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Histological Evaluation of Three New Monoclonal Anti-Cytokeratin Antibodies. 1. Normal Tissues.

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Abstract—*Three new monoclonal anti-cytokeratin antibodies (mabs) potentially useful in cancer research and clinical diagnosis have been evaluated in immuno-histochemistry on cryostat sections of a broad variety of normal human tissues. A45-B/B3 reacts with all cells containing cytokeratins (epithelia and mesothelia). This mab positively identifies epithelial cells of any kind, and it may serve in differentiating carcinomas from tumours of mesenchymal origin. A53-B/A2 recognizes an individual cytokeratin, No.* 19, *and stains preferably mesothelia, urothelium, and bile duct epithelium. This antibody is suited to discriminate between different epithelial lineages. A51-B/H4 reacts with a subgroup of cytokeratins (probably including Nos.* 14, 8 *and/or* 18). *It is positive with most epithelia but negative with keratanized stratified epithelium. This antibody shows an interesting, but up to now unexplained, cross-reactivity with nuclei of certain non-epithelial cells. All three mabs also react with epithelial cells from at least three animal species.*

INTRODUCTION

IN RECENT years, the intermediate filament system of vertebrate cells has increasingly attracted the interest of cell biologists and pathologists. Although their function(s) is still largely unknown, intermediate filaments provide tissue and differentiation specific markers which are remarkably stable even under conditions where other markers are lost, e.g. during malignant transformation or cultivation *in vitro* [1–4]. Desmin filaments, for instance, can be found only in muscle cells, and cytokeratin filaments only in cells of epithelial or mesothelial origin. Conventional (polyclonal) antisera against intermediate filaments have been successfully used in histochemical studies, but monoclonal antibodies (mabs) are for obvious reasons the reagents of choice. This applies especially to the analysis of epithelial cells. Different types of epithelia are characterized by typical sets of individual members of the cytokeratin family of proteins [5, 6]. By use of mabs which recognize epitopes specific for individual cytokeratins one should be able to distinguish and analyse the different epithelial lineages by simple immunohistochemical techniques. The obvious precondition is the availability of mabs with the desired specificity. Up to now many anti-cytokeratin mabs have been described with more-or-less overlapping specificities covering subsets of these proteins [5, 7, 8 and others], but only a few have been found to be restricted to a single cytokeratin [9, 10, 21]. In this paper we present histological data on a mab recognizing an individual cytokeratin (no.19) and compare it with a broad spectrum anti-cytokeratin mab reacting with all epithelia. A third anti-cytokeratin mab whose precise specificity has still to be established is also included in this study. We first examine the reactivity of these mabs with normal tissues in order to confirm, extend or revise conclusions drawn from immunofluorescence tests performed with cell lines and to define the range of possible applications of these mabs in immnohistochemistry. In a forthcoming paper we will investigate their application to cancer research and tumour diagnosis.

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MATERIALS AND METHODS

Monoclonal antibodies

The antibodies were raised in BALB/C mice by immunization with live MC-7 mammary carcinoma cells as described [11]. Table 1 lists basic properties of the hybridoma clones used in this study. For antibody production, clones were cultured in RPMI 1640 medium containing 10 or 15% horse serum, and the cell-free supernatants (after addition of 0.1% NAN_3) were used for immunohistochemistry.

Table 1. Properties of monoclonal antibodies used in this study

Denomination of the hybridoma clone	Fusogen	Ig Class (mouse)	Specificity	Ref
A45-B/B3	PEG	IgG1	Cytokeratins (broad spectrum)	11
A53-B/A2	Electric field	IgG2a	Cytokeratin No. 19 (40 kD)	14
A51-B/H4	PEG	IgG2a	Cytokeratin	—

The immunogen used was MCF-7 (human mammary carcinoma cell line) containing cytokeratins 8, 18 and 19 [1].

Tissue sections

Tissue specimens obtained from surgery or autopsy were mounted in OCT medium (Miles Labs., Elkhart, U.S.A.) and stored in liquid nitrogen. Special attention was paid to quick sampling. Cryostat sections (4 μm) were cut by means of a Reichert Frigocut microtome (Vienna, Austria) and air-dried.

