Monoclonal Antibodies to Human Intermediate Filament Proteins

II. Distribution of Filament Proteins in Normal Human Tissues

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Monoclonal antibodies generated against different human intermediate filament (IF) proteins were assayed on fixed, embedded tissue by the biotin–avidin–immunoperoxidase method for evaluation of the tissue specificity of these antibodies. An antibody (43βE8) made to fibroblast IF protein stains mesenchymal tissue such as endothelium, histiocytes, stromal fibroblasts, and Schwann cells but does not stain epithelium, skeletal muscle, lymphocytes, or neurons. Three different anti-cytokeratin antibodies decorate epithelium in three unique patterns. One (35βH11) stains all nonsquamous epithelium but fails to recognize squamous epithelium. Antibody 34βE12 stains the full thickness of squamous epithelium and ductular epithelium but does not react with hepatocytes, pancreatic acinar cells, proximal renal tubules, or endometrial glands. Antibody 34βB4 stains only the suprabasal portion of squamous epithelium. None of these three anti-cytokeratin antibodies reacts with nerve or mesenchymal tissue. Two anti-neurofilament antibodies recognize only neurons, failing to react with epithelial or mesenchymal tissue. We conclude that these anti-intermediate filament antibodies can be used as tissue-specific markers. Neoplasms retain the same intermediate filament patterns as the normal parental tissue; therefore, these antibodies can be used as diagnostic aids in surgical pathology. (Am J Pathol 1984, 114:309–321)

ANTIBODIES to intermediate filament proteins can be used as tissue-specific reagents and are therefore useful tools in diagnostic surgical pathology.[1,2] We have recently described a panel of monoclonal antibodies to human intermediate filament proteins that react with different human cell lines *in vitro*.[3] These antibodies were made to cytoskeletons from fibroblasts and a human hepatocellular carcinoma line and to proteins from stratum corneum. Antibody 17βG3 and a more recently produced hybridoma with apparently identical antigenic specificity, 43βE8, both recognize the intermediate filament protein of human fibroblasts (58 kd) and other mesenchymal cells and cross-react with glial fibrillary acidic protein (GFAP) (52 kd). These antibodies do not react with many epithelial cell lines. Antibody 35βH11, generated against hepatoma cytoskeleton, specifically recognizes a 54 kd cytokeratin protein present in many epithelial lines but absent from fibroblasts in culture. An antibody made to stratum corneum, 34βE12, recognizes the 57 kd and 66 kd cytokeratin proteins of human stratum corneum and stains only epidermoid carcinoma cells in culture.[3] Here we describe the reactivity of these antibodies on fixed, embedded human tissue and demonstrate that they can identify different tissues *in vivo*. We also describe new monoclonal antibodies to neurofilament proteins, which, together with the antibodies previously defined, form a panel that can be a powerful diagnostic tool in the analysis of human tumors.

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Materials and Methods

Source of Antigens and Production of Antibodies

Vimentin was prepared from human foreskin fibroblasts as previously described.[3] Cytokeratins from stratum corneum and Hep3B, a human hepatocellular carcinoma cell line, were also prepared as previously described.[3] A purified sample of rat 200 kd protein was provided by Dr. F. T. Chiu, Department of Neurology, Albert Einstein College of Medicine, Bronx, New York.

Hybridoma antibodies to intermediate filament proteins were generated according to the basic outline of Köhler and Milstein[4] as modified by Nowinski et al.[5] The description of the antibody isolation has been published elsewhere.[3]

Immunocytochemistry

Small fragments (up to 0.3 cu cm) of human tissues were obtained from the Surgical Pathology and Autopsy Service of the University Hospital, University of Washington, Seattle, the latter within 12 hours of death. They were immersed in Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) or in "Methacarn" fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid) overnight, then transferred to absolute ethanol (1 hour) and to xylene (1 hour) and then embedded in paraffin at 54 C. Subsequently, $6\text{-}\mu$ sections were cut; and the sections deparaffinized and rehydrated by sequential immersions into xylene, absolute ethanol, 70% ethanol, and phosphate-buffered saline (PBS), pH 7.4. Sections were then overlaid with diluted ascitic fluids corresponding to the clones of specific-antibody-producing hybridoma cells (30 minutes). All ascites fluids described herein were used at dilutions ranging from 1:1000 to 1:8000. The ABC-immunoperoxidase system[6] was then employed with the use of a nickel chloride color modification scheme adapted from Hsu and Soban.[7] Briefly, with intervening washes in PBS, sections were serially incubated in 1:500 dilutions in PBS of biotinylated rabbit anti-mouse IgG, corresponding to a dilution of 0.003 mg/ml (Vector Laboratories, Burlingame, Calif; 30 minutes); avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) at a 1:1 ratio, diluted 1:100 each in PBS (30 minutes), as supplied by the manufacturer; 200 ml of a 0.1% solution of 3,3′-diaminobenzidine (DAB, Sigma Chemical Company, St. Louis, Mo) in 0.05 M Tris buffer, pH 7.6 (10 minutes) to which had been added 0.75 ml of 3% H_2O_2 and 1.0 ml of an 8% $NiCl_2$ solution. For slides to be incubated with antibody 43βE8 only, following deparaffinization there was a 2-minute incubation period at room temperature with a 0.01% solution in PBS of Pronase (70,000 proteolytic units/g; Calbiochem-Behring, San Diego, Calif). Sections were counterstained with methyl green and dehydrated in sequential alcohol solutions, and then mounting media and coverslips were applied.

Western Blot Analysis

Tissue obtained from autopsy specimens was washed in TN buffer (10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.5 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and homogenized in 1% sodium dodecyl sulfate (SDS), 0.05 M Tris, pH 6.8, containing 7.7 mg/ml dithiothreitol (DTT). The ratio of tissue to homogenizing buffer was approximately 1/10 ml. The homogenate was boiled for 10

Table 1 — Reactivity of Different Monoclonal Antibodies With Different Intermediate Filament Proteins by the Immunoblot Method

Antibody	Immunogen	Vimentin (58 kd)	Hepatoma cells 54 kd protein	Keratin from stratum corneum				GFAP	Neurofilament proteins		
				66	57	51	49		200	160	68
17βG3	Vimentin	+	–	+	+	–	–	+	–	–	–
43βE8	Vimentin	+	–	ND	ND	ND	ND	+	–	–	–
35βH11	54 kd protein of Hep3B cells	–	+	–	–	–	–	–	–	–	–
34βE12	Keratin from stratum corneum	–	–	+	+	+	+	–	–	–	–
34βB4		–	–	+	+	+	+	–	–	–	–
31γA11	200 kd rat neurofilament	+	–	–	–	–	–	–	+	+	+
31αF3		–	–	–	–	–	–	–	+	+	–

ND, not done.

