 105.

Antibody OV-TL 12/30 also was found to specifically react with keratin 7. This antibody has been shown in a previous study[15] to stain intermediate filament structures in cultured ovarian carcinoma cells and mesothelial cells (Figure 1f). Figure 1g shows immunoblotting studies with OV-TL 12/30 on gel electrophoretically separated cytoskeletal preparations of human cell lines, including HeLa, RT4, and A431, as well as on a keratin preparation of human foreskin. Also a vimentin containing bovine lens cytoskeletal preparation was run on a similar gel (Figures 1i to k).

From these experiments it is clear that OV-TL 12/30 reacts with a 54-kd protein band in the cell lines, but not with the foreskin keratins or with vimentin. Reincubation of immunoblots with antibody RCK 102 (specific for keratins 5 and 8; Figure 1h and data presented below) indicated that the keratin polypeptide detected by OV-TL 12/30 has a molecular weight that is slightly higher than keratin 8, but less than that of keratin 5. When comparing Figures 1i to k, it is obvious that the OV-TL 12/30 antigen has a molecular weight slightly less than that of vimentin. Two-dimensional immunoblots further substantiated the keratin 7 character of this protein (Figures 1l and m).

Antibody OV-TL 12/5 also stained a filamentous network in the cultured ovarian carcinoma cells, described in Materials and Methods, and mesothelial cells in the indirect immunofluorescence assay (Figure 1n). In immunoblotting studies this antibody could be shown to react with cytoskeletal proteins of epithelial, but not of nonepithelial cells and tissues, for example tissue of the eye lens. Several protein bands recognized by OV-TL 12/5 migrate in the positions between keratins 5 and 19 in keratin containing preparations from RT4, esophagus, as well as lung and bladder cancer (Figure 1o). Two-dimensional immunoblotting studies on RT4 cytoskeletal preparations showed that OV-TL 12/5 reacts with keratins 7 and 19 (Figures 1p to r). Immunoblotting studies on TR146 cytoskeletal preparations (known to contain keratins 5, 6, 8, 14, 17, and 18; see Quinlan et al[2]) revealed that in these cells OV-TL 12/5 reacted with keratins 5 and 14 (Figures 1s to u). This was confirmed by reincubation of the immunoblots with antibodies specifically recognizing several other basic and acidic keratins (see legends to Figures 1q, r, t, and u). In conclusion, OV-TL 12/5 recognizes epitopes in keratins 5, 7, 14, and 19.

Antibody RCK 102 has been briefly described before.[9,20] In many cultured epithelial cells this latter antibody reacts with keratin filaments. Immunoblotting results on such cell lines show that RCK 102 reacts with keratin 8 (Figure 1v and w). Using skin keratin preparations and cytoskeletal extracts of tumors containing, among others, keratin 5, indicated that RCK 102 also recognizes this latter keratin protein (Figures 1v to x).

No other keratins were recognized by the antibody. Also two-dimensional immunoblotting of cell lines (Figure 1y), normal tissues (for example, human foreskin; Figure 1z), and tumors (bladder transitional cell carcinomas and lung carcinomas; see Broers et al[8]) revealed that RCK 102 recognizes only keratins 5 and 8.

Monoclonal Keratin Antibody Reactivity Patterns in Normal Human Tissues

The results of immunohistochemical staining experiments (using either the indirect immunofluorescence or immunoperoxidase technique) on normal human epithelial tissues using most of the antibodies described above are summarized in Ramaekers et al[9] and in Schaafsma et al.[20] In general, nonepithelial tissues such as connective tissue, blood vessels and blood cells, lymphoid tissue, spleen, nerve tissue, and muscle tissue (both smooth and striated) were negative with the keratin antisera. However, occasionally we have observed keratin staining in pat-

ches of smooth muscle cells, especially in myometrium. Also occasionally stromal cells in the prostate showed keratin reactivity.

Broadly Cross-reacting Antibodies RCK 102 and OV-TL 12/5

Antibody RCK 102 stained virtually all epithelial cell types, with the exception of the suprabasal keratinizing cells in the epidermis and the seminiferous tubules in the testis. This latter cell type has been described not to contain keratins at all.[23] The keratinizing epidermal cells were, however, positively stained with antibody OV-TL 12/5. This monoclonal antibody shows reactivity with most of the epithelial cells tested, although no staining reaction was observed in cardiac glands, acini and islet cells of the pancreas, hepatocytes, or in the proximal tubules of the kidney. These differences in the reaction patterns of RCK 102 and OV-TL 12/5 can be explained by the observation described above that OV-TL 12/5 does not react with keratin 8.