Immunohistology

Unfixed sections were used throughout. In immunofluorescence, the sections were rinsed with phosphate buffered saline, pH 7.2, (PBS), and incubated with supernatant containing anti-cytokeratin mab or (as a negative control) an irrelevant mab for 30 min at room temperature. After two wash steps with PBS, they were incubated with FITC labeled sheep anti-mouse globulin diluted 1 : 20 (Staatliches Institut für Immunpräparate und Nährmedien, Belin). The sections were washed again and mounted in PBS/glycerol containing *p*-phenylenediamine [12]. Alternatively, a modified PAP technique was performed as described in [13]. In brief, the following sequence of incubations was employed: normal goat serum (1 : 100); monoclonal anti-cytokeratin antibody; goat anti-mouse globulin (1 : 20); monoclonal mouse anti-peroxidase antibody (BL-POD); peroxidase; 3,3′-diaminobenzidine. The sections were counterstained with Mayer's hematoxylin and mounted in Canada balsam.

RESULTS

Reactivity with cell lines

The cytokeratin specificity of the three mabs was concluded from immunofluorescence studies performed on cell lines. They stained a typical intracellular filament structure which was restricted to cell lines derived from epithelia or carcinomas. Up to now no proteins other than cytokeratins are known which fit into this pattern. Since all three mabs were raised against MCF-7 cells which contain cytokeratins Nos. 8, 18 and 19 [1], the epitopes recognized could be expected to be specific for these or common to all members or a subgroup of the cytokeratin family. Whereas mab A45-B/B3 reacted with all epithelial cell lines, A53-B/A2 did not [14]; it was negative with the epidermoid carcinoma cell lines HeLa and A-431 which lack cytokeratin 19 [1]. The antigen distribution of the third mab, A51-B/H4, was similar to that of A45-B/B3 among cell lines (unpublished results) except for a weak nuclear staining in certain mesenchymal cell lines. Intermediate filament or nuclear staining seemed to exclude each other. Lymphoid cells were completely unreactive.

Reactivity with normal human tissues

The results of extensive immunohistological studies with all three anti-cytokeratin mabs on cryostat sections of normal human tissues are summarized in Table 2. Therefore only general aspects of some illustrative examples will be commented on here.

The skin is an organ with a rather complicated cytokeratin pattern changing during cellular differentiation; this results in characteristic sets of cytokeratins in the different layers of the epidermis, reflected by the staining pattern seen with our mabs. A45-B/B3 stains all epidermal cells. Intercellular bridges as visible in Fig. 1(a) (compare Fig. 2a) indicate cytokeratin filament stretching to desmosomes. A51-B/H4 reacts predominantly with basal cells but not, or only very weakly, with upper layers of the epidermis (Fig. 1c), whereas A53-B/A2 does not bind at all to adult skin (Fig. 1b). However, this mab is positive with fetal skin and with basal cells of squamous epithelia other than epidermis (e.g. uterine cervix, Fig. 2b). This is consistent with the fact that the expression of this 40 kD cytokeratin in squamous epithelia is variable, depending on localization and developmental stage [15, 16].

In the liver the situation is different. This organ contains two unrelated epithelial cell types, hepa-

Table 2. Reactivity of three monoclonal anti-cytokeratin antibodies with normal human tissues

Tissue	A45-B/B3	A53-B/A2	A51-B/H4
Epidermis*			
Basal layer	+	−	++
Spinous layer	+++	−	(+)
Granular layer	+++	−	−
Cornified layer	++	−	−
Hair follicles	+	+	+
Sebaceous gland	+	−	+
Sweat gland (duct cells and secretory cells)	+++	+	+
Anal canal epithelium	+	−	+
Tongue epithelium			
Basal layer	+++	++	+++
Other layers	+++	(+)	−
Salivary glands*			
Serous acinar cells	++	−	−
Mucinous cells	++	−	−
Ducts	++	++	−
Esophagus, stratified squamous epithelium			
Basal cells	++	(+)	++
Lumenal strata	++	(+)	−
Stomach			
Foveolar epithelium	++	(+)	−
Fundus glands	+	−	−
Corpus glands	+	−	−
Duodenum, mucosa	+++	(+)	++
Brunner's glands†	+	−	(+)
Small intestine, mucosa*	++	(+)	(+)
Colon, mucosa†	+	+	(+)
Gall bladder			
Mucosal epithelium	++	+	+
Liver			
Hepatocytes	+++	−	+
Bile ducts	+++	++	−
Pancreas*			
Duct epithelium	++	++	++
Acinar epithelial cells	++	(+)§	++
Islet cells	+	−	−
Lung			
Bronchial musosa	++	+	++
Bronchial glands	++	−/+	−/+
Pleura mesothelium	+++	++	+
Tracheal epithelium	++	+	−
Kidney			
Glomerulus	−	−	−
Bowman's capsule epithelium	(+)	(+)	−
Proximal convoluted tubules‡	+	−	−
Distal convoluted tubules	++	+++	−
Collecting ducts	++	+	−
Urothelium	+++	+++	(+)
Prostate	++	+	+++

