Immunofluorescence Microscopic Evaluation of the Intermediate Filament Expression of the Adrenal Cortex and Medulla and Their Tumors

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Normal adrenal glands (10 specimens) and adrenal gland tumors (58 cases) were immunohistochemically evaluated for different types of intermediate filament (IF) proteins. Some of the normal cortical cells showed cytokeratin positivity, and no positivity was seen for epidermal keratin or other types of IF. In the adrenal medulla, neurofilament positivity was seen in nerve axons, some ganglion cells, and chromaffin cells; and cytokeratin-positive cells could not be detected. Only the vascular and connective tissue elements showed vimentin positivity in both cortical and medullary areas. In half of the cortical carcinomas (13/25), cytokeratin-positive tumor cells were found. Furthermore, vimentin-positive tumor cells were present in 10 of 25 cases, in some of them together with cytokeratin-positive cells. Thus, the results show heterogeneity among the adrenal cortical carcinomas. Interestingly, many benign adrenal cortical tissues and some carcinomas lacked immunoreactivity for all types of IF, suggesting a poorly developed IF system in these tissues. In contrast to adrenal cortical tumors, pheochromocytomas contained neurofilamentlike immunoreactivity. These results reflect the different cellular nature of adrenal cortical and medullary tumors, which apparently can be distinguished from each other with antibodies to intermediate filament proteins. (Am J Pathol 1985, 118: 360–366)

INTERMEDIATE FILAMENTS (IFs), cytoplasmic structural constituents of eukaryotic nucleated cells, are composed of five biochemically and immunologically distinct subunit proteins.[1-3] The IF proteins are expressed in cells under genetic control according to the cellular differentiation. Thus, keratins/cytokeratins form the intermediate filaments of epithelial cells, vimentin of fibroblasts, and related nonmuscular mesenchymal cells, desmin of muscle cells, neurofilament proteins of neural cells, and glial fibrillary acidic protein (GFAP) of glial cells.[1-3] Also, tumor cells are known to express the intermediate filament type found in their putative parental cells.[4-6] Therefore, antibodies to IFs are useful in the characterization of both normal tissues and tumors.[4-6]

In this study, we have evaluated the expression of intermediate filament proteins in normal adrenal glands and adrenal tumors. Cytokeratin appeared to be present in many adrenal cortical cells and cortical tumors, whereas neurofilamentlike immunoreactivity was characteristic of both normal adrenal medullary cells and pheochromocytomas.

Materials and Methods

Tissue Material

Normal adrenal glands were obtained at extrafascial nephrectomies performed because of renal carcinomas (9 cases) or at fresh autopsies (1 case). Two adrenal cortical adenomas, 6 carcinomas, and 3 pheochromocytomas were obtained at frozen sections. Pieces from one additional cortical carcinoma and 2 additional pheochrocytomas were fixed in absolute ethanol and embedded in paraffin.[7] Paraffin blocks from formalin-fixed tissues of 8 adrenal cortical hyperplasias, 3 cortical adenomas, 18 cortical carcinomas, and 15 pheochromocytomas were available from the files of the Department

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of Pathology, University of Helsinki. All the adrenal cortical hyperplasias and adenomas were from patients with Cushing's syndrome. Only three of the 25 adrenal cortical carcinomas had hormonal symptoms (Cushing's syndrome). All pheochromocytomas were preoperatively diagnosed because of their secretion of catecholamines.

The histopathologic diagnoses of the tumors included in this study were based on the morphologic evaluation of paraffin sections stained with hematoxylin and eosin. The pheochromocytomas were often composed of lobulated clusters of cells with clear cytoplasm separated by vascular septa, while in cortical carcinomas, similar compartmentalization was lacking. Cortical carcinomas often showed considerable cellular pleomorphism and giant cells, lacking in pheochromocytomas. Seven cortical carcinomas and five pheochromocytomas were studied by electron microscopy. All pheochromocytomas were found to contain typical granules with a small dense core and a large empty space, resembling catecholamine granules.[8,9] Such granules were absent in cortical carcinomas.

