THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXVIII APRIL, 1961 NUMBER 4

ELECTRON MICROSCOPIC STUDIES OF NORMAL AND PROLIFERATED BILE DUCTULES

FENTON SCHAFFNER, M.D., AND HANS POPPER, M.D.

From the Department of Pathology, The Mount Sinai Hospital, New York, N.Y.

Tubules lined by flat or cuboidal epithelium connect bile canaliculi between the liver cells with the smallest bile ducts in the portal tracts. They lie partly in the portal tract and partly in the parenchyma, mainly in its periportal zone, and have been designated as cholangioles, canals of Hering, ampullas or intermediate pieces. The name "bile ductule" seems to be preferable [1] to differentiate it from the smallest interlobular duct accompanied by terminal branches of portal vein and hepatic artery. Under abnormal circumstances in both human beings and experimental animals, these ductules exhibit excessive proliferation, appear in a variety of shapes, and the cells may be quite undifferentiated ("oval cells").[2] The proliferation is usually associated with the accumulation of inflammatory cells, and the combined lesion has been designated as "ductular cell reaction." [3] The derivation of normal and particularly of proliferated ductules has not been established. They have been assumed by some to originate from liver cells, and by others to be derived from pre-existing ducts or ductules. Connections between ductules and liver cell plates have been demonstrated repeatedly by injection methods.[4] Histochemical reactions,[5,6] including staining for various phosphatases,[7] have shown them to differ from liver cells. Since interpretations have been controversial, we have utilized electron microscopy in order to investigate the ductules in man and in representative examples of experimental hepatic injury in animals (a) to determine the basic cytology of these structures

Supported by Research Grant A-3846 Path. from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service, and by the United States Army Medical Research and Development Command under Contract DA-49-007-MD-790.

Presented in part at the Annual Meeting of the American Society of Experimental Pathology, Chicago, April 13, 1960.

Accepted for publication, November 28, 1960.

which might permit conclusions as to their function; (b) to compare their appearance with that of neighboring liver cells and mesenchymal cells; and (c) to describe their relation to the surrounding stroma.

Material and Methods

Liver sections containing ductules were chosen for this study from collected specimens prepared for electron microscopy. The human tissues were from cases of acute viral hepatitis (2 cases), subacute hepatitis (1 case), postnecrotic cirrhosis (2 cases), primary biliary cirrhosis (2 cases), Wilson's disease (1 case), idiopathic hemochromatosis (1 case), nonspecific portal inflammation (2 cases), extrahepatic biliary obstruction (2 cases), and chlorpropamide-induced cholestasis (1 case). The animal specimens came from 2 normal rats and 1 normal dog, 1 rat fed a low choline-low protein-high fat diet [8] for 3 months, 4 rats fed a diet containing 0.5 per cent ethionine [9] for 1 to 2 months, and 1 fed a diet with 5,000 ppm. of Aramite [10] for 1 month.

Liver tissue was obtained with a Menghini biopsy needle [11] from both patients and animals and immediately fixed in a 1.5 per cent aqueous osmium tetroxide solution adjusted to pH 7.4 with sodium hydroxide. In animals this was done at a laparotomy preceding sacrifice. Tissue was dehydrated and embedded in a mixture of methyl and n-butyl methacrylate and sectioned with the Porter-Blum microtome. After mounting on copper grids, some of the sections were floated face down for 20 minutes on a 1 per cent solution of phosphotungstic acid for staining collagen fibers.[12] The sections were examined and photographed with a Philips EM 100 electron microscope. Thicker sections were examined with the phase contrast microscope or with the light microscope after staining with chromotrope aniline blue. Paraffin sections from the same biopsy specimens were examined by conventional methods.