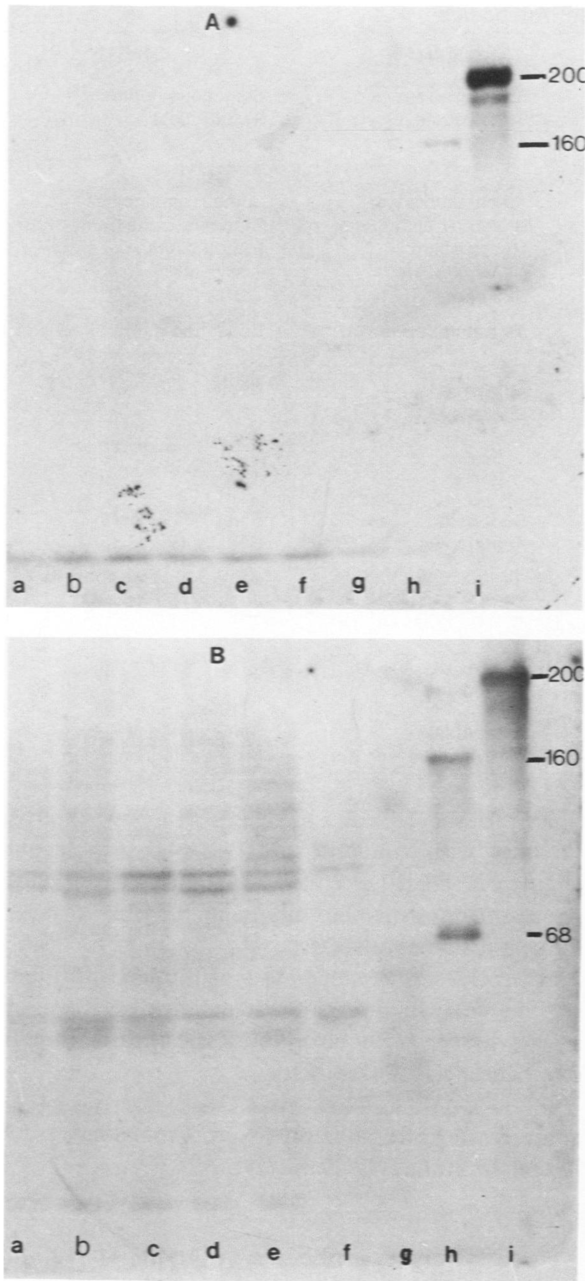

Figure 1 – Immunoblots of anti-neurofilament antibodies on tissue culture cells and preparations of rat neurofilament proteins. **A** – Hybridoma 31αF3. **B** – Hybridoma 31γA11. Confluent monolayers of tissue culture cells were solublized in 1% SDS, 0.05 M Tris, pH 6.8, and underwent electrophoresis on 8% polyacrylamide gels. Approximately 5–10 μg of protein were added per well. Lane *a*, human fibroblasts; lane *b*, Hep3B, human hepatocellular carcinoma; lane *c*, A431, human epidermoid carcinoma; land *d*, HM 919, human melanoma; lane *e*, HM 1804, human melanoma; lane *f*, rhabdo 1, human rhabdomyosarcoma; lane *g*, keratin from stratum corneum; lane *h*, neurofilament preparation from rat brain, lane *i*, purified 200 kd neurofilament preparation from rat brain.

minutes and centrifuged at 5000 rpm for 10 minutes at 4 C. The supernatant was diluted 1:10 in SDS sample buffer containing DTT and underwent electrophoresis on an 8% polyacrylamide gel.[8] Proteins were electrophoretically transferred onto nitrocellulose paper overnight at 100 mamp and the antigens identified as previously described.[3]

Results

Characterization of Antibodies

Anti-Vimentin

Two hybridoma antibodies, 43βE8 and 17βG3, were made to cytoskeletal preparations from human fibro-

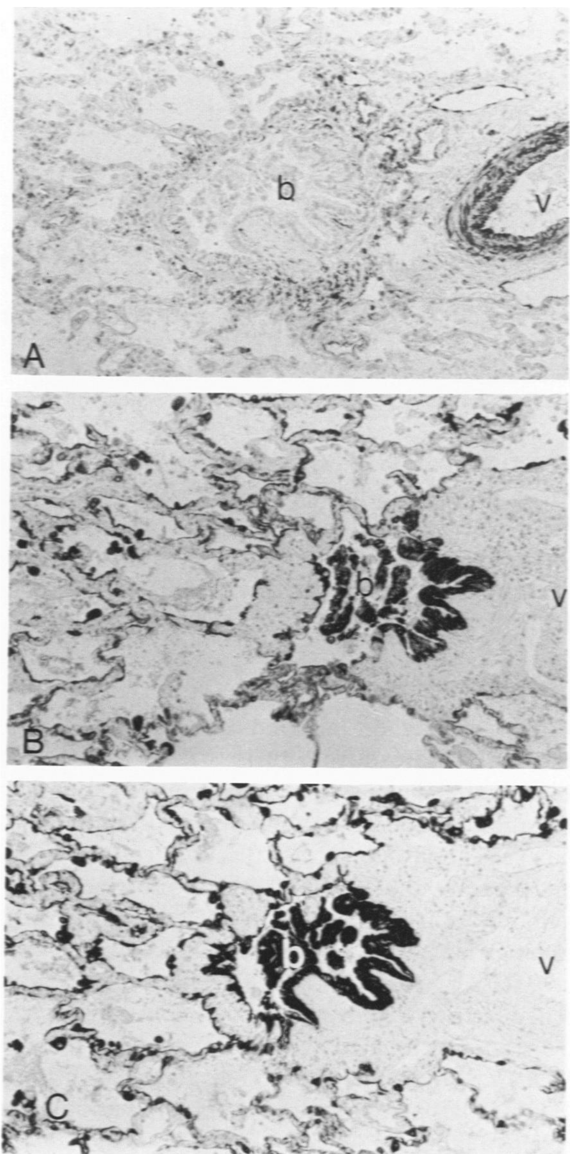

Figure 2 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human lung. *b*, bronchiole; *v*, blood vessel. **A** – Anti-vimentin antibody 43βE8. Vascular smooth muscle cells, endothelial cells, and connective tissue cells only are positive. (×100) **B** – Anti-cytokeratin antibody 34βE12. Bronchiolar epithelium and pneumocytes only are positive. (×100) **C** – Anti-cytokeratin antibody 35βH11. Bronchiolar epithelium and pneumocytes only are positive. (×100) Both cytokeratin antibodies define similar subsets of cells in this organ.

Table 2—Reactivity of Normal Tissues With Intermediate Filament Antibodies

	43βE8	35βH11	34βE12
Skin	Blood vessels Endothelium Fibroblasts Nevus cells	Sweat glands Sweat ducts	Squamous epithelium Sweat ducts
Lung	Blood vessel endothelium Alveolar histiocytes	All pneumocytes Bronchial epithelium Mesothelium	Some pneumocytes Bronchial epithelium Mesothelium
Liver	Sinusoidal lining cells Blood vessel endothelium	All hepatocytes Bile ducts	Bile ducts
Kidney	Glomeruli: endothelium Occasional tubular epithelial cells	All tubular epithelia	Collecting duct epithelia only
Pancreas	Blood vessel endothelium	Acinar cells Ductal cells	Ductal cells
Spleen/lymph node	Capillary endothelium Dendritic cells Some histiocytes	–	–
Breast	Stromal fibroblasts Endothelium	Duct cells Acinar cells	Some acinar cells Duct cells
Thyroid	Endothelium	All follicular epithelia	Some follicular epithelia
Skeletal muscle	Interstitial fibroblasts; endothelial cells	–	–
Cardiac muscle	Interstitial fibroblasts; endothelial cells	–	–
Gastrointestinal tract: small and large bowel	Lamina propria Blood vessel endothelium	All epithelia Mesothelium (serosa)	Some epithelia Mesothelium (serosa)

blasts. Both antibodies recognize a 58 kd protein present in fibroblasts and other mesenchymal cells and also identify glial fibrillary acidic protein (GFAP) in "immunoblot" experiments.[3] These antibodies do not react with cytokeratins or neurofilament proteins (Table 1).

Anti-Cytokeratin

Three cytokeratin antibodies will be described. One, 35βH11, arose from fusions using cytoskeleton from a human hepatoma cell line (Hep3B) as antigen. This antibody identifies a 54 kd protein in the hepatoma cells and other epithelial cells in culture. It does not recognize the 58 kd fibroblast protein, keratins from stratum corneum, GFAP, or neurofilament proteins (Table 1). The other two antibodies, 34βE12 and 34βB4, were made to solubilized human stratum corneum. In "Western blot" experiments, both recognize stratum corneum proteins of molecular weight 66 kd and 57 kd. They fail to recognize vimentin, the 54 kd hepatoma protein, GFAP, or neurofilament proteins (Table 1).