Antibodies

Antibodies to epidermal keratin were raised by immunizing rabbits with keratin polypeptides isolated from human plantar callus as described previously.[10,11] These antibodies react with all squamous and some glandular epithelial cells, but not with hepatocytes, acinar cells of salivary glands and pancreas, or renal tubular cells. The three mouse monoclonal cytokeratin antibodies used in this study have been characterized elsewhere.[6,12,13] These antibodies, raised against cytokeratins purified from a pig renal tubular cell line (LLCK-PK), show different reactivity with various epithelial cells. PKK 1 antibodies chiefly react with simple epithelial cells and stain also acinar cells of salivary glands and pancreas, renal tubular cells, and hepatocytes.[6,12,13] These antibodies react with hair shaft epithelia but not with the interfollicular epidermis. The PKK 2 antibodies react with basal cells of epidermis and myoepithelial cells, but not with acinar cells of salivary glands, hepatocytes, or renal tubular cells. PKK 3 antibodies have an exclusive reactivity to simple epithelial cells and stain acinar cells in salivary gland and pancreas, renal tubular cells, and hepatocytes. The use of the whole set of cytokeratin antibodies was only possible in frozen sections, while PKK 1 antibodies could be applied on the formalin-fixed and paraffin-embedded material also. Rabbit antibodies to vimentin (isolated from human cultured fibroblasts) and mouse monoclonal antibodies to vimentin (isolated from cultured pig kidney epithelial cell line [LLC-PK]) have been previously characterized.[10,11,15] These antibodies react *in situ* with various mesenchymal cells but not with epithelial cells. Two monoclonal antibodies to neurofilaments (antigen purified from bovine spinal cord) have been recently characterized elsewhere.[6] NF 1 antibodies react in western blotting only with the 200-kilodalton subunit protein of human neurofilaments, while NF 2 antibodies react both with the 70-kilodalton and the 200-kildalton subunit proteins of human neurofilaments. These antibodies react with only neural cells. Some tumors, including those which were negative for other types of IFs, were studied with mouse monoclonal antibodies to glial fibrillary acidic protein (antigen isolated from human spinal cord), which had exclusive reactivity with glial cells,[6] and with rabbit antibodies to chicken gizzard desmin, only reacting with muscle cells.[10,16,18] The latter antibodies were a kind gift from Dr. R. A. Badley (Bedford, UK).

Immunostaining

The frozen sections were fixed in methanol (−20 C, 10 minutes), rinsed with phosphate-buffered saline, and used as such for the immunostaining. The paraffin sections from alcohol-fixed material were used for staining after a careful deparaffinization. The paraffin sections from formalin-fixed material were, in addition, exposed to 0.4% pepsin in 0.01 N HCl for 30 minutes at 37 C for enhancement of the antigenic exposure prior to the staining.[17,18] The primary antibodies were used at a concentration of 25–50 μg/ml. After a thorough washing in PBS, the specimens were exposed to fluorescence isothiocyanate (FITC)-coupled goat anti-rabbit

Table 1—Intermediate Filament Expression in the Adrenal Cortex and Medulla and Their Tumors

	Keratin	Cytokeratin	Vimentin	Neurofilaments
Normal cortex	0/10	10/10	0/10	0/10
Cortical hyperplasia	(0/8)	(4/8)	(0/8)	(0/8)
Cortical adenoma	0/2 (0/3)	1/2 (2/3)	0/2 (1/3)	0/2 (0/3)
Cortical carcinoma	0/7 (3/18)	3/7 (10/18)	3/7 (7/18)	0/7 (0/18)
Normal medulla	0/10	0/10	0/10	10/10
Pheochromocytoma	0/5 (0/15)	0/5 (0/15)	0/5 (0/15)	5/5 (5/15)

The presence of vimentin in stromal cells is not included in the table. The figures without parentheses refer to cases studied in frozen sections or alcohol-fixed cases, and the figures in parentheses refer to those studied by the use of formalin-fixed material.