Observations

Normal Ductules

Since ductules were not seen in the small electron microscopic specimens of normal human liver, the normal ductules in the rat and dog are described. In cross section, with the light microscope, the ductule appeared as an acinar structure lined by cuboidal epithelium. By electron microscopy it consisted of 3 to 10 cells, each about 10 μ in diameter, arranged around a 1 to 3 μ lumen (Figs. 1, upper, and 2, upper left). The ductular cell border directed toward the lumen exhibited 5 to 30 projecting microvilli; these measured 0.3 μ in length and 0.1 μ in thickness. They were somewhat shorter than the canalicular microvilli of liver cells but were of similar thickness. Fine filaments appeared to traverse the length of the microvillus, but no definite central canal was seen. The filaments appeared to connect with an irregular cytoplasmic network which was much finer than the endoplasmic reticulum and was well recognized only in optimal specimens (Fig. 3). Occasional cells also showed bleb formation replacing microvilli along part of the lumen surface (Fig. 2, upper left). The nucleus was round to oval, about 5 μ in diameter, and occupied the central half of the cell. Nuclear chromatin appeared homogeneous, and a small and irregular nucleolus was usually eccentric.

Within the cytoplasm, 5 to 10 small mitochondria appeared in cross section. These were considerably smaller than the mitochondria in parenchymal liver cells and had few cristae. Ergastoplasm was scant, and ribonucleoprotein or Palade granules appeared as a few scattered clusters not necessarily near endoplasmic reticulum. Between adjacent cells radiating from the lumen of the ductule, the borders were straight. Canaliculi draining near-by liver cells occasionally extended between two ductular cells (Fig. 2, upper right). The outer border of the ductule was gently curved and devoid of microvilli. It was separated by a thin light zone about 500 Å in width from an electron-dense homogeneous layer of about equal width. This was enveloped by another less distinct and narrower light zone. The dense layer was continuous around the entire ductular structure; it resembled a basement membrane and, on occasion, was duplicated (Fig. 2, lower half). External to the outer light zone of the membrane, small bundles of collagenous fibrils were occasionally seen; similar fibrils were not found inside the basement membrane. Adjacent to the ductule a lymphatic space, one or two small blood vessels and a few mesenchymal cells were manifest.

In the patient with Wilson's disease, a normal intralobular ductule was encountered. This exhibited microvilli projecting into the lumen (Fig. 4). A few mitochondria were noted in each cell, and one cell contained a fat droplet. A few fibrils lay outside the basement membrane.

Ductular Proliferation Without Inflammation or Fibrosis

The fatty liver of the rat on a low protein-high fat diet exhibited many ductules. The cells in these were smaller and flatter than in the normal rat. The lumens were more frequently cut tangentially or even longitudinally, suggesting that they were no longer cylindrical but were rather flattened or irregular. Increased numbers of microvilli projected into the lumens although they were the same size and shape as in the normal liver. The nuclei were oval, with the long axis parallel to the lumen; nucleoli were small. Mitochondria were sparse, but ergastoplasm seemed to be increased in amount. Neither fibrils nor mesenchymal cells were in proximity to the thin basement membrane. The ductule usually lay in the space of Disse, which extended between sinusoidal endothelium and the liver cell margins. No comparable ductules were seen in the human tissues.

Ductular Proliferation with Periductular Inflammation of Short Duration

In rats receiving ethionine or Aramite, ductular proliferation began within 2 to 3 weeks. These ductules were larger than normal and occa-

sionally had larger though rounded lumens (Fig. 1, lower). The cells varied in size and shape, but microvilli were present. Occasionally the entire lumen surface of a cell formed a bleb. The nuclei were often irregularly shaped. Some mitochondria contained increased numbers of cristae. The basement membrane was unaltered but was surrounded by a continuous layer of intertwined fibrils and many parenchymal cells. The periodicity of the fibrils was that of collagen. Some mesenchymal cells were large and contained phagosomes and lipofuscin granules. The relations to lymphatics and blood vessels were preserved. Neither fibrils nor mesenchymal cells lay within the space encircled by the basement membrane.

In acute viral hepatitis somewhat similar ductules were found although with a greater amount of periductular fibrosis. In some instances surface bleb formation was more prominent. Focally, the basement membrane was separated from the ductular cell. Since the surrounding structures were intact, this empty space presumably was not artifact but probably represented edema. In several patients small black globules, probably lipid, lay in the ductular cell cytoplasm. In addition, vacuolated or dense granular bodies similar to the lipofuscin pigment granules in liver cells were seen. Reticulated and osmiophilic areas were noted in some ductular cells in jaundiced patients. These resembled bile imbibition in parenchymal cells.