Anti-Neurofilament 200 kd Protein

Antibodies 31γA11 and 31αF3, both generated to a 200 kd protein of rat neurofilaments, identify the 200 kd and 160 kd neurofilament proteins (Figure 1A and B; Table 1). Additionally, 31γA11 recognizes the third triplet protein (68 kd) and demonstrates weak cross-reactivity with vimentin and two higher-molecular-weight proteins (Figure 1B). No staining of GFAP or any other intermediate filament protein is noted. These antibodies also decorate the same neurofilament peptides in Western blots of human brain (data not shown). In immunocytochemical analysis of human tissue, both antibodies specifically identify only neural tissue (see below).

Analysis of Antibodies on Fixed, Embedded Tissue: Immunocytochemistry

Antibodies to Cytokeratin

Three different antibodies (34βB4, 34βE12, and 35βH11) react only with epithelial cells, each manifesting a unique pattern of reactivity.

Antibody 35βH11, which specifically identifies a 54 kd cytokeratin protein in many epithelial cell lines, reacts with all nonsquamous epithelia. In the lung, it delineates the flattened pneumocytes as well as the bronchiolar epithelium (Figure 2C, Table 2), while failing to react with stromal cells, histiocytes, and blood vessels. All tubular cells (proximal, distal, and collecting ducts) are positive in the kidney; glomeruli, stroma, and vessels are negative (Figure 3C, Table 2). In the liver, it identifies the hepatocytes as well as the bile ducts, highlighting the bile canaliculi in the former (Figure 4C, Table 2); again, nonepithelial

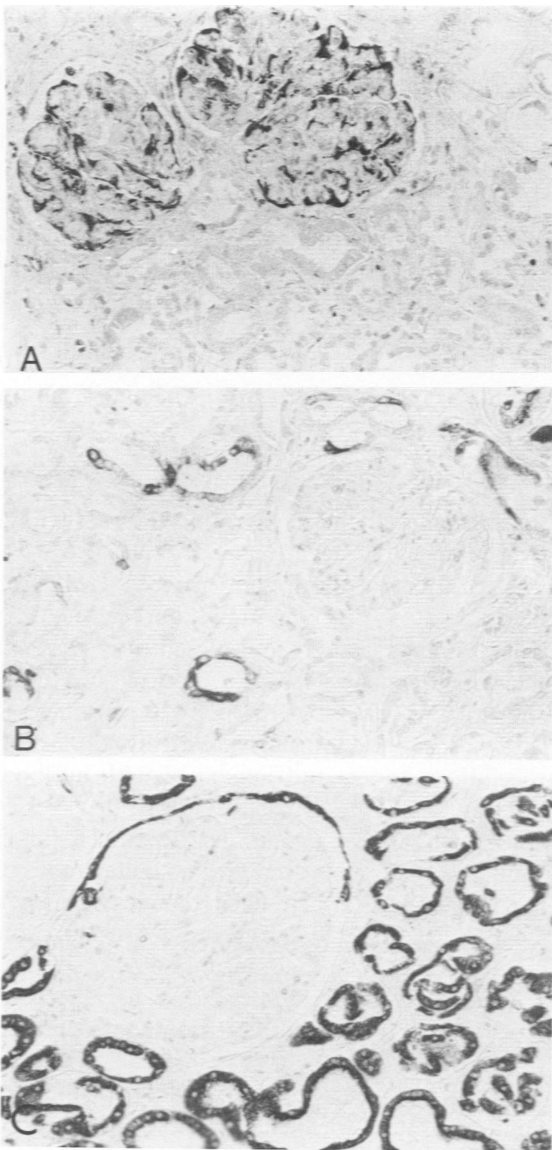

Figure 3 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human kidney. A – Anti-vimentin antibody 43βE8. Glomerular cells show strong positivity, probably corresponding to endothelial cells and/or epithelial cells. Mesangial cells appear negative. Also positive are scattered cells in the interstitium, but no tubular epithelial cells appear positive here, although some show dotlike activity (see text). (×100) B – Anti-cytokeratin antibody 34βE12. A subset of tubular epithelial cells only is positive, probably corresponding to collecting ducts. Glomerular cells are all non-reactive. (×100) C – Anti-cytokeratin antibody 35βH11. All tubular epithelial cells are positive. Also positive are parietal epithelial cells of glomerular tufts, whereas cells within glomeruli are all nonreactive. (×100)

structures are unstained. The epithelial cells and mesothelium of the gastrointestinal tract are decorated, but the smooth muscle, connective tissue cells, and blood vessels are nonreactive (Figure 5C, Table 2). The antibody also stains the cells of the thyroid, parathyroid, pituitary gland, salivary gland (Figure 9C), and some cells in the adrenal cortex.

Significantly, stratified squamous epithelium (skin, esophagus, etc.) is completely unstained by this antibody (Figure 6C). Additionally, organs without epithelial cells such as the cerebral cortex, cerebellum, spleen, thymus, and lymph nodes are also nonreactive. Thus, this antibody is unique in that it recognizes only nonsquamous epithelium.

Antibody 34βE12, generated against human stratum corneum, reacts strongly with the full thickness of all stratified squamous epithelium (skin, larynx,

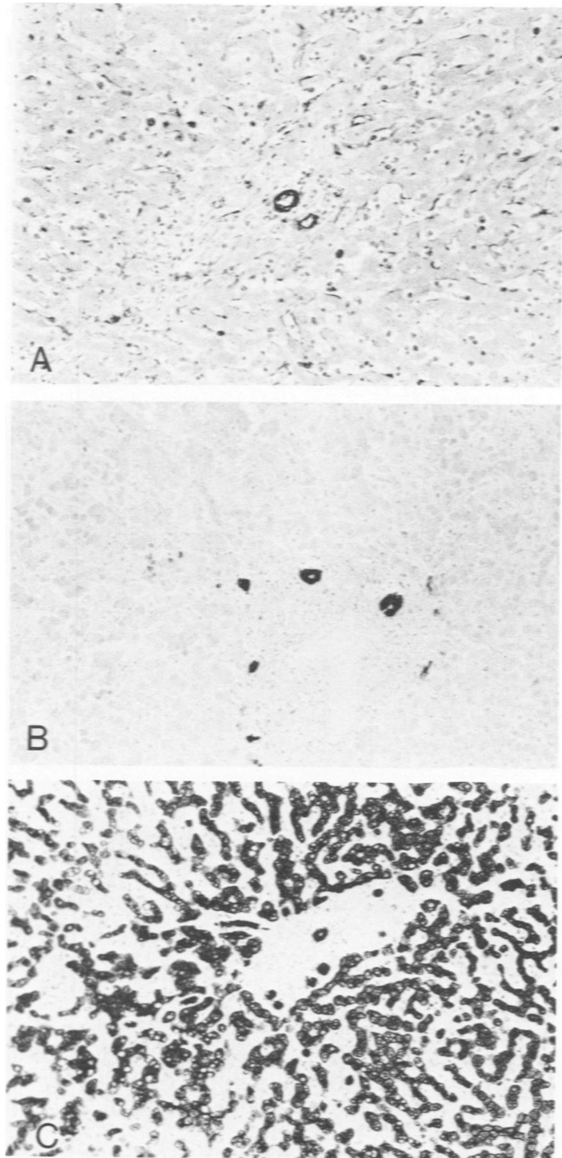

Figure 4 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded human liver. A – Anti-vimentin antibody 43βE8. Notice the positivity of blood vessels and sinusoidal lining cells; hepatocytes and bile ducts are nonreactive. (×100) B – Anti-cytokeratin antibody 34βE12. Bile ducts are the only positive structures. (×100) C – Anticytokeratin antibody 35βH11. All hepatocytes and bile duct epithelia are positive; sinusoidal lining cells are negative. (×100)