As a result, a combination of both antibodies allows the detection of virtually all keratin-containing epithelia.

Keratin 7 Antibodies RCK 105 and OV-TL 12/30

The antibodies specifically recognizing keratin 7 (RCK 105 and OV-TL 12/30) showed reactivity patterns in the normal tissues, essentially as described before.[8,9,20] In brief, both antibodies reacted in the columnar and glandular epithelium of the lung, cervix, breast, bile ducts, collecting ducts in prostate, mesothelium and surface epithelium of the ovary, the epithelium lining the fallopian tubes, rete epithelium in the testis, and epididymis epithelium. In the kidney, glomeruli and proximal tubules were negative. An increasing number of cells became positive for the keratin 7 antibodies when going from the distal ducts to the collecting tubules. Also transitional epithelium in the bladder was found to be strongly positive, although in a heterogeneous fashion.[20]

In general, no reaction was found with RCK 105 in (keratinizing) squamous epithelia, myoepithelium, hepatocytes, or the epithelia lining the gastrointestinal tract and prostatic acinar epithelium. OV-TL 12/30 reacted in a similar way, with the exception that the surface epithelium of the stomach and duodenum, as well as myoepithelial cells, were positive with this antibody. Because cardiac and gastric glands were not stained by OV-TL 12/30, this antibody may be used to distinguish between surface epithelium, which is positive, and glands in the stomach and duodenum, which are negative. On the other hand, this antibody discriminates between surface epithelium of the colorectal part and the small intestine, which is negative, and stomach epithelium, which is positive. With respect to the myoepithelial cells it should, however, be stated that positivity or negativity in these flattened cells is often difficult to interpret.

Another striking difference in the staining patterns of RCK 105 and OV-TL 12/30 was observed in the prostate, in which OV-TL 12/30 stained part of the luminal and basal cells of the acini, while RCK 105 reacted occasionally with some luminal cells. With respect to the nonreactivity of the keratin 7 antibodies in hepatocytes of normal liver, it should be stated that in patients with acute and chronic cholestasis we have observed keratin 7 expression in hepatocytes.[24]

Keratin 18 Antibodies RGE 53, RCK 106, and CK 18-2

All three keratin 18 antibodies reacted with glandular and ductal epithelia, including those of the gastrointestinal tract, prostate, and kidney and with hepatocytes and mesothelium as described before.[6,9] No reaction was seen in squamous epithelia, most myoepithelial cells, and most basal cells in the cervix, prostate, and lung.

In the transitional epithelium of the bladder only the superficial umbrella cells reacted positively with RGE 53, while antibodies RCK 106 and CK 18-2, in addition, also stained the basal and intermediate cell layer of the bladder epithelium.[20]

Monoclonal Keratin Antibody Reactivity Patterns in Malignant Human Tissues

The results of the immunohistochemical studies on human malignant epithelial tissues are summarized in Table 1 and illustrated in Figures 2 to 4. In general, malignancies of nonepithelial origin, such as (non-Hodgkin) lymphomas, fibrous histiocytomas, leiomyosarcomas, rhabdomyosarcomas and other soft tissue tumors, including Ewings' sarcoma and epithelioid cell sarcoma, as well as melanoma were negative with all keratin antibodies applied. Occasionally, keratin-positive cells were observed in such nonepithelial malignancies (see, for example, Guelstein et al[22]), and in the tumor stroma (Figure 2l).

Broadly Cross-reacting Keratin Antibodies (left panels in Figures 2 to 4)

Antibody RCK 102 gave a positive reaction in virtually all the 365 primary and metastatic epithelial neoplasms tested, including squamous cell carcinomas of different degrees of differentiation, transitional cell carcinomas, adenocarcinomas, hepatocellular carcinoma, (small cell) anaplastic carcinomas, mesotheliomas, and carcinoids. Antibody OV-TL 12/5, tested in a smaller series of carcino-