Long Standing Ductular Proliferation

Many normal-sized ductules were found in rats given ethionine for two months or longer. The lumens were small and round or elongated; microvilli were normal. The nuclei were quite irregular and occasionally eccentric. The cytoplasm was generally disrupted and occasionally contained finely granular electron-dense deposits. These resembled ferritin [13] although they were not clearly contained within organelles. The basement membrane often was not a sharp thin line but a smudgy band measuring up to 0.2 μ in thickness. Surrounding the ductule were many mesenchymal cells and a tangled mass of fibers in large bundles. Lymphatic spaces were not discerned, and no near-by blood vessels were found. Mesenchymal cells were smaller than ductular cells and had irregular nuclei with scant cytoplasm. Fine filaments lacking periodicity and not impregnated by phosphotungstic acid occasionally extended from the cytoplasm through the cell wall and were intermixed with collagen fibrils outside the cell. These cells had the characteristic crenated nuclei of Kupffer cells, but the cytoplasm differed by the absence of phagocytized content. Fibroblasts were not seen.

Similar ductules were seen in livers with postnecrotic cirrhosis, hemochromatosis, nonspecific portal inflammation and chronic cholangitis secondary to common duct stone (Fig. 5). These also had small, round lumens and were surrounded by thickened basement membranes, fiber bundles and inflammatory cells. Lymphatics or blood vessels were at a distance. The nuclei often varied considerably in size, shape and density in the same ductule. Nucleoli were larger than in rats, and occasionally two appeared in a single nucleus. In hemochromatosis, ferritin[13] was present in the ductular cell cytoplasm. Only in this condition were the ductular cell nucleoli prominent.

Canalicular-Ductular Junction

In one patient with inactive postnecrotic cirrhosis, two ductular cells lay in close apposition to two parenchymal cells to which they bore no similarity (Fig. 6). The cell borders between ductular and parenchymal cells were straight, and neither a space nor a basement membrane separated them. A lumen containing microvilli was evident between the two ductular cells and another between the two ductular cells and one of the parenchymal cells. The cytoplasm of the parenchymal cell had the usual abundant and orderly profiles of endoplasmic reticulum and showed numerous Palade granules as well as many normal mitochondria containing fairly dense material. The ductular cells appeared empty; they had little endoplasmic reticulum and few small mitochondria with scant content. Ductular cells were separated from the tissue space by a thin basement membrane which extended over the surface of the two liver cells for a distance of 10 to 20 μ and then ended in a frayed fashion (Fig. 7). The extension of the basement membrane seemed to serve as an anchor holding the ductule in place. Microvilli, normally present on the sinusoidal surface of the parenchymal cell, could be seen under the basement membrane.

Bleb Formation in the Lumen Membrane of the Ductular Epithelium

In some instances with more rapid ductular proliferation (a rat with Aramite intoxication, a 10-year-old child with very active postnecrotic cirrhosis, and an infant with severe subacute hepatitis and cholangiolitis) numerous blebs projected into the ductule lumen (Fig. 8). The blebs usually involved the entire lumen surface of an affected cell. The cell membrane appeared lifted up, the bleb containing cell sap and a few Palade granules. Organelles were not seen. When bleb formation was extensive, blebs were also encountered in the parenchymal cell canalicular lumens.

Discussion

The electron microscope shows the ductular cell to have a far less elaborate cytoplasmic structure than the parenchymal cell, indicating a less complex metabolic function. The paucity and smallness of mitochondria suggest little energy production, and the sparsity of endoplasmic reticulum reflects minimal biosynthesis.[14] Despite great individual variations in the same ductule under abnormal circumstances, the appearance of the cells differs from that of liver cells. Intermediate forms between the two types of cells were not seen although ductules were in process of excessive proliferation. These findings do not support the concept that ductular cells proliferating in postnatal life originate from liver cells by modulation.[15] Rather, the viewpoint that they are derived from pre-existing ductular cells is favored.[16] This does not negate the possibility that during embryonal development ductular cells may develop from liver cells.[17] The most distinctive feature of the ductule is the basement membrane; this is lacking about liver cell plates except where the membrane seems to serve as an anchor at the point of ductule origin. Even when surrounded by excessive fiber deposit, liver cells do not have a basement membrane although the existence of such a membrane is suggested in conventional sections stained with the periodic acid-Schiff stain.[18] An apparently continuous membrane impregnated with silver stain, as judged by very thin sections examined by both light and electron microscopy, results from overlap of individual collagen fibers.