Table 2 cont'd overleaf

Table 2 cont'd

Tissue	A45-B/B3	A53-B/A2	A51-B/H4
Uterus*			
Myometrium	–	–	–
Corpus endometrium			
Basal cells	++	++	–
Other cells	+	–	–
Cervix endometrium glandular cells	++	(+)	–
Portio uteri			
Basal cells	(+)	++	(+)
Lumenal strata	+++	–	–
Mammary gland			
Epithelium of ducts	++	+/–	(+)
Myoepithelial cells	+	–	–
Placenta			
Amnion epithelium	++	+++	–
Trophoblast epithelium*	++	++	++
Mesothelium	++	+++	(+)
Thyroid gland			
Epithelium	++	–	–
Colloid	(+)	–	–
Adrenal gland			
Cortex and medulla	+	–	–
Brain			
Nervous and glial tissue	–	–	–
Ependymal cells	–	–	–
Meninges	–	–	–
Choroid plexus cells*	+++	–	+
Corneal epithelium	++	+	+
Spleen	–	–	–
Thymus			
Epithelial reticular cells	++	++	(+)
Hassall's bodies	++	++	++
Lymphocytes	–	–	–
Macrophages	–	–	–
Lymph nodes	–	–	–
Muscle tissues	–	–	–
Connective tissue	–	–	–
Synovial tissue	–	–	–

*PAP technique.
†Apical parts.
‡Distal parts.
§Focal.
Evaluation of intensity: – = no reaction; (+) = weak or uncertain; + = clearly positive; ++ = moderate reaction; +++ = strong reaction.

tocytes and bile duct epithelia, which can be distinguished by their reactivity towards A53-B/A2. Since hepatocytes are known to lack cytokeratin 19 [1], their non-reactivity with A53-B/A2 was to be expected. A45-B/B3 (Fig. 4) stains bile duct epithelium as well as hepatocytes, and A51-B/H4 reacts weakly with hepatocytes but not with bile ducts.

Other organs where A53-B/A2 and A51-B/H4 are suited to characterize and distinguish different epithelial populations are kidney (Fig.5), digestive tract and mammary gland.

A53-B/A2 is especially brilliant in simple epithelia and mesothelia. A51-B/H4, besides undoubtedly defining epithelial cytoskeletal structures, also stains nuclei of certain mesenchymal cells, probably those with proliferating capacity, but not lymphoid cells. In a few cases it appeared that epithelial cell nuclei were also stained with A51-B/H4 (Fig. 2c). Again, intermediate filament