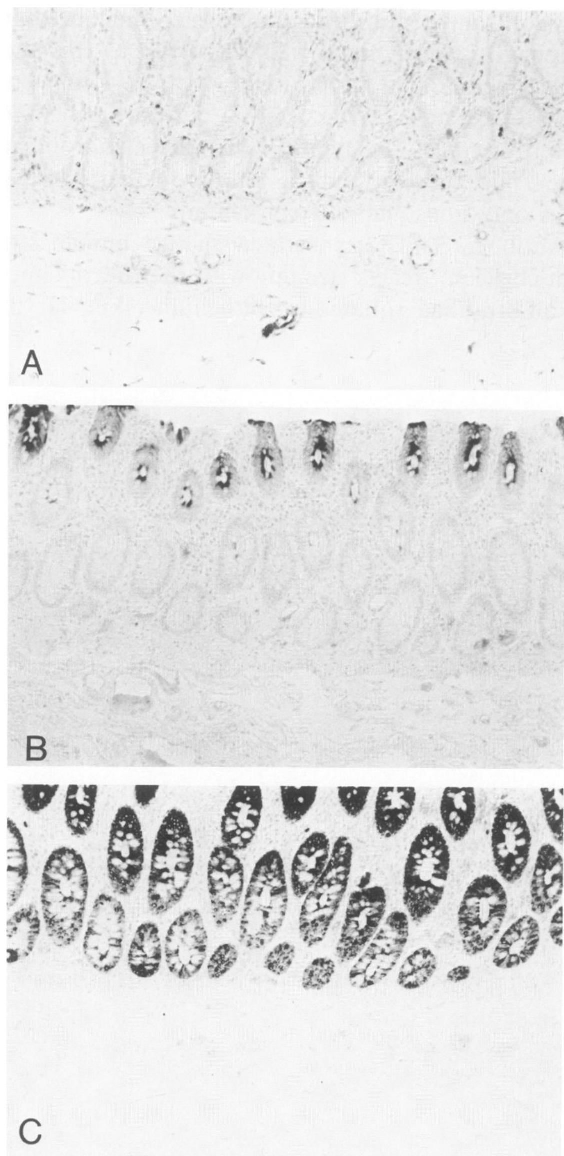

Figure 5—Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human colon. **A**—Anti-vimentin antibody 43βE8. Vascular endothelium and connective tissue cells only are positive. Notice the nonreactivity of lymphoid cells in the lamina propria and smooth muscle cells in the muscularis. (×100) **B**—Anti-cytokeratin antibody 34βE12. Only superficial epithelial cells are positive. (×100) **C**—Anti-cytokeratin antibody 35βH11. Glandular epithelial cells are all positive. (×100)

esophagus, ectocervix, etc.) (Figure 6B). It also stains some but not all nonsquamous epithelium. For example, hepatocytes are unstained; whereas bile ducts are markedly positive (Figure 4B, Table 2). Similarly, pancreatic acinar cells are nonreactive, whereas pancreatic ducts are reactive. In the lung, pneumocytes and bronchial epithelial cells are positive (Figure 2B, Table 2). In the skin, the squamous epithelium as well as the sweat duct epithelium is positive. In other epithelial organs, such as the small bowel, salivary gland, and prostate, only a portion of the cells are positive (Figures 7B and 9B, and Table 2). In general, the positive cells tend to have a more basal location within the gland, best depicted in the prostate (Figure 7B). Only the collecting duct epithelium is positive in the kidney; proximal tubules are negative (Figure 3B). This antibody also recognizes the cytokeratins present in mesothelium and thymic epithelium. Nonepithelial tissues fail to react with antibody 34βE12.

Antibody 34βB4, also generated against stratum corneum, reacts exclusively with differentiated squamous epithelium. In the skin, esophagus, and ectocervix, the suprabasal cells are positive, whereas the basal layer is totally negative (Figure 6D, Table 2). All other tissues, including all nonsquamous epithelium, are completely negative.

To be certain that the antibodies recognize the same proteins in intact tissue as in cultured cells, Western blot experiments were done using autopsy tissue as a source of antigen. Tissue was solubilized in 1% SDS, 7.7 mg/ml DTT, 0.05 M Tris, pH 6.8, run on an 8% polyacrylamide gel, transferred electrophoretically to nitrocellulose paper, and incubated with antibodies 35βH11 and 35βE12. Antibody 35βH11 identifies a 54 kd protein in Hep3B, a human hepatocellular carcinoma line,[3] and also decorates a similar molecular weight protein in kidney, liver, pancreas and colon mucosa (Figure 8A, Lanes a, b, c, e). No 54 kd protein is identified in adrenal, colon muscularis, spleen, or lymph node (Figure 8A, Lanes d, f, g, h), all of which do not react with the antibody immunocytochemically. Lower molecular weight proteins are also identified by this antibody, but we do not know whether these are unique proteins or breakdown products of the 54 kd protein.

Antibody 34βE12 identifies proteins of 66 kd and 57 kd in extracts of stratum corneum (Figure 8B, Lane j) and identifies proteins of 66 kd and 57 kd in all tissues tested, even those that do not react with the antibody in the biotin-avidin-peroxidase assay (spleen, lymph node, colon muscularis). This finding is consistent with the results with tissue culture cells, in which this antibody decorated only the intermediate filaments of an epidermoid carcinoma cell line (A431) by immunofluorescence but identified proteins of 66 kd and 57 kd in all cells tested, even those negative in immunofluorescence assays.[3] We do not understand the reasons for this discrepancy. We conclude that antibodies 34βE12 and 35βH11 recognize antigens in whole tissue similar to those they recognize in tissue culture cells.

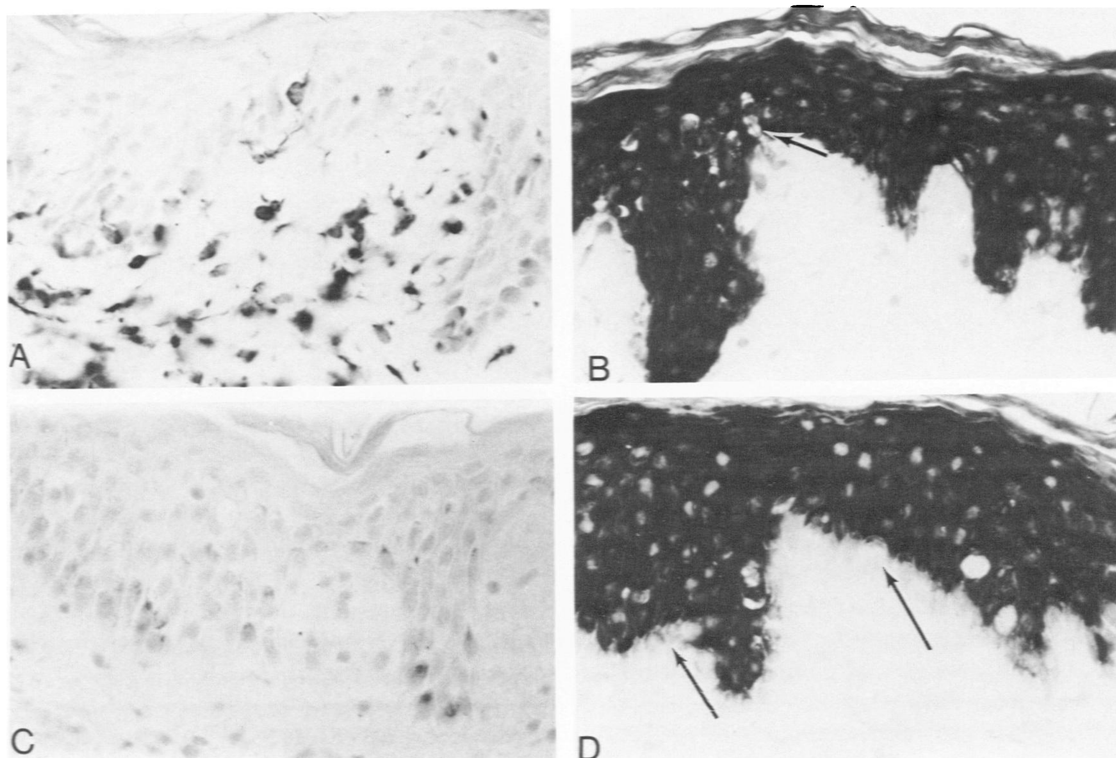

Figure 6 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human skin. **A** – Anti-vimentin antibody 43βE8. Dermal fibroblasts, endothelial cells, melanocytes, and nevus cells at the dermal-epidermal junction are positive, whereas the epithelium is negative. (×160) **B** – Anti-cytokeratin antibody 34βE12. Full thickness of squamous epithelium is positive, while dermal cells are all negative. Note "holes" in squamous epithelium (*arrow*) corresponding to vimentin-containing melanocytes. (×160) **C** – Anti-cytokeratin antibody 35βH11. Squamous epithelium and all dermal cells are nonreactive. (×160) **D** – Anti-cytokeratin antibody 34βB4. Suprabasal squamous epithelium only is positive. Notice the nonreactivity of basal cells (*arrows*).