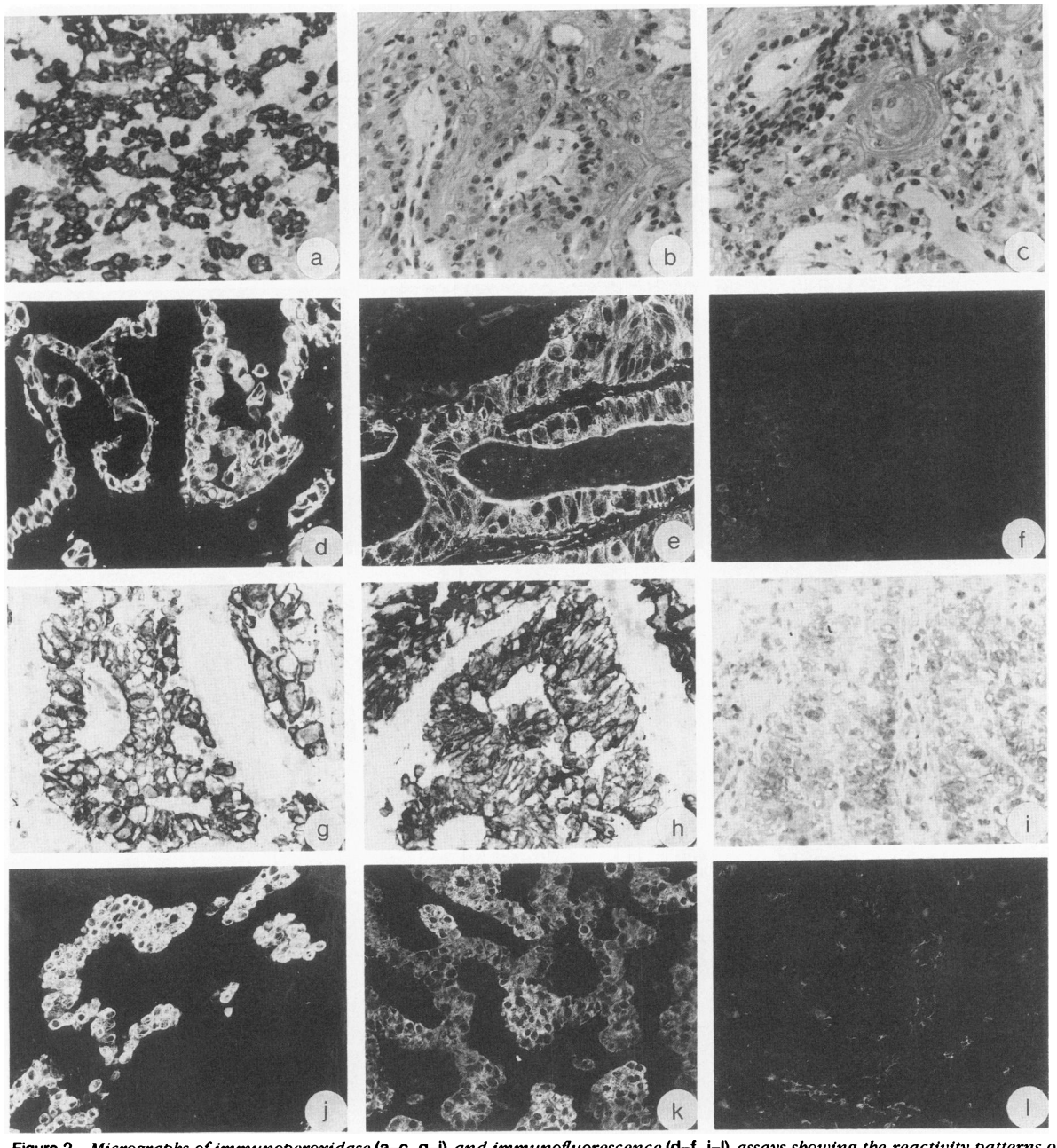

Figure 2. *Micrographs of immunoperoxidase* **(a–c, g–i)** *and immunofluorescence* **(d–f, j–l)** *assays showing the reactivity patterns of RCK 102* **(a** *and* **g)**, *OV-TL 12/5* **(d,** *and* **j)**, *RCK 106* **(b, e, h, k)**, *RCK 105* **(c, i, l)**, *and OV-TL 12/30* **(f)** *in a squamous cell carcinoma of the vulva* **(a–c)**, *an adenocarcinoma of the stomach* **(d–f)**, *an adenocarcinoma of the colon* **(g–i)**, *and an intestinal carcinoid* **(j–l)** *(magnifications × 150–300).*

mas, showed a similar staining pattern. However, in some tumors this antibody was only partly positive, or stained less cells than RCK 102, probably due to the fact that it does not recognize keratin 8. Negative reactions with both reagents were obtained in anaplastic seminoma, known not to contain keratins, and in two cases of adrenal pheochromocytoma. Antibody OV-TL 12/5 was, furthermore, found to be negative in two cases of renal cell carcinoma, in two cases of breast carcinoma, and in one case of a small-cell anaplastic carcinoma of the lung in which RCK 102 was positive.