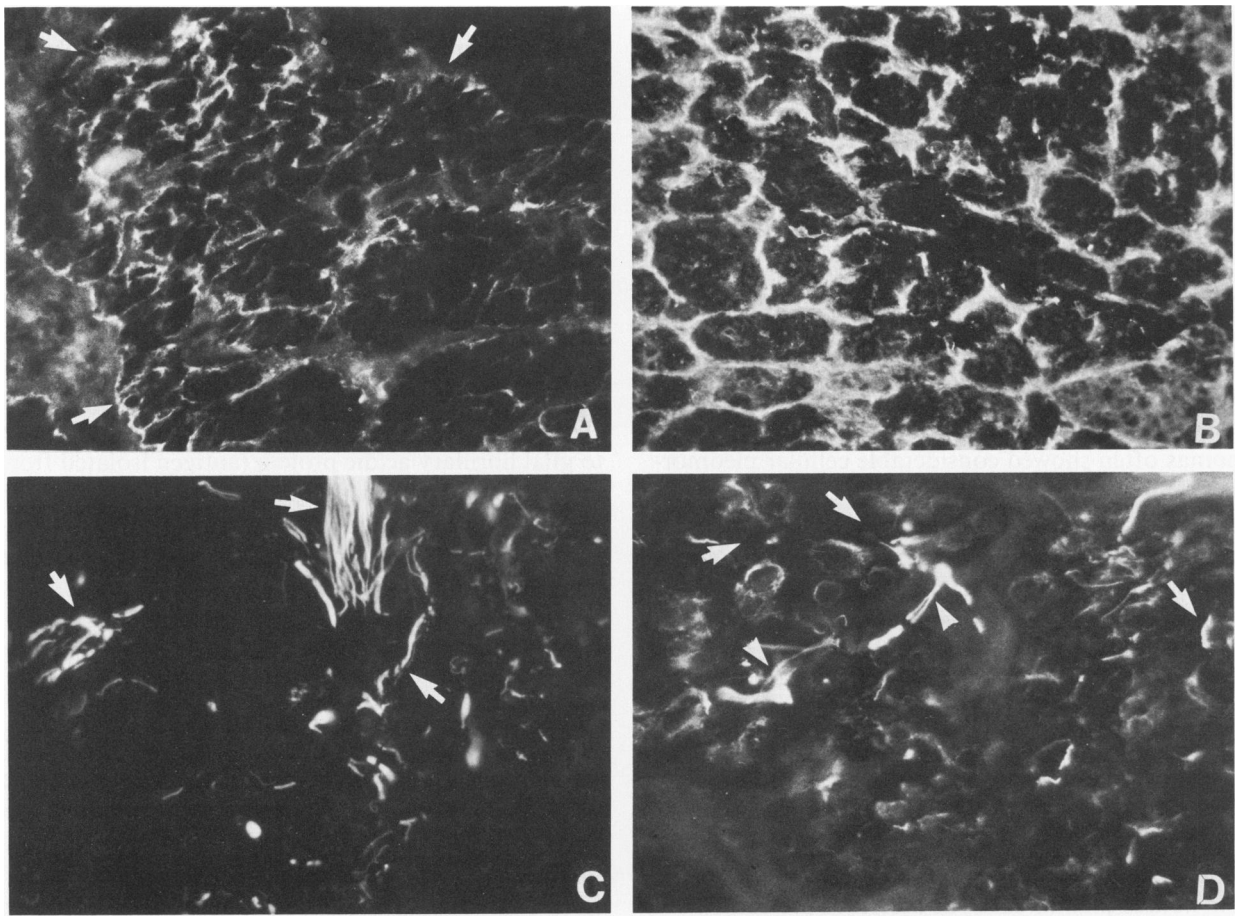

Figure 1—Normal adrenal gland. Many adrenal cortical cells show PKK 1 cytokeratin positivity in the peripheral cytoplasm. The area of positively stained cells is limited by *arrows* (**A**). Antibodies to vimentin label only the septal cells (**B**). NF 1 antibodies to neurofilaments preferentially stain the axons, marked by *arrows* (**C**), while NF 2 antibodies stain both axons (*arrowheads*) and many chromaffin cells (*arrows*) (**D**). (**A**, **C**, and **D**, ×500; **B**, ×200)

immunoglobulin G (IgG, 1:40, Cappel Laboratories, Cochranville, Pa) or to FITC-coupled goat anti-mouse IgG (1:40, Cappel Laboratories). Some cortical carcinomas were studied by the double immunofluorescence technique by the use of rabbit antibodies to vimentin as described above, followed by monoclonal antibodies to cytokeratin and tetramethylrhodamine isothiocyanate (TRITC)-coupled goat anti-mouse IgG (Cappel Laboratories). The slides were mounted in veronal-buffered glycerol (1:1, pH 8.4) and viewed in a Zeiss Universal microscope equipped with filters for FITC and TRITC fluorescence.