The structural features common to both ductular and liver epithelium are the microvilli, which extend from the surfaces exposed to bile flow into the lumens of ductules and those of hepatic cell canaliculi. The basal surfaces of ductular epithelium are straight and rest on basement membranes while the sinusoidal surfaces of liver cells are thrown into irregularly projecting microvilli which extend into the pericellular tissue spaces and occasionally into the sinusoidal blood stream. Since liver cells secrete bile and ductule cells apparently do not, microvilli probably do not reflect secretion of bile but represent a function common to both cells, presumably secretion or reabsorption of water. While the appearance of the basal surface of ductular epithelium indicates considerably less exchange activity than the sinusoidal surface of liver cells, water transport is suggested by the close proximity to lymphatic spaces and blood vessels. Several reported observations imply the secretion of fluid by ductular epithelium. Secretin increases the flow of a bile containing bicarbonate but one poor in bile salts. If biliary content exemplified by bile salts is formed in liver cells, bicarbonate-containing fluid may be

secreted by the ductular epithelium.[19,20] Moreover, in the face of ductular proliferation, as in subacute ethionine intoxication, bile flow is increased but the bile salt concentration is low, thus supporting the view that the ductule secretes water.[21] Finally, in other intoxications, water, bilirubin and dye excretion are affected in different fashions.[22,23] A role in bile secretion has been ascribed to the ductules. Bleb formation has been related to apocrine secretion, and this has also been seen in liver cell bile canaliculi.[24] However, the significance of these blebs requires further investigation. Evidence for reabsorption by ductules can also be cited. That the gallbladder can resorb water has long been known. In intrahepatic cholestasis and in hamartomas (Meyenberg complexes), inspissation of bile in canaliculi and ductules bespeaks water removal. Ductules may therefore determine the final water and electrolyte content of hepatic bile.

With light microscopy it may be difficult to recognize ductular epithelium. In experimental animals, particularly, the cells may appear flattened and resemble fibroblasts or appear oval or clustered like mesenchymal cells. However, by electron microscopy their true nature is readily revealed by the demonstration of microvilli and basement membranes. The close proximity of inflammatory cells and fibers to proliferating ductules comprised of irregular epithelial cells ("ductular cell reaction") suggests a causal interrelationship. It may be claimed that portal inflammation leads to ductular proliferation. On the other hand, it is likely that under abnormal circumstances a factor which provokes growth of bile ductules may be excreted into the bile, possibly by damaged parenchymal cells.[25] If this escapes into the surrounding stroma, possibly as part of a fluid interchange mechanism, it may prove to be an irritant, thus accounting for the inflammation demonstrated.

Summary

Intrahepatic biliary ductules in man and experimental animals were investigated in normal and abnormal livers by electron microscopy. The ductular cells varied greatly, particularly under abnormal circumstances, and differed from liver cells in the appearance of the cell organelles and the presence of basement membranes. Cells intermediate between ductular and parenchymal elements were not encountered. Thus, there was no evidence that the former were derived from the latter in postnatal life. The occurrence of microvilli in the lumens of liver cell canaliculi and of ductules also indicates a common function, presumably the regulation of water content of bile.