Keratin 7 Antibodies (right panels in Figures 2 to 4)

In general, antibody RCK 105 was positive in carcinomas that are derived from the normal epithelial tissues also positive for this antibody. Similarly, carcinomas derived from epithelia that were negative for RCK 105 did

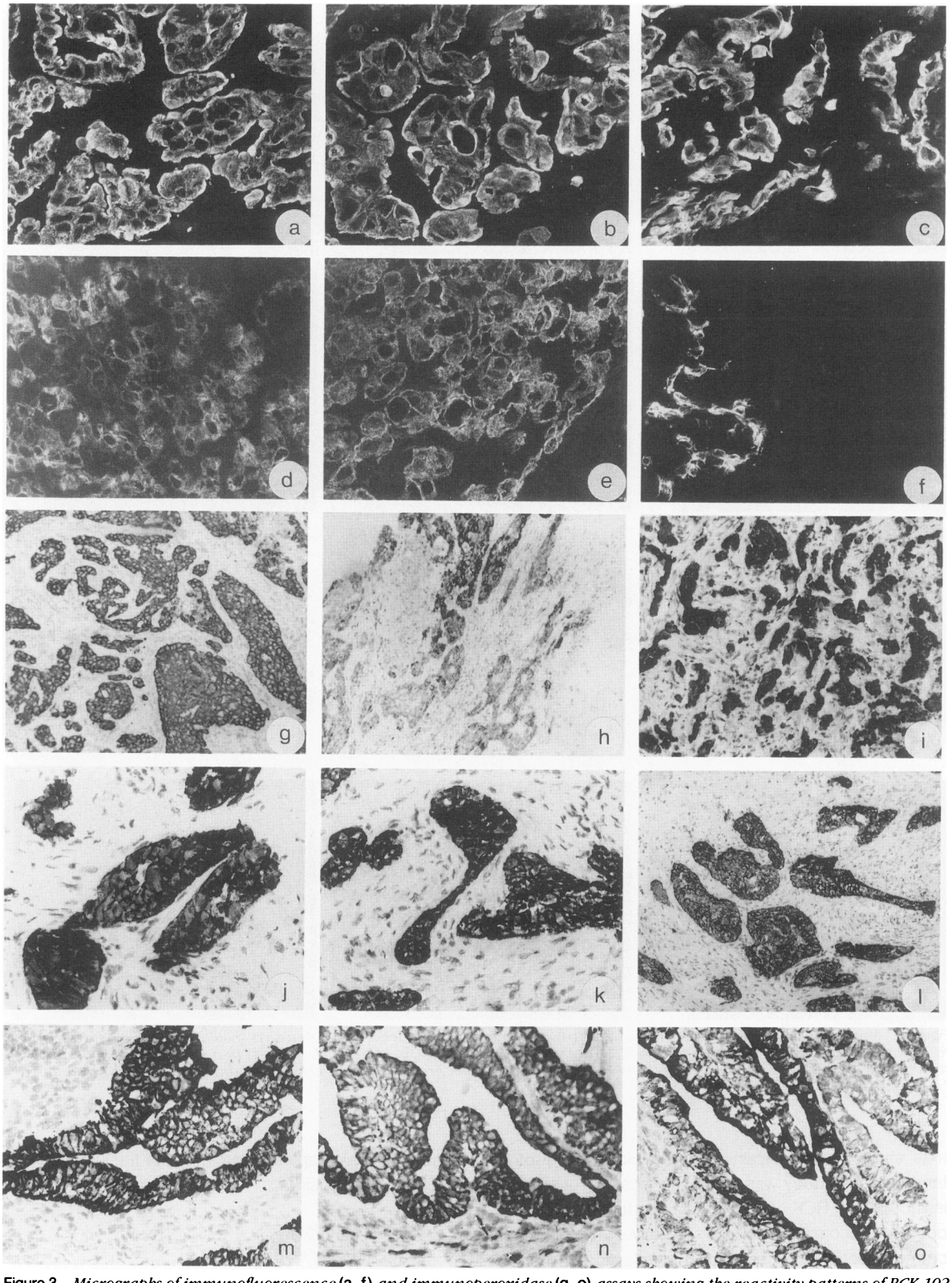

Figure 3. *Micrographs of immunofluorescence* **(a–f)** *and immunoperoxidase* **(g–o)** *assays showing the reactivity patterns of RCK 102* **(a, g, j, m)**, *OV-TL 12/5* **(d)**, *RCK 106* **(b, e, k, n)**, *RGE 53* **(h)**, *OV-TL 12/30* **(c, f)**, *and RCK 105* **(i, l, o)** *in a pancreas carcinoma* **(a–c)**, *a hepatoblastoma* **(d–f)**, *an invasive breast carcinoma* **(g–i)**, *an ovarian carcinoma* **(j–l)**, *and an endometrioid carcinoma* **(m–o)** *(magnifications × 150–300).*