Results

The results are summarized in Table 1.

Normal Adrenal Glands

Frozen sections of normal adrenal glands revealed positive cortical cells only with PKK 1 and PKK 3 cytokeratin antibodies. The cytokeratin positivity was often found in parallel trabeculas extending from the outer cortex to the border of medulla, while a large number of cortical cells were negative. The cytokeratin positivity was characteristically observed as a thin peripheral cytoplasmic rim (Figure 1A). The positivity was not clearly restricted to any of the functional zones in the adrenal cortex. Both antibodies to vimentin labeled stromal and vascular endothelial cells but not cortical or medullary cells (Figure 1B). NF 1 monoclonal neurofilament antibodies mainly labeled axons in the medulla (Figure 1C), while the NF 2 antibodies labeled both axons and some ganglion cells and chromaffin cells in the medulla (Figure 1D). No cortical cells were positive for neurofilaments. No GFAP positivity was found in the adrenal gland.

Adrenal Gland Hyperplasia and Adenoma

About half of the cases showed scattered cytokeratin-positive cells, but most cells were negative. The tumor

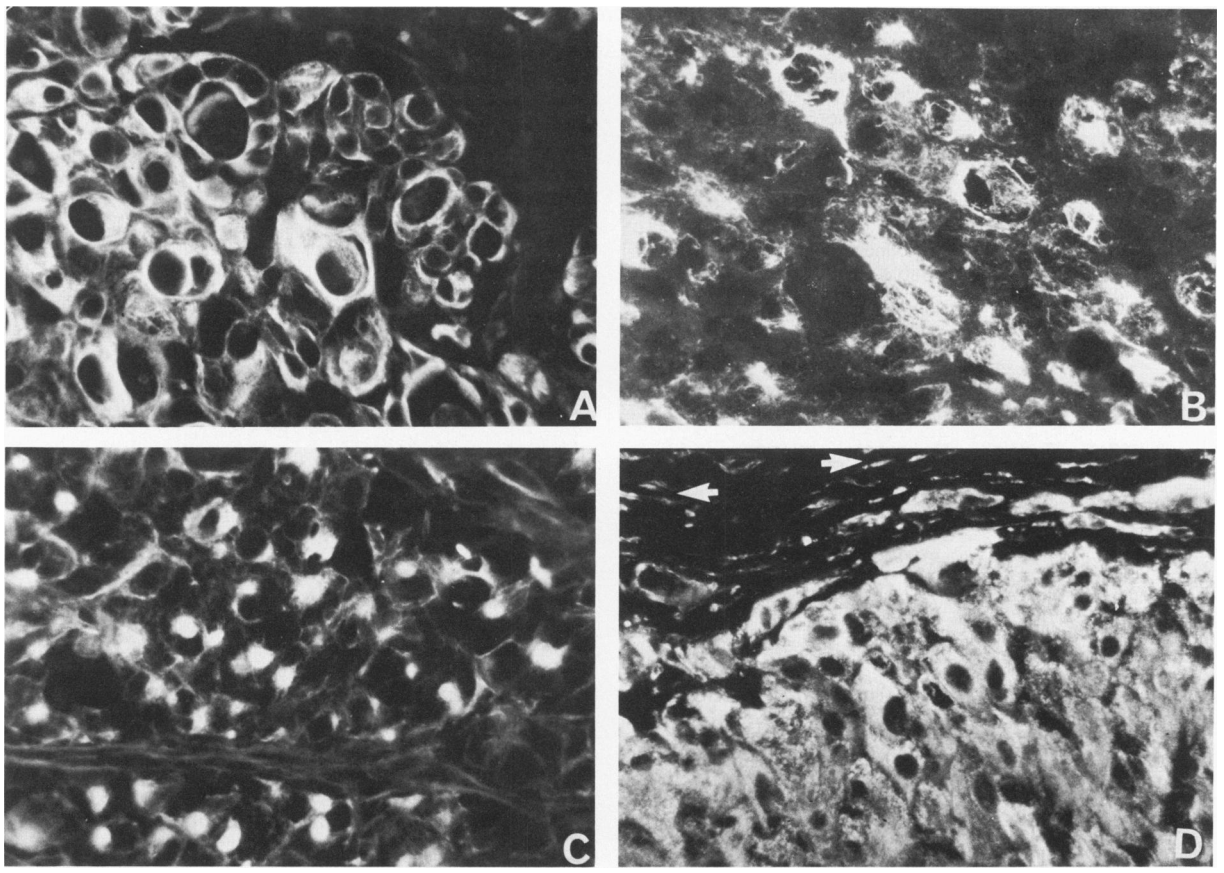

Figure 2—Adrenal cortical carcinomas show varying reactivities with PKK 1 cytokeratin antibodies: in some cases, all cells show diffuse cytoplasmic positivity (**A**), while some cases show fibrillary cytoplasmic fluorescence in scattered cells (**B**). Inclusionlike cytoplasmic collections of cytokeratin are sometimes observed in adrenal cortical carcinomas (**C**). Most tumor cells are vimentin-positive in some cortical carcinomas. Note also the vimentin positivity of the stromal cells (*arrows*) (**D**). (**A–D**, ×500)

cells were negative for epidermal keratin, vimentin, and neurofilaments in all cases. No desmin or GFAP could be detected in the tumor cells lacking cytokeratin.

Adrenal Cortical Carcinoma

Cytokeratin-positive tumor cells could be detected in 13 of 25 cases with PKK 1 cytokeratin antibodies. The number of cytokeratin-positive cells varied in adrenal cortical carcinomas. Rarely, most cells were cytokeratin positive (Figure 2A), whereas, in many tumors, only scattered positive cells presenting fibrillar cytoplasmic (Figure 2B) or tuftlike bright positivity (Figure 2C) were found. The cytokeratin positivity, often confined to a minority