REFERENCES

1. POPPER, H., and SCHAFFNER, F. Liver: Structure and Function. Blakiston Div., McGraw-Hill Book Co., Inc., New York, 1957, p. 103.
2. FARBER, E. Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene and 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res.*, 1956, **16**, 142–148.
3. POPPER, H.; KENT, G., and STEIN, R. Ductular cell reaction in the liver in hepatic injury. *J. Mt. Sinai Hosp.*, 1957, **24**, 551–556.
4. HERXHEIMER, G., and THÖLLDTE, M. Regeneration und Hypertrophie (Hyperplasie) der Leber. In: Handbuch der speziellen pathologischen Anatomie und Histologie. HENKE, F., and LUBARSCH, O. (eds.). J. Springer, Berlin, 1930, Vol. 5, Verdauungsdrüsen, Pt. 1, Leber, Chapt. 11, pp. 988–1037.
5. MELNICK, P. J. Polycystic liver; analysis of seventy cases. *A.M.A. Arch. Path.*, 1955, **59**, 162–172.
6. ROSENHOLTZ, M. Use of the histochemical demonstration of aminopeptidase to distinguish small bile ducts and hepatic parenchymal cells. (Abstract) *Gastroenterology*, 1960, **38**, 794.
7. WACHSTEIN, M. Enzymatic histochemistry of the liver. *Gastroenterology*, 1959, **37**, 525–537.
8. HOFFBAUER, F. W. Fatty cirrhosis in the rat. I. A method of grading specimens. *A.M.A. Arch. Path.*, 1959, **68**, 160–170.
9. SINGER, E. J.; HUTTERER, F.; KENT, G.; ZAK, F. G., and POPPER, H. Hepatic fibrosis; chemical and histologic studies during subacute ethionine intoxication. *A.M.A. Arch. Path.*, 1959, **68**, 103–112.
10. POPPER, H.; and STERNBERG, S. S.; OSER, B. L., and OSER, M. The carcinogenic effect of Aramite in rats; a study of hepatic nodules. *Cancer*, 1960, **13**, 1035–1046.
11. PARETS, A. D.; SCHAFFNER, F., and BLACK, H. Percutaneous liver biopsy with the Menghini needle. *Am. J. Digest. Dis.*, 1959, N.S. **4**, 693–699.
12. WATSON, M. L. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. & Biochem. Cytol.*, 1958, **4**, 475–478.
13. RICHTER, G. W. A study of hemosiderosis with the aid of electron microscopy; with observations on the relationship between hemosiderin and ferritin. *J. Exper. Med.*, 1957, **106**, 203–218.
14. NOVIKOFF, A. B., and ESSNER, E. The liver cell; some new approaches to its study. *Am. J. Med.*, 1960, **29**, 102–131.
15. LEDUC, E. H. Cell modulation in liver pathology. *J. Histochem.*, 1959, **7**, 253–255.
16. UNGAR, H., and GOLDBERG, M. G. The nature of bile duct proliferations in the liver following ingestion of DL-ethionine. Lab. Invest., 1959, **8**, 1523–1534.
17. HORSTMANN, E. Entwicklung und Entwicklungsbedingungen des intrahepatischen Gallengangsystems. *Arch. f. Entwicklngsmech. d. Organ.*, 1939, **139**, 363–392.
18. POPPER, H.; PARONETTO, F., and BARKA, T. PAS-positive structures of nonglycogenic character in normal and abnormal liver. *Arch. Path.*, 1960, **70**, 300–313.
19. SPERBER, I. Secretion of organic anions in the formation of urine and bile. *Pharmacol. Rev.*, 1959, **11**, 109–134.
20. WHEELER, H. O.; RAMOS, O. L., and WHITLOCK, R. T. Electrolyte excretion in bile. *Circulation*, 1960, **21**, 988–996.

21. POPPER, H.; SCHAFFNER, F.; HUTTERER, F.; PARONETTO, F., and BARKA, T. Parenchymal fibrogenesis: the liver. *Ann. New York Acad. Sc.*, 1960, **86**, 1075–1088.

22. ANDREWS, W. H. H. Excretory function of the liver; a re-assessment. *Lancet*, 1955, **2**, 166–169.

23. VANLERENBERGHE, J.; MILBLED, G., and GUERRIN, F. Étude du mécanisme sécrétoire de la cholérèse. *Path. et Biol.*, 1960, **8**, 1231–1234.

24. KITANI, T. Morphologic Studies on Cholestasis. Pamphlet of the Department of Internal Medicine, Osaka University Medical School, Japan; distributed at presentation at the first meeting of the International Association for the Study of the Liver, London, April 25–26, 1960.