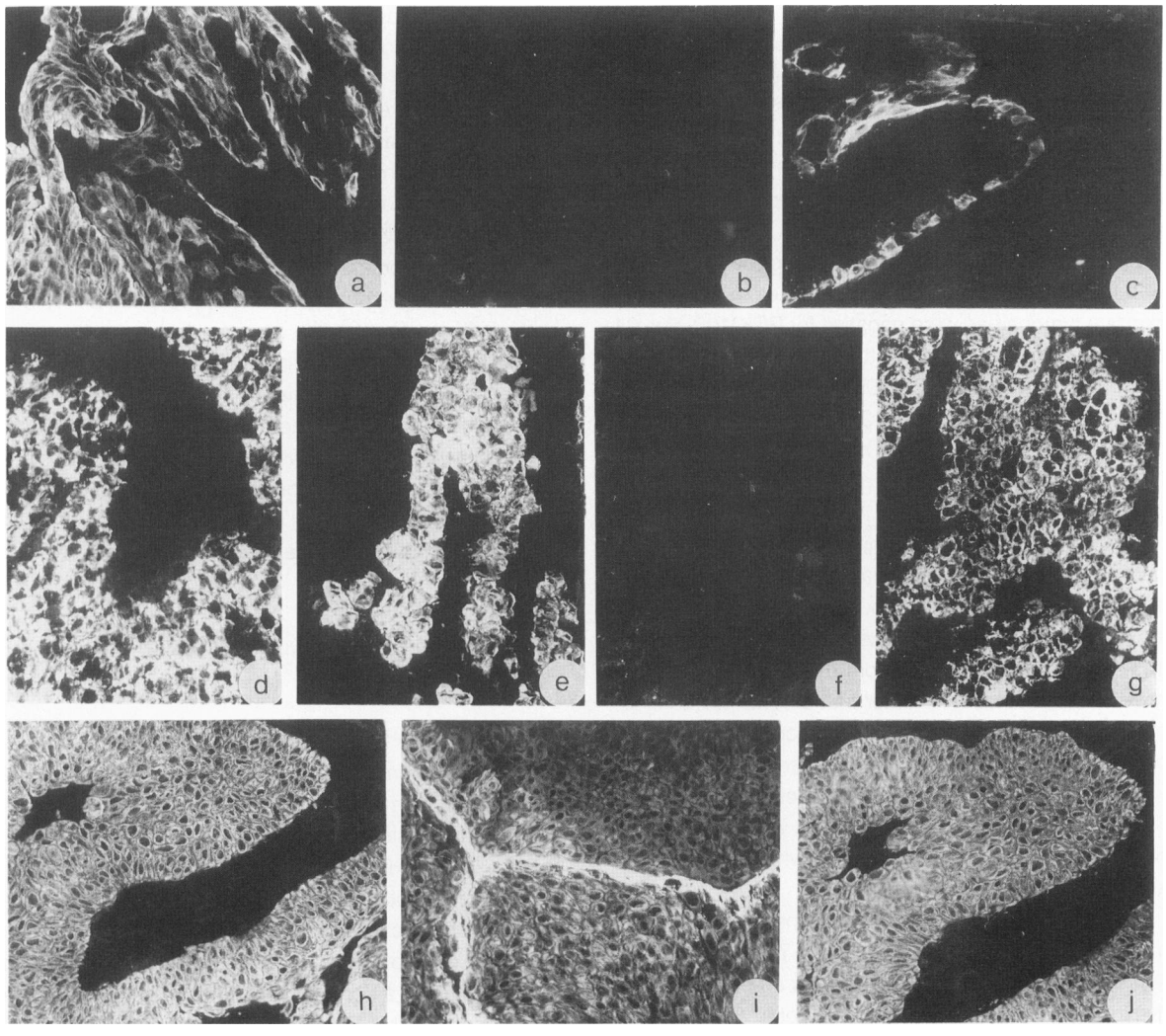

Figure 4. *Micrographs of immunofluorescence assays showing the reactivity patterns of OV-TL 12/5* **(a)**, *RCK 102* **(d, h)**, *RGE 53* **(e, i)**, *RCK 105* **(b, f, g, j)**, *and OV-TL 12/30* **(c)** *in a prostate adenocarcinoma* **(a–c)**, *a renal cell carcinoma (Grawitz tumor;* **d–g** *), and a transitional cell carcinoma of the urinary bladder* **(h–j)** *(magnifications × 150–300).*

not, in general, react with this reagent. For example, primary and metastatic squamous cell carcinomas in diverse tissues were normally negative for RCK 105. However, exceptions to this rule were encountered in squamous cell carcinomas of the lung in which 2 of 10 cases were completely reactive, and 3 of 10 cases showed focal areas of RCK 105 positivity. The other keratin 7 antibody OV-TL 12/30 showed a positive reaction in even more of the pulmonary squamous cell carcinomas, with 3 of 10 cases completely positive and focal reactions in 4 other cases. This unexpected staining reaction is probably a result of the well-known heterogeneous composition of these tumors, as extensively described before.[8] Strikingly, also in a lymph node metastasis of a squamous cell carcinoma, antibodies RCK 105 and OV-TL 12/30 showed different staining patterns in that OV-TL 12/30 gave a positive reaction in some of the tumor cells.

Adenocarcinomas of the lung were completely positive for the keratin 7 antibodies, with some small areas negative for RCK 105. The adenocarcinomas of the gastrointestinal tract were, in general, negative for RCK 105 with the exception of some tumors in which sporadic positive cells were found.

Again, similar to what has been observed in the squamous cell carcinomas, OV-TL 12/30 stained more of these gastrointestinal adenocarcinomas than RCK 105. For example, three stomach carcinomas were partly or completely positive for OV-TL 12/30. Furthermore, two carcinoids of the small intestine showed a positive reaction in some of the tumor cells with OV-TL 12/30.

Carcinomas of the pancreas were positive for both RCK 105 and OV-TL 12/30, with one case partly positive for RCK 105.

In the malignant tumors of the liver three different staining patterns were observed with the keratin 7 antibodies.