Anti-Vimentin Antibody

Antibody 43βE8 generated against fibroblast intermediate filament protein reacts specifically with many types of mesenchymal cells such as Schwann cells, vascular smooth muscle cells, endothelial cells, other "stromal cells," and tissue histiocytes. This antibody strongly reacts with vascular smooth muscle of small vessels. The smooth muscle cells of larger arteries are variably stained. This antibody fails to react with lymphocytes, which contain vimentin[1] (Table 2). We do not understand the lack of reactivity with lymphocytes. It is unlikely that fixation "removes" the immunoreactive material from lymphocytes because frozen sections of unfixed lymph nodes also do not react with antibody 43βE8 (data not shown). Additionally, lymphocytes react with antibodies capable of recognizing more than one type of intermediate filament protein; thus, these cells definitely contain intermediate filaments (data not shown).

In the skin, the antibody leaves the squamous epithelium unstained but strongly reacts with dermal capillary endothelium and dermal fibroblasts. It also stains melanocytes at the dermal-epidermal junction (Figure 6A, Table 2). Lymphoid organs such as the spleen and lymph node demonstrate positivity of the large vessels, microvasculature, and dendritic and epithelioid histiocytes (Table 2). In parenchymal organs such as the liver, the sinusoidal lining cells and blood vessels are positive; all epithelium is negative (Figure 4A). Reactivity of tissue stromal cells in other parenchymal organs, such as pancreas, is variable, perhaps as a function of the inconspicuous cytoplasm of these cells. Only the vasculature is prominently stained in these organs. The kidney is remarkable in that cells within the glomeruli (presumably epithelial and endothelial cells) are positive (Figure 3A). The vast majority of tubular epithelia are negative; however, the proximal tubular epithelium demonstrates occasional "dotlike" positivity. No other epithelium shows similar positivity.

This antibody recognizes GFAP in "immunoblot" experiments. Sections of cerebellum show that Berg-

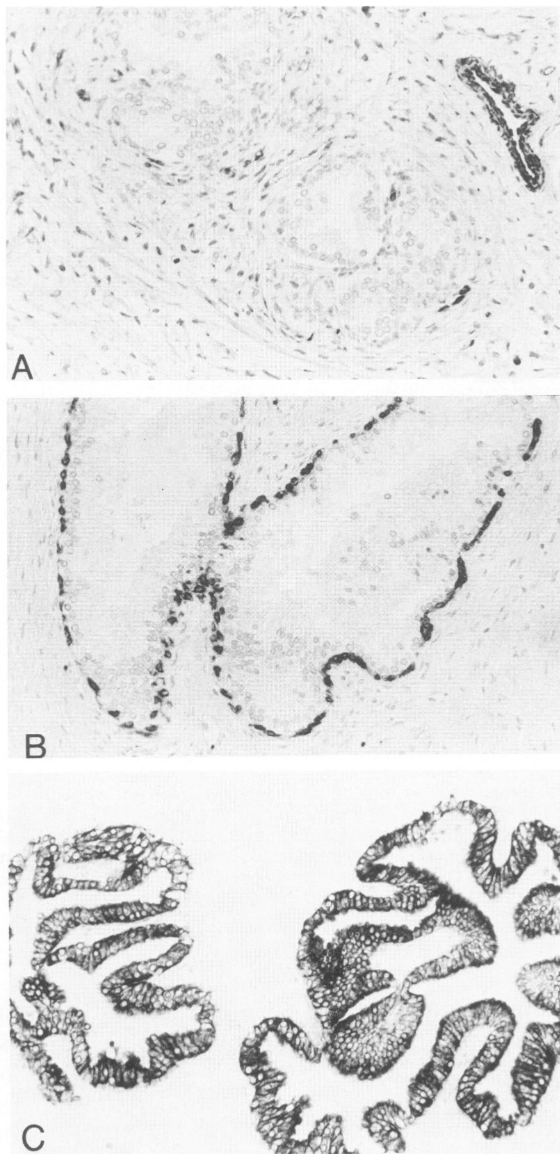

Figure 7—Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human prostate. **A**—Anti-vimentin antibody 43βE8. Blood vessels and stromal cells are positive, but the epithelium is negative. (×160) **B**—Anti-cytokeratin antibody 34βE12. Basal glandular epithelial cells only are positive. (×160) **C**—Anti-cytokeratin antibody 35βH11. The total thickness of the epithelium only is positive. (×160)

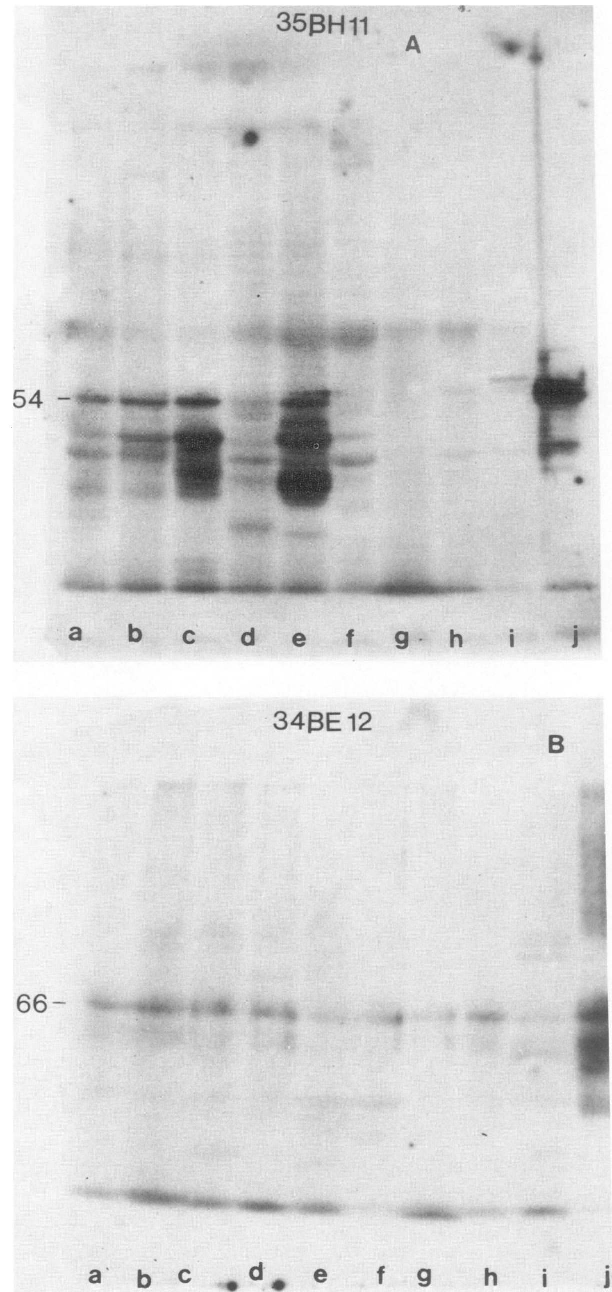

Figure 8—Immunoblots of anti-cytokeratin antibodies on intact human tissues. Tissue was solubilized as described in Materials and Methods section, and samples were applied to 8% polyacrylamide gels. Approximately 10-20 μg of protein was added per well. **A**—35βH11. **B**—34βE12. Lane *a*, kidney; lane *b*, liver; lane *c*, pancreas; lane *d*, adrenal; lane *e*, colonic mucosa; lane *f*, colonic muscularis; lane *g*, spleen; lane *h*, lymph node; lane *i*, *in vitro* human fibroblasts; lane *j*, *in vitro* Hep3B cells (hepatocellular carcinoma). In **B**, lane *i* contains A431, human epidermoid carcinoma cells instead of human fibroblasts, and lane *j* contains stratum corneum keratin instead of Hep3B cells.

mann glial cells and some GFAP-containing structures are positive, though the distribution of positive cells is clearly distinct from those that are GFAP- or neurofilament-positive (Figure 10).