of tumor cells, was also evaluated by immunoperoxidase staining (Vectastain) in three cases. Epidermal keratin antibodies revealed positive cells only in 3 of 25 cases. Immunostaining with antibodies to vimentin revealed positive neoplastic cells in 10/25 cases, and vimentin was found as the only type of IF in 4 tumors. In double immunofluorescence stainings, the vimentin-positive cells appeared to lack cytokeratin. Minor numbers of vimentin-positive cells were observed in 8 additional cases, and such cells were often smaller than the tumor cells, possibly representing infiltrating macrophages. Neurofilament-positive cells could not be observed in cortical carcinomas. All types of IFs, including desmin and GFAP, were lacking in 5/25 adrenal cortical carcinomas studied. The results on the seven cortical carcinomas studied at frozen sections are presented in detail in Table 2. In these cases, the whole set of cytokeratin antibodies could be used.

Pheochromocytoma

Both frozen sections (n=3) and alcohol-fixed specimens (n=2) showed neurofilament-positive tumor cells with NF 1 and NF 2 antibodies, and cytokeratin-positive tumor cells could not be seen (Figure 3). Typically, a larger number of positive cells were observed with the NF 2 antibodies than with the NF 1 antibodies. In paraffin sections from formalin-fixed specimens, neurofilament-positive tumor cells could be detected in 5 of 15 cases (as tested with the NF 1 antibodies). Vi-

Table 2—Detailed Presentation of the Intermediate Filament Expression in the 7 Adrenocortical Carcinomas Studied in Frozen Sections or Alcohol-Fixed Material

Case	Age	Sex	Hormonal disorders and tumor size	Keratin	Cytokeratin			Vimentin	Neurofilaments
					PKK 2	PKK 1	PKK 3		
1	35	Female	No hormonal symptoms	–	–	–	–	+ +	–
2	69	Female	Cushing's syndrome Hypercorticolism, 200 g	–	–	–	–	+ +	–
3	26	Male	No hormonal symptoms, 750 g	–	–	–	–	–	–
4	38	Female	No hormonal symptoms, 10×5×5 cm (local recurrence)	–	+ +	+ +	+ +	–	–
5	18	Female	No hormonal symptoms, 1100 g	–	–	+	+	+	–
6	62	Female	No hormonal symptoms, 950 g	–	–	–	–	–	–
7	71	Female	No hormonal symptoms, 700 g	–	–	+	+	–	–

+, low number of positive cells; + +, high number of positive cells.

mentin-positivity could not be found in pheochromocytoma cells. However, some isolated vimentin-positive cells occurred near the septa of the tumor-cell nests.