Five of eight hepatocellular carcinomas were negative with RCK 105 and showed some sporadic positive cells with OV-TL 12/30. Two out of these eight cases were positive with both antibodies, and in one case only OV-TL 12/30 was positive, while RCK 105 showed staining in some cells. In a case of hepatoblastoma RCK 105 was again negative, while OV-TL 12/30 was partly positive (see also van Eyken et al[25]).

Breast carcinomas of all types were generally positive with the keratin 7 MAbs. In 9 of 80 cases, however, the invasive tumor areas were negative for RCK 105. The 24 cases in which the reaction patterns of OV-TL 12/30 were compared to those of RCK 105 showed one extra case that was negative for OV-TL 12/30, while this tumor was partly positive for RCK 105. The epithelial tumors of the female genital tract also were positive for both MAbs, albeit only partly in some cases.

All tumors of the male genital tract examined by us, particularly prostate carcinoma, were negative for RCK 105 or showed only sporadic positive cells. However, OV-TL 12/30 again showed reactivity in more cells than RCK 105. With respect to the tumors of the urinary tract we have observed that the greater part of renal cell carcinomas is negative for the keratin 7 antibodies, with 2 of 27 cases positive and a sporadic positive reaction in 6 cases with RCK 105 and in 6 additional cases with OV-TL 12/30. All four cases of Wilm's tumors, as well as two cases of adrenal carcinoma were negative when examined for keratin 7 expression.

However, transitional cell carcinomas of the urinary tract were generally positive for keratin 7, with the exception of 6 of 59 cases that were partly or completely negative for RCK 105 (see also Schaafsma et al[26]).

Of the neuroendocrine tumors examined, two carcinoids of unknown origins were found to be negative, while also lung carcinoids were mainly unreactive for RCK 105. However, 3 of 10 of these latter tumors showed a focal or complete positivity with this MAb (see also Broers et al[8]). Part of the small-cell anaplastic carcinomas of the lung examined in this study were, to a relatively large extent, positive for RCK 105 (2 of 6 cases) and OV-TL 12/30 (3 of 6 cases).