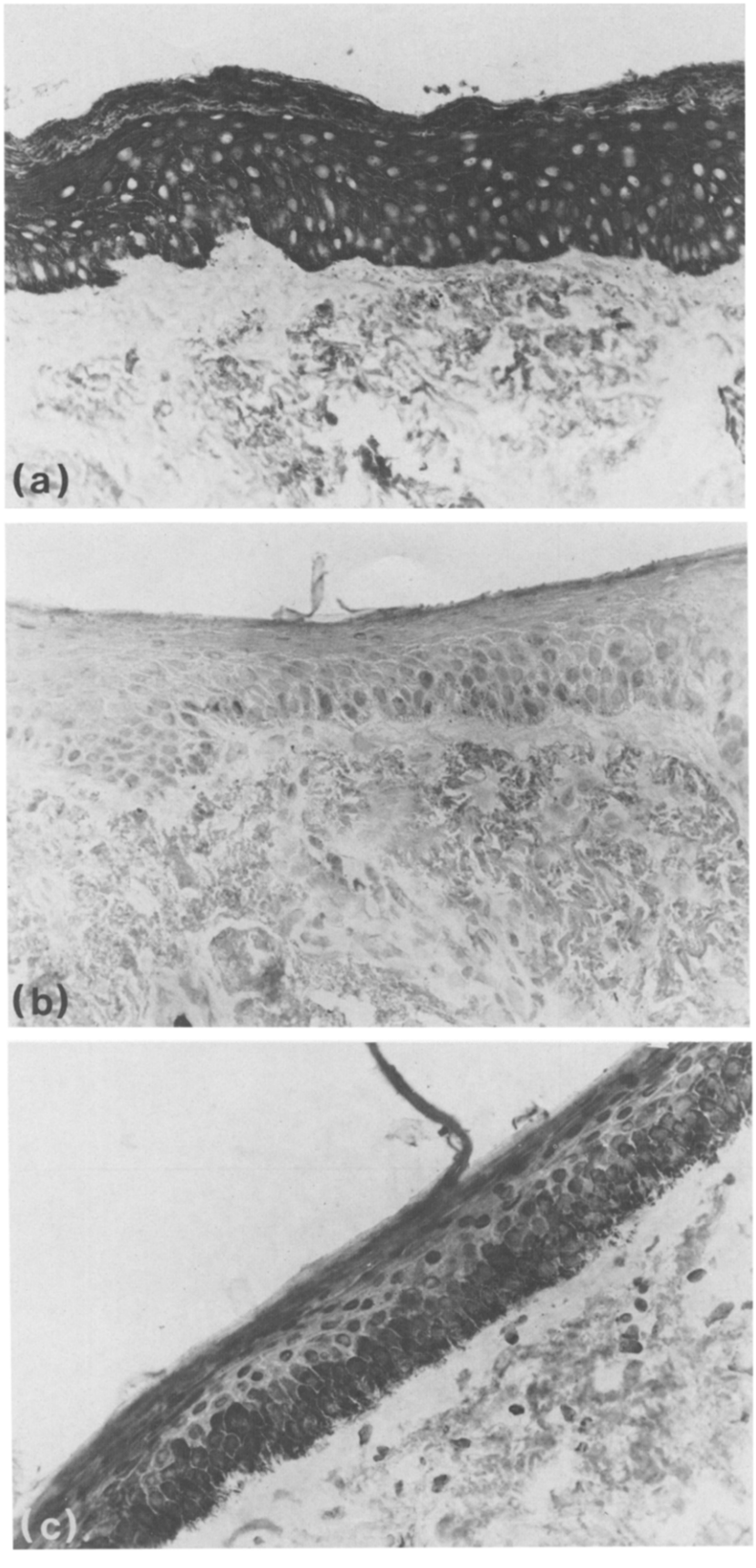

Fig. 1. *Cryostat sections of normal adult human skin reacted with monoclonal anti-cytokeratin antibodies. PAP technique*, 280 ×. (*a*) *A*45-*B*/*B*3 (*broad spectrum*) *stains all epidermal cell layers.* (*b*) *A*53-*B*/*A*2 (*cytokeratin* 19) *shows no staining with adult epidermis.* (*c*) *A*51-*B*/*H*4 *reacts mainly with basal cells.*