Discussion

In this study we evaluated the expression of IF proteins in the adrenal gland and its tumors. The results show distinct differences in the expression of IF proteins in the adrenal cortical and medullary tissues and their tumors. Thus, the normal cortex and many cortical tumors showed cytokeratin positivity, while the medulla and pheochromocytomas lacked cytokeratin and showed neurofilament positivity. In addition, many adrenocortical carcinomas showed vimentin-positive tumor cells.

Cytokeratins are a chemically and immunologically heterogeneous group currently subdivided to 19 polypeptides, which are differentially localized in various epithelial cells.[19,20] Some of the cytokeratins are expressed preferentially in the epidermis and other squamous epithelia, some are widely distributed, and some occur mainly in simple epithelia.[20] The heterogeneity of epithelia with respect to their cytokeratin expression is reflected in the results of immunostaining with different types of cytokeratin antibodies.

Our results show that adrenal cortical cells contain cytokeratins present only in simple epithelia.[6,20–23] The cytokeratin content demonstrates the true epithelial character of adrenal cortical cells, in line with the recent results of van Muijen et al,[24] obtained with monoclonal cytokeratin antibodies.

The adrenal cortical carcinomas also showed cytokeratin-positive cells in half of the cases. Also, the presence of vimentin in many adrenal cortical carcinomas indicates the acquisition of a new intermediate filament type upon the malignant transformation. A similar expression of both cytokeratin and vimentin is known in mixed tumors of salivary glands[25,26] and has recently been described for some renal[12,27] and thyroid[28] carcinomas, too. In their IF expression the adrenal cortical carcinomas show heterogeneity, which, however, appeared to be difficult to correlate with the histologic patterns of these tumors.

Many adrenal cortical cells, as observed in both normal and neoplastic tissues, did not react with any antibodies to cytokeratin or other IFs. It is possible that these cells contain cytokeratin in such low amounts that it is undetectable by the immunofluorescence technique. However, immunoperoxidase studies also revealed cytokeratin positivity in a restricted number of tumor cells in the cortical carcinomas studied by this technique. The possibility that normal and neoplastic adrenal cortical cells contain a cytokeratin type not detectable with the antibodies used might also be considered. This seems improbable, however, because of the very comprehensive reactivity of the combination of epidermal keratin and cytokeratin antibodies in various epithelial cells and organs.[6,11,12,28] Finally, it is also possible that many adrenal cortical cells and corresponding tumor cells totally lack IFs. Cells lacking IF are known, for example, among ganglion cells of the nervous system.[4] Recently, the lack of IFs was also shown by Nadakavukaren et al[29] in a cultured adrenocortical carcinoma cell line.