Anti-Neurofilament Antibodies

Antibodies 31γA11 and 31αF3, generated against rat 200 kd protein, stain neuronal processes in central nervous system tissue (Fig. 10B, Table 2). In addition, peripheral nerves in various organs react with these antibodies, whereas the surrounding nonneuronal tissues are negative. Neither antibody reacts with neuronal cell bodies within the gray matter of the central nervous system.

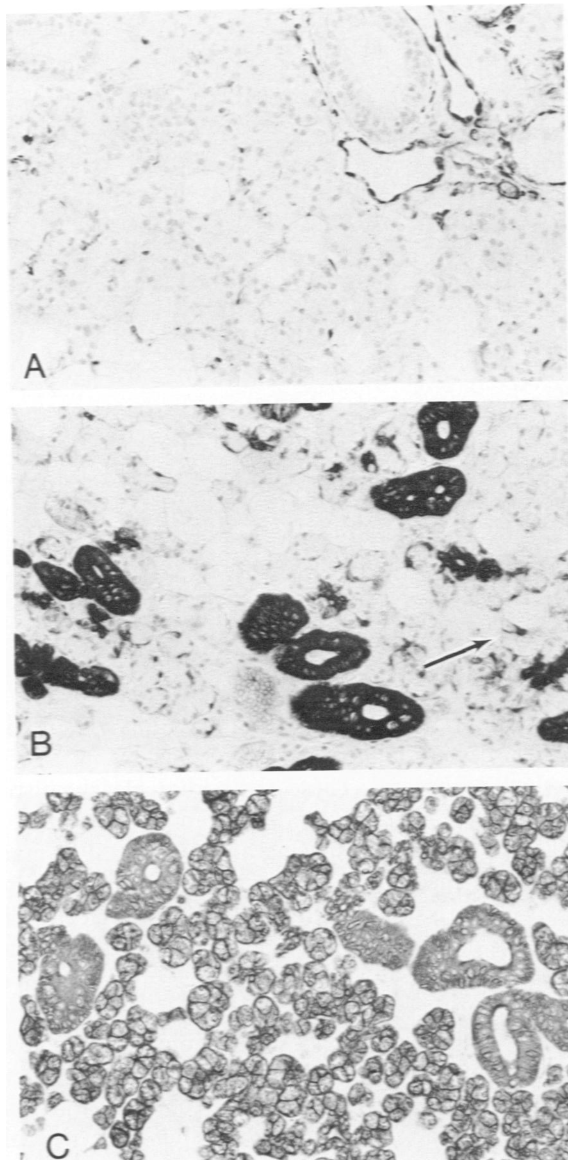

Figure 9 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human salivary gland. **A** – Anti-vimentin antibody 43βE8. Vascular endothelium and connective tissue cells only are positive. (×160) **B** – Anti-cytokeratin antibody 34βE12. Ductular epithelium only is positive, together with intercalated duct processes (*arrow*). (×160) **C** – Anti-cytokeratin antibody 35βH11. Acinar and ductal epithelia are equally positive. (×160)

Immunocytochemical Studies of Tumor Specimens

The reactivity on normal tissues suggests that these antibodies might be able to distinguish among different tumors. To test this hypothesis, we analyzed an unselected series of human tumors appropriately fixed in Carnoy's or Methacarn. A more detailed report of this study is currently in preparation. Preliminary results are shown in Table 3 and Figures 11-13.

In general, the tumors behaved in a way similar to that of their nonneoplastic parental tissue with respect to reactivity with the antibodies (Table 3). For example, virtually all squamous cell carcinomas of skin, lung, and nasopharynx mimic the pattern of reactivity of normal squamous epithelium: uniformly positive with anti-cytokeratin antibody 34βE12, focally positive with antibody 34βB4, and negative with antibody 35βH11, anti-vimentin antibodies, and anti-neurofilament antibodies (Figure 11). Indeed, squamous cell carcinomas are the only tumors found to be positive with the anti-cytokeratin antibody 34βB4. Antibody 35βH11 fails to stain most squamous tumors; however, some cervical epidermoid carcinomas exhibit variable staining with antibody 35βH11. We attribute this to the origin in metaplastic (squamous) endocervical epithelium, which also stains positively with this antibody. Normal ectocervical squamous epithelium does not react with antibody 35βH11.

Nonsquamous epithelial tumors can be divided into two categories: those that react with only 35βH11 and those that react with both 35βH11 and 34βE12.

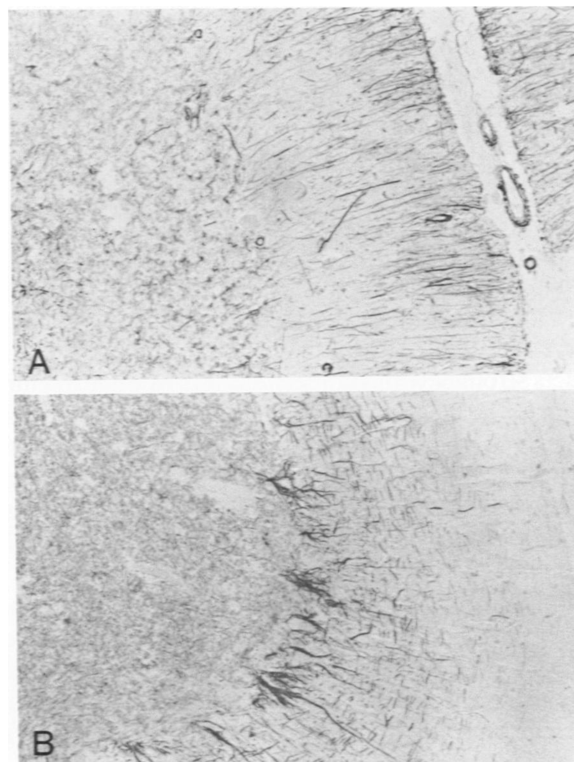

Figure 10 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human cerebellum. **A** – Anti-vimentin antibody 43βE8. Notice the positive staining of the blood vessels and Bergmann glial fibers (*center*). (×100) (B) Anti-neurofilament antibody 31γA11. Notice the nonreactivity of blood vessels and glial cells and positive staining of basket cells surrounding Purkinje cells (*center*). (×100)

Table 3 – Reactivity of Monoclonal Antibodies With Human Tumors

			Antivimentin	Anticytokeratin			Antineuro-filaments
Tumor designation	Source	N*	43βE8	35βH11	34βE12	34βB4	31γA11
Squamous cell carcinoma	Lung	5	–†	–	+	+	–
	Skin	4	–†	–	+	+	–
	Nasopharynx	3	–†	–	+	+	–
	Cervix	6	–†	±	+	+	–
Adenocarcinoma	Colon	3	–†	+	±	–	–
	Thyroid	2	–†	+	±	–	–
	Breast	6	–†	+	+	–	–
	Pancreas	1	–†	+	+	–	–
	Cholangiocarcinoma	2	–†	+	+	–	–
	Hepatocellular	2	–†	+	–	–	–
	Endometrium	4	–†	+	–	–	–
	Kidney	2	–†	+	–	–	–
Giant cell tumor	Bone	4	+	–	–	–	–
Meningioma	Brain	2	+	–	–	–	–
Sarcoma	Lung	2	+	–	–	–	–
Fibrous histiocytoma	Retroperitoneum	3	+	–	–	–	–
Lymphoma	Lymph nodes	8	–	–	–	–	–
Neuroblastoma	Adrenal	3	–	–	–	–	–

* Number of cases observed.
† Tumor stroma only positive with antivimentin antibody 43βE8.