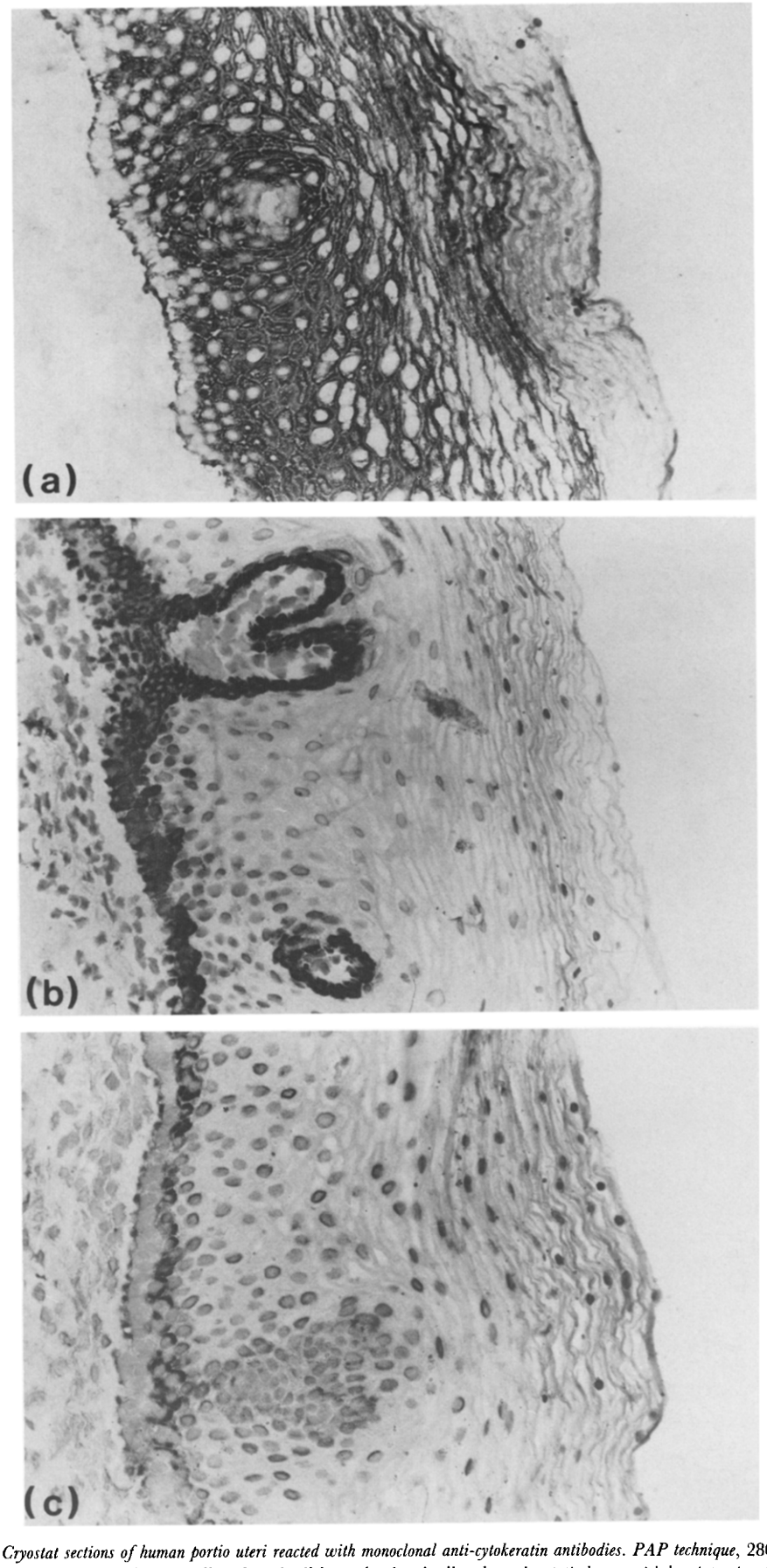

Fig. 2. Cryostat sections of human portio uteri reacted with monoclonal anti-cytokeratin antibodies. PAP technique, 280 ×. (*a*) *A45-B/B3 (broad spectrum) stains all epidermal cell layers but basal cell and parakeratotic layers with less intensity.* (*b*) *A53-B/A2 (cytokeratin 19) reacts only with basal cells.* (*c*) *A51-B/H4 stains basal cells and some nuclei of other epidermal cells.*