Finally, of the nine mesotheliomas examined in frozen sections with RCK 105, seven cases were completely or partly positive, one case was negative, and one case showed a few scattered positive cells. When tested in smears of fine needle aspirates obtained from patients with pleural fluid containing mesothelioma, 9 of 12 cases were positive and 2 cases were negative for RCK 102, while in one case the tumor cells were partly positive.

Keratin 18 Antibodies (middle panels in Figures 2 to 4)

As reported before,[6] the antibodies to cytokeratin 18 (RGE 53 and RCK 106 were tested in most cases, while CK 18-2 was applied only occasionally) generally do not stain squamous cell carcinomas. An exception to this rule, however, is found in cases of pulmonary squamous cell carcinoma, especially in which the moderately and poorly differentiated tumors become partly positive for RGE 53 and even more so for RCK 106 (see also Broers[8]).

Adenocarcinomas of all tissues and their metastases, as well as hepatocellular carcinomas and hepatoblastomas, mesotheliomas, carcinoids, and small-cell anaplastic carcinomas are positive for the CK 18 antibodies, albeit only partly in a few cases.

A significantly different staining pattern of the three CK 18 antibodies was observed in transitional cell carcinomas of the urinary tract. There RGE 53 showed an extensive staining pattern, however, with varying intensities and preference for the superficial cell layers in grades 1 and 2 tumors, as reported earlier.[26,27] RCK 106 and CK 18-2 normally stained all the tumor cells in these cases.[26]

Anti-keratin 7 as an Aid in Routine Histopathology

To demonstrate the usefulness of cytokeratin 7 antibodies in the differential diagnosis of tumor cases in which it was difficult or impossible to reach a satisfactory conclusion on the basis of routine histology on the one hand, and to show the limitation of these reagents in routine pathology on the other hand, we illustrate 14 separate diagnostic problem cases (Table 2), which are not included in Table 1. In 12 of these cases the keratin 7 expression pattern allowed a conclusive diagnosis, or restricted the number of possible diagnoses. In several instances these immunohistochemical results were supported by clinical data. However, in two cases (Table 2, cases 13 and 14), one a poorly differentiated carcinoma and one an undifferentiated carcinoma, the cytokeratin 7 staining pattern did not correlate with the final diagnosis. In these cases the combined histopathologic diagnoses of several experienced pathologists, as well as immunocytochemical results with specific tumor markers supported the diagnosis of endometrial carcinoma and mixed Müllerian tumor, respectively.

Discussion

Keratin immunocytochemistry has proved to be a valuable additional technique in the routine diagnosis of cancers that pose problems on morphologic examination. Broad-spectrum (polyclonal rabbit or mouse monoclonal) keratin antisera can be used to separate epithelial from nonepithelial malignancies.[28] Recently the development of chain-specific keratin MAbs has allowed the immuno-

cytochemical distinction between different types of normal epithelia and as a result also between different types of epithelial malignancies. For example, MAbs to keratin 18 can distinguish between adenocarcinomas (which are generally keratin 18 positive) and most squamous cell carcinomas (which are generally keratin 18 negative; for exceptions see Broers et al[8]).

Recently the use of different keratin 7 MAbs by several authors [8, 10–12, 20, 24–26, 29–31] has revealed that the expression of this keratin type is restricted to a subgroup within the glandular types of normal human epithelia. As a consequence MAbs to keratin 7 can apparently distinguish between different types of adenocarcinomas. For example, colorectal carcinomas and hepatocellular carcinomas were found to be negative for keratin 7, while pancreatic carcinomas and bile duct carcinomas were positive.[10,11] However, van Eyken et al,[30] after studying an extended series of hepatocellular carcinomas, found that these tumors can express keratin 7, albeit in a variable number of cells. On the other hand, some carcinomas of the ovary, as well as some ductal breast carcinomas, which were expected to be positive, showed no or only heterogeneous keratin 7 expression[10, 32]; see also van Niekerk et al[14] for a heterogeneous reaction pattern in the ovarian carcinoma cell line OTN 14.