The former group includes hepatocellular carcinomas, endometrial carcinomas, renal carcinomas, and pituitary adenomas (Figure 12). Additionally, gastrointestinal carcinomas and thyroid carcinomas exhibit much greater reactivity with antibody 35βH11 than with antibody 34βE12. These results are identical with the staining of the corresponding normal tissue by these antibodies.

Carcinomas of ductal origin, including breast, pancreas, and bile duct, are decorated by both anti-cytokeratin antibodies 35βH11 and 34βE12. This result is again expected because the ductal epithelium in the normal tissue of these organs also reacts with both of these antibodies. The anti-vimentin antibody 43βE8 reacts strongly with giant cell tumors of bone, meningiomas, fibrous histiocytomas, and other sarcomas (Figure 13). These tumors fail to react with the anti-cytokeratin and anti-neurofilament antibodies. Lymphomas fail to react with antibody 43βE8, even though lymphoid cells are thought to contain vimentin.[1]

No tumors are positive with anti-neurofilament antibodies, including three neuroblastomas studied. We conclude that this battery of antibodies can distinguish among different tumors and that they are therefore useful reagents in the practice of surgical pathology.

Discussion

We have exploited the differences in intermediate filament proteins in various tissues to generate tissue-specific intermediate filament monoclonal antibodies to be used as diagnostic reagents in the practice of surgical pathology. These antibodies clearly distinguish mesenchymal, neural, and epithelial tissues.

The mesenchymal-specific antibody, 43βE8, made to the intermediate filament protein of human fibroblasts (58 kd), stains endothelium, Schwann cells, histiocytes, and "stromal fibroblasts" in tissue sections. The antibody also stains the smooth muscle cells of small blood vessels and variably stains the smooth muscle of larger vessels. No staining of epithelial cells, skeletal muscle, or axons of the central or peripheral nervous system is observed. We do not understand the lack of reactivity with lymphocytes, cells which should contain vimentin.[1] One possible explanation is that there are slight tissue-specific differences in vimentin, and this antibody only recognizes one type of vimentin.

Three anti-cytokeratin antibodies manifest different tissue specificity. Antibody 35βH11, made to a 54 kd protein in human hepatocellular carcinoma cells, reacts with all nonsquamous epithelium and by Western blot experiments also identifies a 54 kd protein in epithelial tissue. This antibody does not react with squamous epithelium, mesenchymal tissue, or nervous tissue. It is therefore very specific for nonsquamous epithelium. Antibody 34βE12, made against stratum corneum, reacts with squamous epithelium and some, but not all, nonsquamous epithelium. No reactivity is observed with mesenchymal or nervous tissue. Antibody 34βB4, also generated to stratum corneum, stains only the suprabasal portions of

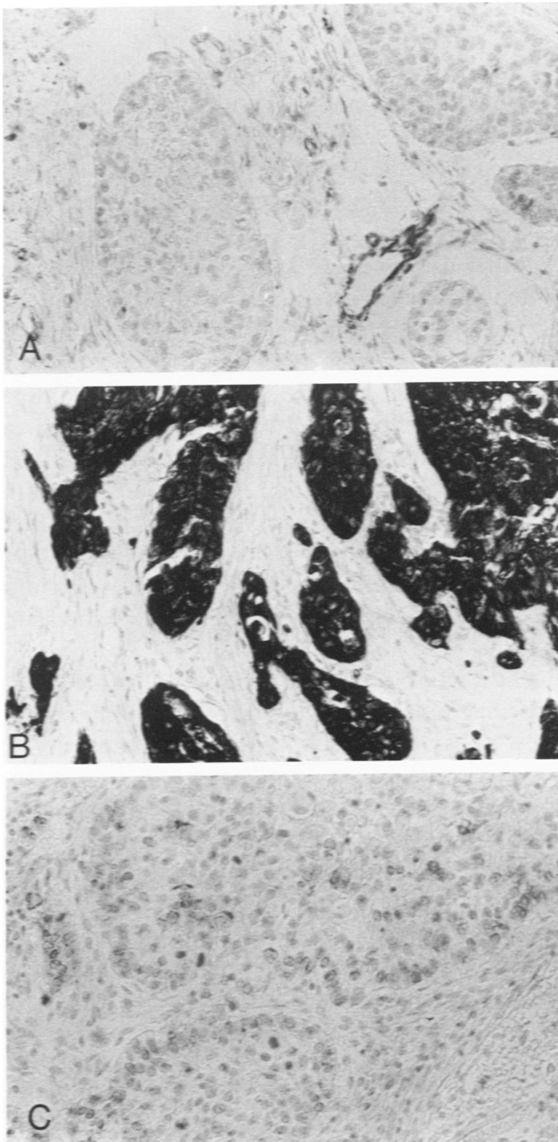

Figure 11 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human squamous cell carcinoma of lung. **A** – Anti-vimentin antibody 43βE8. Tumor cells are nonreactive; only stromal cells and vascular endothelia are positive. (×160) **B** – Anti-cytokeratin antibody 34βE12. Tumor cells are strongly positive. (×160) **C** – Anti-cytokeratin antibody 35βH11. Tumor cells are nonreactive. (×160)

squamous epithelium. Both anti-stratum corneum antibodies recognize peptides of 66 kd and 57 kd in all tissues examined, even those that do not stain in immunocytochemical assays. We do not understand the reason for this observation. In nonreactive tissue, the proteins may be "masked" or have a different intracellular localization than in cells stained by these antibodies.

The anti-neurofilament antibodies were made to rat 200 kd protein and recognize the 200 and 160 kd proteins in immunoblot experiments. One antibody, 31γA11, also decorates the 68 kd protein of neurofilaments. These antibodies are highly specific for axons; they do not stain mesenchymal or epithelial tissue.

Diagnostic Use of These Antibodies

Tumors resemble their nonneoplastic parental tissue with respect to expression of intermediate filament proteins.[1,2] For example, nonepithelial neoplasms, such as sarcomas, lymphomas, and neural