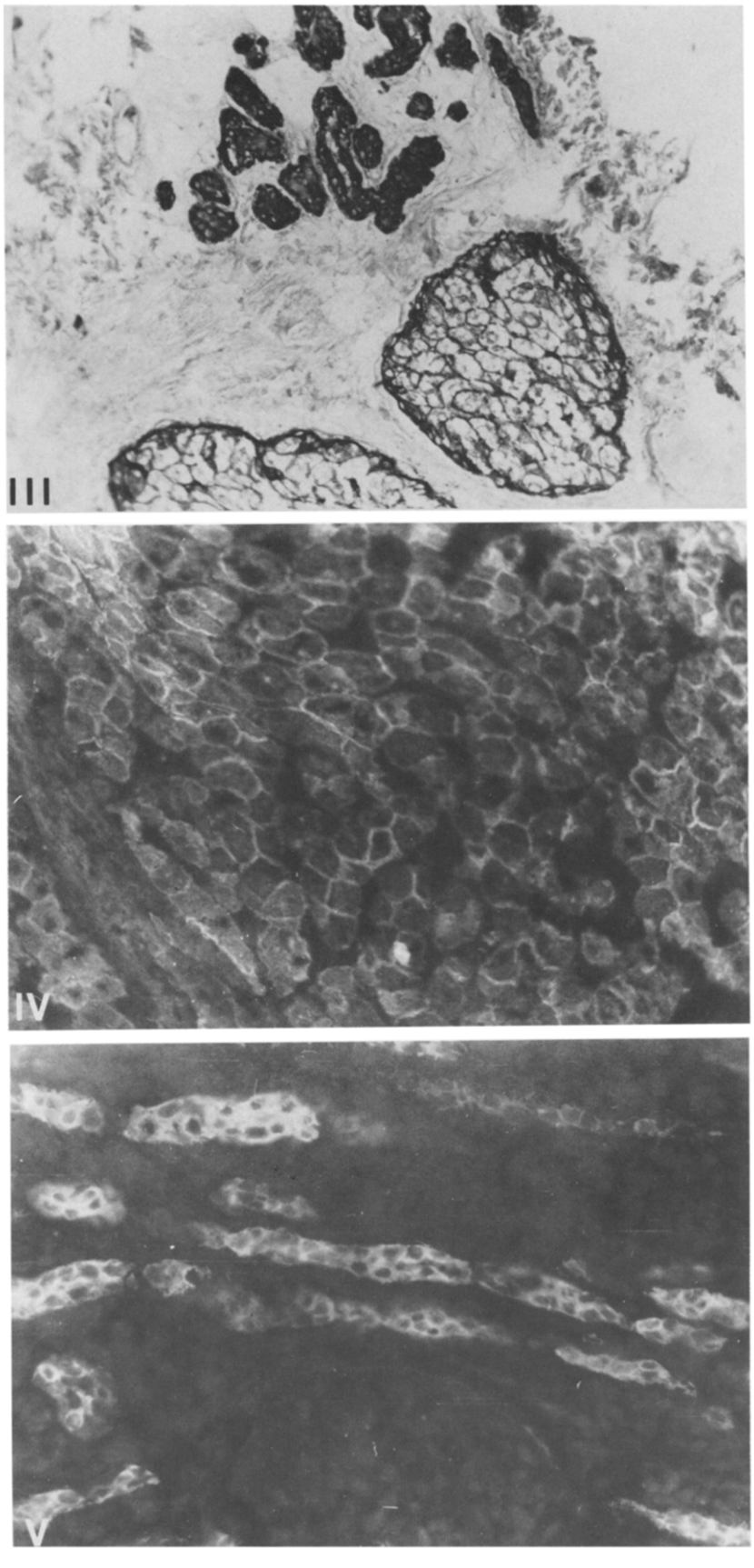

Fig. 3. *Reaction of A45-B/B3 (broad spectrum cytokeratin) with sweat and sebaceous glands in cryostat section of normal adult human skin. PAP technique*, 280 ×.

Fig. 4. *Reaction of A45-B/B3 (broad spectrum cytokeratin); with hepatocytes in cryostat section of normal human liver. Immunofluorescence*, 280 ×.

Fig. 5. *Reaction of A53-B/A2 (cytokeratin 19) with digital tubules (Henle's loop) in cryostat section of normal human kidney. Immunofluorescence*, 280 ×.

Table 3. Reactivity of three monoclonal anti-cytokeratin antibodies with animal tissues

Species	Tissue	A45-B/B3	A53-B/A2	A51-B/H4
Guinea-pig				
	Liver			
	Hepatocytes	+++	–	+
	Bile ducts	+++	(+)	+
	Kidney			
	Tubules	++	(+)	+
	Bowman's capsule epithelium	++	–	+
	Stomach			
	Glands	++	–	+
	Corneal epithelium	+++	–	–
	Esophagus			
	Basal cells	++	–	–
	Other cells	–	–	–
	Tongue epithelium	+	–	+
	Lip	+	–	+
Mouse				
	Liver			
	Hepatocytes	+++	–	N.D.
	Bile ducts	++	++	N.D.
Rat				
	Liver			
	Hepatocytes	+++	–	N.D.
	Bile ducts	++	(+)	N.D.

N.D.: not done. Evaluation of intensity: see Table 2.

and nuclear staining seemed to exclude each other. This phenomenon needs further examination.