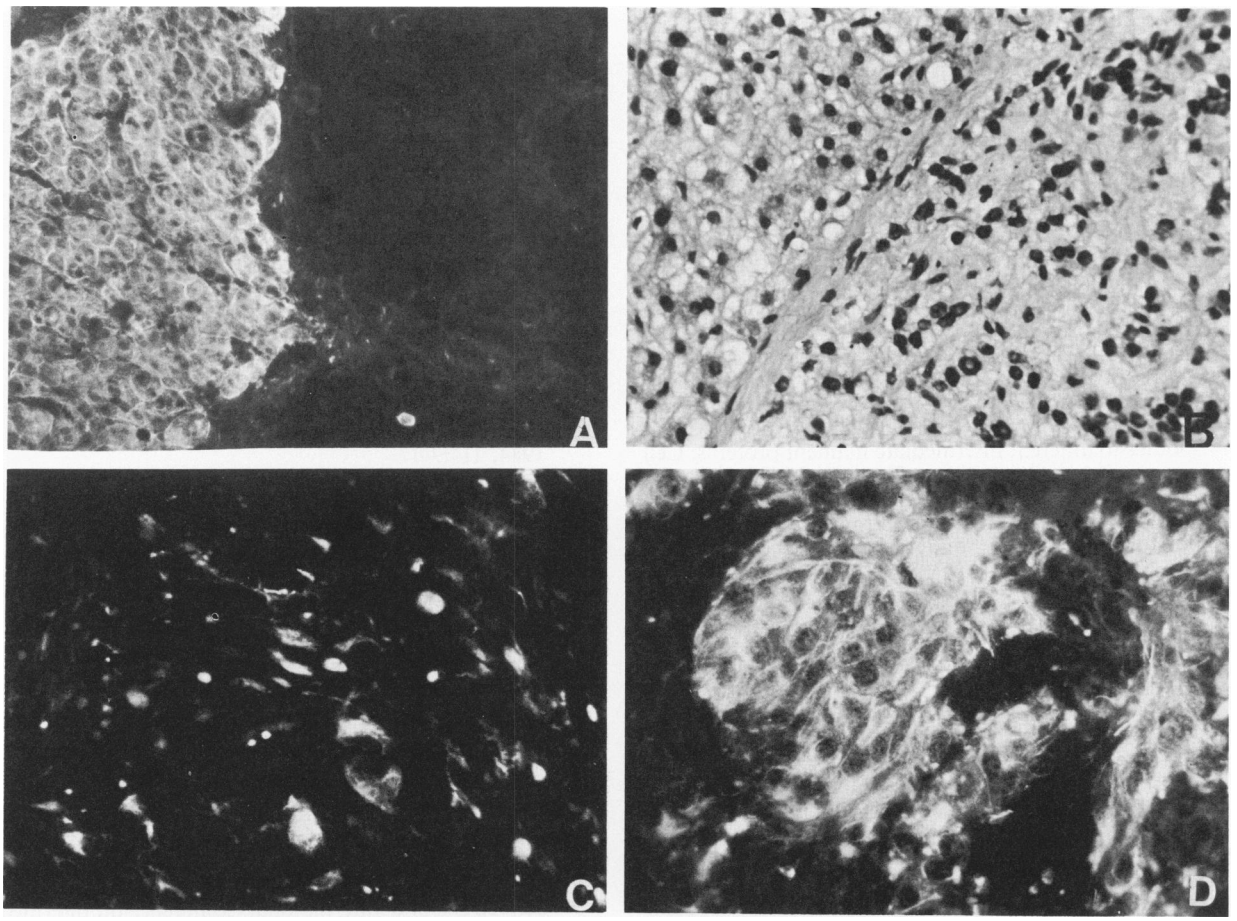

Figure 3—A pheochromocytoma metastatic to liver is cytokeratin-negative, in contrast to the hepatocytes. (**A**). A section stained with hematoxylin and eosin from a similar area shows hepatocytes to the left and clusters of tumor cells to the right (**B**). NF 1 neurofilament antibodies react with some tumor cells in the cellular lobules (**C**), while NF 2 antibodies stain the majority of the tumor cells (**D**). (**A–D**, ×500)

Adrenal medullary cells contained only neurofilamentlike immunoreactivity in contrast to the adrenal cortical tissue. This is in line with the neural nature of adrenal medullary tissue, composed of ganglion cells, chromaffin cells, and nerve axons.[30] Adrenal medullary tissues have also been reported to contain cytokeratin, as assessed with monoclonal antibodies, but this result may be due to difficulties in proper definition of the medullary and cortical tissues.[24] In line with the expression of only neurofilaments in adrenal medulla, pheochromocytomas contained neurofilament positivity.[31–33] We found more neurofilament-positive cells in pheochromocytomas with antibodies recognizing both the 70-kilodalton and the 200-kilodalton neurofilament (NF) subunit proteins than with the antibodies only reacting with the 200-kilodalton NF protein. This finding is similar to the recent report by Lee and Page[34] showing more 70-kilodalton than other NF proteins in rat pheochromocytoma cells.

The content of cytokeratin or vimentin in cortical tumors versus neurofilaments in pheochromocytomas can be used as a diagnostic aid in surgical pathology. The differential diagnosis between cortical carcinomas and pheochromocytomas can sometimes be difficult[35,36]; in fact, 2 of our cortical carcinomas with cytokeratin positivity had originally been thought malignant pheochromocytomas.

In conclusion, adrenal cortical tissues and their tumors have epithelial characteristics because they contain cytokeratin, while adrenal medullary tissues and their tumors have neural chracteristics because of their containing neurofilaments. Some adrenocortical cells and their tumors appear to lack all types of IFs, in contrast to most other cells and tissues.

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