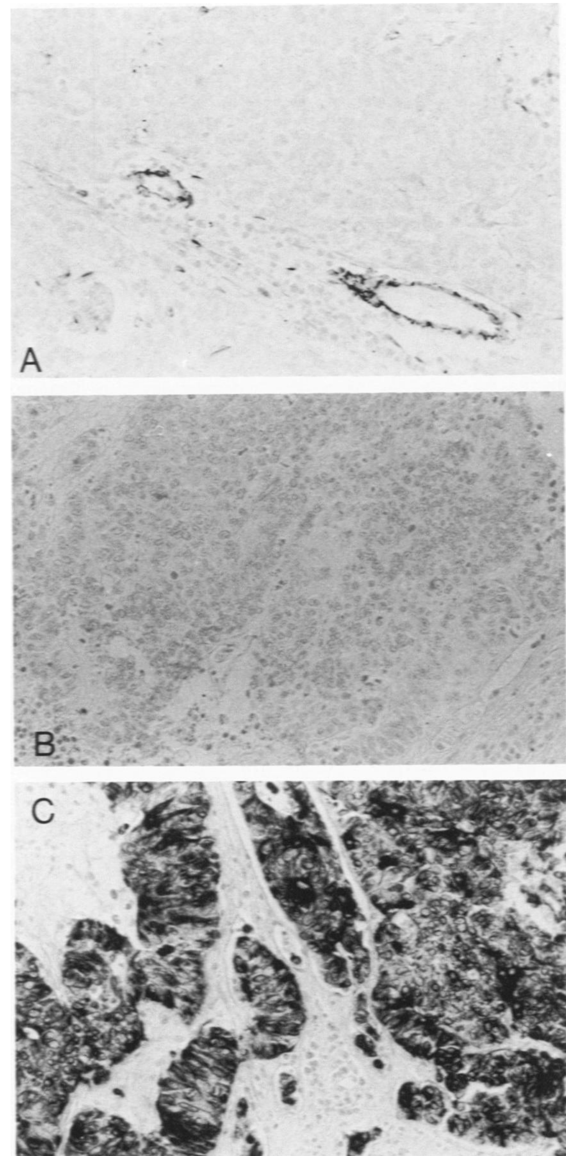

Figure 12 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human moderately well differentiated adenocarcinoma of endometrium. **A** – Anti-vimentin antibody 43βE8. Tumor cells are nonreactive; only stromal and endothelial cells are positive. (×160) **B** – Anti-cytokeratin antibody 34βE12. Tumor cells are nonreactive. (×160) **C** – Anti-cytokeratin antibody 35βH11. Tumor cells are strongly positive. (×160)

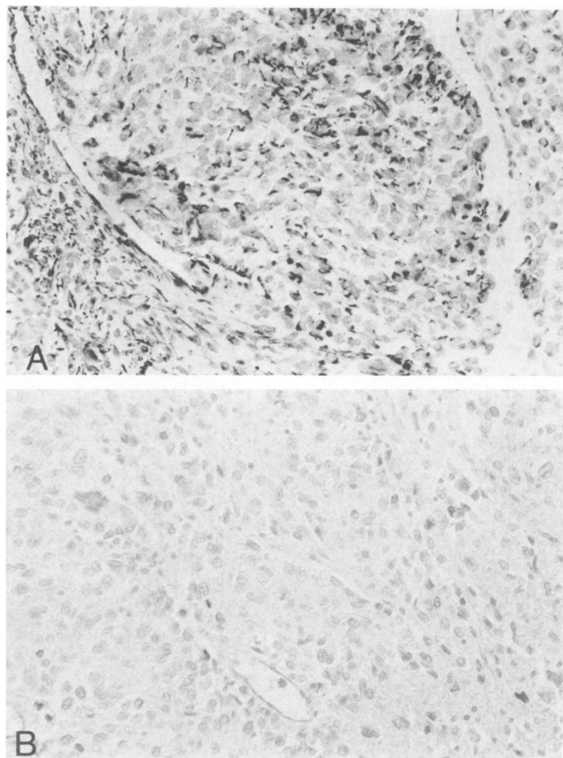

Figure 13 – Biotin-avidin immunoperoxidase staining of Carnoy's-fixed, paraffin-embedded sections of human osteogenic sarcoma. A – Anti-vimentin antibody 43βE8. Tumor cells are positive. (×160) B – Anti-cytokeratin antibody 35βH11. Tumor cells are nonreactive. Tumor cells were also nonreactive with all other anti-cytokeratin monoclonal antibodies.

tumors, do not express epithelial specific cytokeratin molecules as detected by the three cytokeratin antibodies. Similarly, epithelial tumors (carcinomas) display the same pattern of reactivity with the cytokeratin antibodies as the nonneoplastic tissue from which they arose. Therefore, these antibodies can be used to identify specific types of neoplasms.

One case in which these antibodies are useful is in distinguishing carcinomas from lymphomas and sarcomas, because the former react with anti-cytokeratin antibodies 35βH11 or 34βE12, while the latter two do not. Additionally, at least some sarcomas react with the anti-vimentin antibody 43βE8, while epithelial neoplasms do not.

One can use the three anti-cytokeratin antibodies to identify different subtypes of epithelial neoplasms. Antibodies 35βH11 and 34βE12 react differently with squamous epithelium, hepatocytes, pancreatic acinar cells, endometrial glands, and proximal renal tubules and therefore can be used to distinguish tumors arising in these tissues. Squamous carcinomas are positive with 34βE12 and negative with 35βH11. In fact, antibody 35βH11 is positive on all carcinomas except squamous carcinomas (with the exception of a few cervical carcinomas). Antibody 34βB4 can also be used to identify squamous tumors because it reacts with only squamous epithelium. The extent of reactivity depends upon the amount of keratinization present in the tumor, with better differentiated tumors staining more heavily than poorly differentiated squamous tumors.

Another use for these antibodies is in differentiating hepatocellular carcinomas from cholangiocarcinomas, because the former react with only antibody 35βH11 and the latter reacts with both antibodies 35βH11 and 34βE12 (Table 3). One could also differentiate between breast and colon or endometrial carcinomas, because breast lesions react with both antibodies, while colonic and endometrial neoplasms react more strongly with antibody 35βH11. Renal carcinomas also stain with only antibody 35βH11. Therefore, these antibodies may provide useful information with regard to the primary site of metastatic neoplasms.

Puchtler et al[9] noted the superiority of the fixative Methacarn in preserving the cytoskeletal filaments of various cells. Our results further demonstrate the superior preservation of antigenicity by this fixative. We have been successful, in a number of cases, in utilizing some of these antibodies on routine formalin-fixed, paraffin-embedded surgical pathology material with pretreatment of the slides with Pronase (as described in Materials and Methods) for 20 minutes. However, these results have been somewhat unpredictable; Carnoy's or Methacarn-fixed tissue has consistently yielded superior results.

Comparison of Anti-Cytokeratin Antibodies

Approximately 15–20 different keratins have been identified in human tissues,[10] and many antibodies have been generated to these proteins. Anti–cytokeratin antibodies isolated in many laboratories react with squamous epithelium in three unique patterns: basal layer only, suprabasal layers only, and full thickness staining.[11,12] In general, antibodies that recognize lower-molecular-weight keratins localize to the basal portions of the epidermis, whereas antibodies generated against higher-molecular-weight keratins stain the upper portions of the epidermis. Antibodies 34βB4 and 34βE12, described here, exhibit two of the three staining patterns described in the literature: full thickness and suprabasal staining. Both antibodies recognize high-molecular-weight keratins (57 kd and 66 kd). On nonsquamous epithelium, many polyclonal anti-keratin sera stain ductular cells but fail to

stain other types of epithelia.[13,14] Antibody 34βE12 behaves similarly, staining ducts but not hepatocytes, pancreatic acinar cells, or other types of epithelium.

Antibody 35βH11 is unique in that it recognizes only a 54 kd protein restricted to all nonsquamous epithelium. Other antibodies, both polyclonal and monoclonal, identify similar intermediate filament proteins unique to nonsquamous epithelium. Franke et al[13] have described an antiserum to a 55 kd protein in mouse liver that stains hepatocytes and outlines the bile ductules between hepatocytes but does not stain squamous epithelium. This protein appears to be unique to nonsquamous epithelium.[10] Lane[12] has made monoclonal antibodies to cytokeratin from PtK_1 cells that decorate a 45 kd protein in these cells. This antibody also stains nonsquamous epithelium but not squamous epithelium. Finally, Tseng et al[15] have described three monoclonal antibodies to epidermal keratins, some of which recognize proteins of 52, 46, and 40 kd present in nonsquamous epithelium but absent from squamous epithelium. Therefore, it is clear that some cytokeratin proteins are found in only nonsquamous epithelium.

In conclusion, we have described a series of tissue-specific monoclonal antibodies that can distinguish among many different types of tumors. We believe these antibodies will be useful reagents in the practice of surgical pathology.

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