Reactivity with animal tissues

Cytokeratins—like other cytoskeletal proteins—are highly conserved proteins. Reactivity of anti-cytokeratin mabs across species barriers is a rule and could be expected to be the case with our mabs too. The results shown in Table 3 confirm their binding to epithelial cells of at least three animal species (guinea-pig, rat, mouse). Epithelial cell lines derived from rat and bovine tissues have also been found positive (data not shown).

Generally, the results confirm the preliminary conclusions drawn from the screening of a panel of cell lines.

DISCUSSION

Cryostat sections of all available normal human tissues were analysed using three new anti-cytokeratin mabs in immunofluorescence and peroxidase–anti-peroxidase (PAP) techniques.

As a result, these mabs are among the most thoroughly tested anti-cytokeratin mabs available.

A45-B/B3 was found to stain all cells known to contain cytokeratins, e.g. epithelial and mesothelial cells, with no exception, and does not react with any other cell type. This antibody therefore provides an excellent histological reagent for the detection of epithelial or mesothelial cells independent of their cytokeratin composition. There is no need for mixing different mabs as recommended for other less comprehensive anti-cytokeratin mabs [17]. Obviously, a common epitope shared by many, if not all, cytokeratins is recognized by A45-B/B3. This epitope disappears after SDS or urea treatment of cell extracts. Therefore a biochemical characterization has not yet been possible. The antibody A45-B/B3 has already been used to identify epithelial cells in culture and to detect micrometastases of carcinomas in regional lymph nodes (unpublished). Another example is the detection of cytokeratin(s) in human brain Plexus choroideus epithelium, hitherto not yet examined in this respect, by means of mab A45-B/B3 [18] and confirmed by commercial anti cytokeratin mabs [19].

A53-B/A2, on the other hand, is specific for an individual cytokeratin, No. 19 of the catalogue of Moll *et al.* [1]. This was first concluded from the absence of staining in cell lines HeLa and A-431 and has now been confirmed by the results of this

study. Epithelial cells devoid of cytokeratin 19 according to biochemical analyses (adult skin, hepatocytes, cornea) were found negative with A53-B/A2 and *vice versa*. The specificity was confirmed by immunoblotting (Franke, personal communication). This cytokeratin is especially prominent in urothelium, amnion epithelium and mesothelia which are ontogenetically related. The mab A53-B/A2 may thus provide a novel histological reagent suited to differentiate among epithelial lineages. One example is the examination of cirrhotic ductular proliferations [20]. Bartek *et al.* applied two mabs of similar specificity successfully to the study of subpopulations of mammary epithelial cells [21]. Furthermore it may be useful for studies aimed at examining the preconditions of expression of this peculiar cytokeratin [22] as well as its possible function(s). For instance, the expression of cytokeratin 19 in the skin resembles that of an oncofetal antigen because it may be re-expressed in skin tumours ([23] and own results), especially *in vitro* [24, 25]. In the liver (in normal or malignant hepatocytes) this protein is never found [26].

The antigen(s) recognized by the third mab, A51-B/H4, remain to be established. This mab reacts with many, but not all, epithelia. The fact that it stains predominantly basal cells of the skin but not keratinized cells suggests that it recognizes cytokeratin No. 14 (50 kD) [5] in addition to No. 8 and/or 18 as concluded from its reactivity with MCF-7 and HeLa cells (only these cytokeratins are common to both) and with hepatocytes [1]. This antibody, like A45-B/B3, seems to detect a conformation dependent epitope which is destroyed by SDS. An up-to-now unexplained feature of this mab is its cross-reactivity with some nuclear antigen. Since this activity was still found after recloning, and only one isotype could be identified in immunodiffusion, it seems improbable that two antibodies are involved. Furthermore, both reactivities exclude each other, and certain cell populations are unreactive altogether. The analysis of the nuclear antigen detected by A51-B/H4 may be of interest with respect to a possible interrelationship between cytoskeleton and nuclear structures as suggested by some authors [28–31]. It may also shed light on functional aspects of intermediate filaments.

All three mabs can be used with tissues taken from at least some animal species as well. Like most anti-cytokeratin mabs described in the literature they cannot be applied to routine formalin-fixed, paraffin-embedded material. A53-B/A2 can, however, be used with methacarn-fixed, paraffin-embedded tissues. Both imunofluorescence and PAP technique are feasible, the latter being of advantage in tissues with autofluorescent components (e.g. elastic fibers).

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