These inconsistencies have urged us to meticulously test a comprehensive series of epithelial malignancies for the presence of keratin 7. To avoid as much as possible false-negative outcomes as a result of epitope masking we have used two MAbs to keratin 7, which were obtained from different fusions and most probably recognize different epitopes. Next to these keratin 7 MAbs we have used two other groups of keratin MAbs, ie, two broad-spectrum MAbs and three keratin 18 MAbs. Generally, the two broadly cross-reacting antibodies OV-TL 12/5 and RCK 102, can, when combined, separate most carcinomas, which are positive, from nonepithelial malignancies, which are generally negative. Some exceptions of keratin-positive nonepithelial tumors include, for example, leiomyomas and leiomyosarcomas,[33] chondrosarcomas and chondroblastomas.[34] Also malignant nonepithelial components in teratocarcinomas were found to be positive for RCK 102, as well as for RGE 53 (Oosterhuis et al, to be published). To distinguish between different subgroups of carcinomas in problem cases in which routine histologic techniques do not allow a precise diagnosis, antibodies to keratins 18 and 7 have proved to be very useful. The keratin 18 antibodies can, in general, recognize adenocarcinomas, hepatocellular carcinomas and hepatoblastoma, mesotheliomas, transitional cell carcinomas, and neuroendocrine tumors, but normally do not react with squamous cell carcinomas. Squamous cell carcinomas of the lung may be an exception to this rule because an increasing number of keratin 18-positive cells are found in these malignancies with a decreasing degree of differentiation.[8] Similarly, squamous cell carcinomas of the oral cavity and laryngeal area may show keratin 18 positivity, in particular at their front of invasion into surrounding stromal tissue (Schaafsma et al, to be published). Similarly, keratin 7 has been found in poorly differentiated squamous cell carcinomas of the lung.[8]

Both keratin 7 MAbs were tested for their reactivity on frozen sections of normal human tissues and showed some discrepancies in their staining patterns. Antibody OV-TL 12/30 showed a reaction in part of the tongue squamous epithelium, some myoepithelial cells, prostate acini and surface epithelium of stomach and duodenum tissues, which were negative for RCK 105. Similarly, discrepancies were found in the reactivity patterns of both MAbs in epithelial malignancies. Normally, relatively more cases were (partly) positive for OV-TL 12/30 than for RCK 105. This is particularly striking in some cases of squamous cell carcinomas and small cell carcinomas of the lung, GI-tract adenocarcinomas, and hepatocellular carcinomas. These differences in the reactivity patterns of the individual keratin 7 antibodies likely can be explained by epitope masking. Thus although OV-TL 12/30 detects keratin 7 expression more consistently than RCK 105, the latter antibody allows a better immunohistochemical distinction between adenocarcinomas and squamous cell carcinomas, on the one hand, and between different groups of adenocarcinomas on the other hand. However, within the group of RCK 105-negative adenocarcinomas (for example those derived from the gastrointestinal tract or prostate), a positive reaction for OV-TL 12/30 may provide an indication of the origin of such neoplasms in cases of metastases or anaplastic growth. Because OV-TL 12/30 distinguishes between surface epithelium and glandular structures in stomach and duodenum, in contrast to RCK 105, the OV-TL 12/30 antibody might point to whether a stomach cancer is derived from surface epithelium or from glandular cells. In addition, OV-TL 12/30 (in combination with RCK 105) refines the diagnosis of gastrointestinal tract cancer in showing their derivation from colon, small intestine, or stomach duodenum. This phenomenon of epitope masking can also explain the slightly different reaction patterns of the individual keratin 18 antibodies (see also Franke et al[23]).

In general, adenocarcinomas arising from the gastrointestinal tract are negative for the keratin 7 antibodies. Also metastases of such malignancies remain unstained, which is nicely illustrated by stomach and colon adenocarcinomas metastatic to the ovaries.

Similarly, prostate carcinomas, renal cell carcinomas, epithelial liver tumors, and carcinoids of several organs are normally negative for keratin 7. However, some tumors within this group of normally keratin 7-negative neoplastic tissues may show keratin 7 reactivity (also with

RCK 105), ranging from a few scattered positive cells *via* positive tumor areas to homogeneously positive tumors.

In contrast, adenocarcinomas of the lung, pancreas, breast, and female genital tract, as well as transitional cell carcinomas and mesotheliomas are normally positive for keratin 7. Again, exceptions to this rule are found that showed positivity in only part of the tumor cells or which were even completely negative. This became especially apparent in two cases of gynecologic malignancies, illustrating that these latter tumor types need further examination.

Although now we routinely use the keratin antibodies described above to help in the differential diagnosis of (adeno)carcinoma of unknown origin, it is obvious that results obtained with these monoclonal antibodies should be interpreted with care and that exceptions to a general reactivity pattern should be kept in mind when applying these reagents in diagnostic histo- and cytopathology.

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