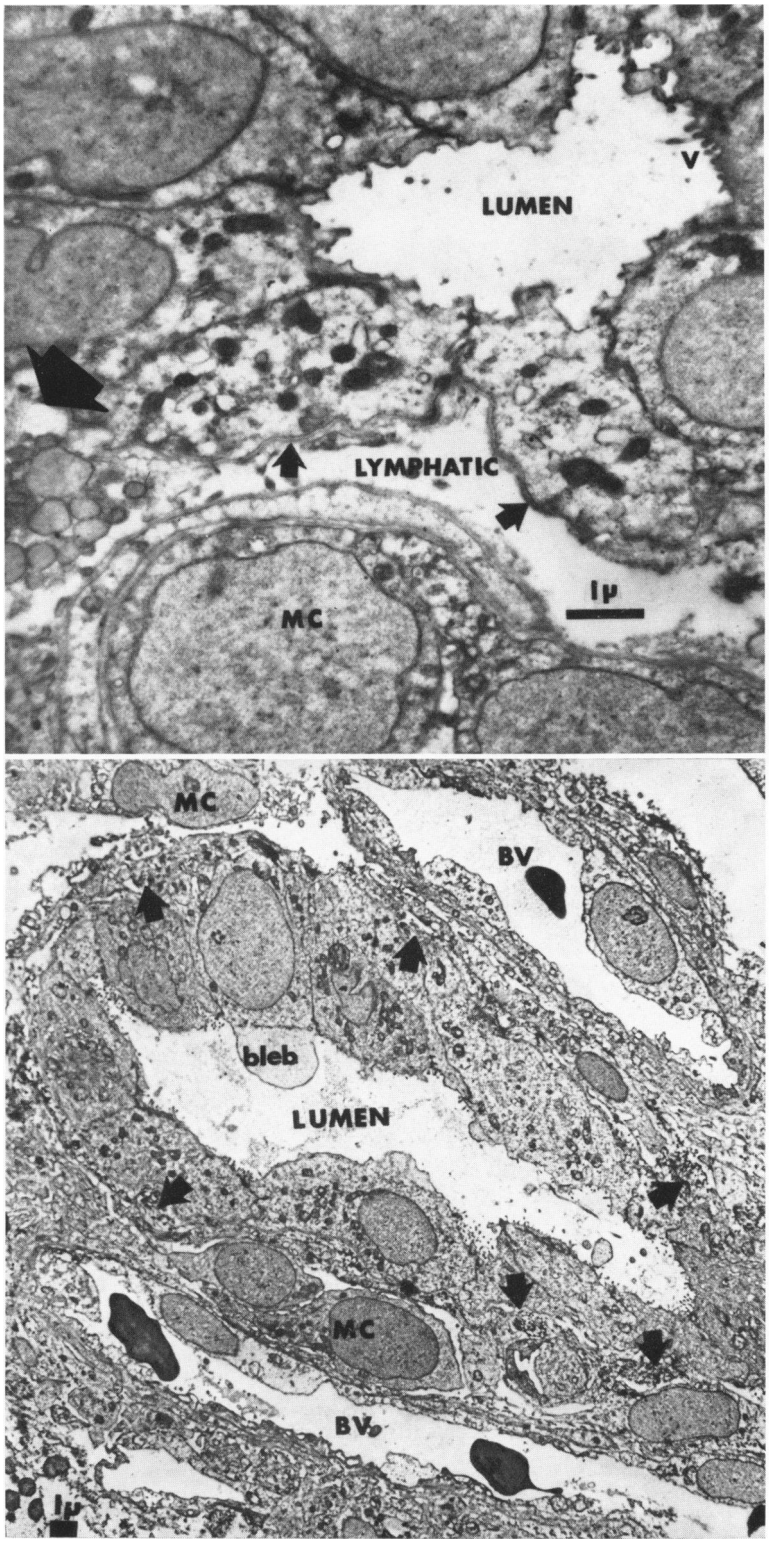
25. GILLMAN, J.; GILBERT, C., and SPENCE, I. Some factors regulating structural integrity of the intrahepatic bile ducts with special reference to primary carcinoma of the liver and vitamin A. *Cancer*, 1954, **7**, 1109–1154.

Thanks are due to the staff of the Cell Research Laboratory of The Mount Sinai Hospital for assistance in the performance of this study.

[*Illustrations follow*]

Legends for Figures

FIG. 1. Electron micrographs of bile ductules in rats. Upper: Ductule in a normal rat; V, microvilli; MC, a mesenchymal cell. The small arrows indicate the basement membrane while the large arrow on the left points to a fiber bundle. × 10,000. Lower: Ductule in a rat fed a diet containing Aramite. The ductular epithelium is surrounded by a basement membrane and an envelope of fibers (arrows), endothelial and mesenchymal cells (MC), and blood vessels (BV); the latter contain erythrocytes. × 3000.



1

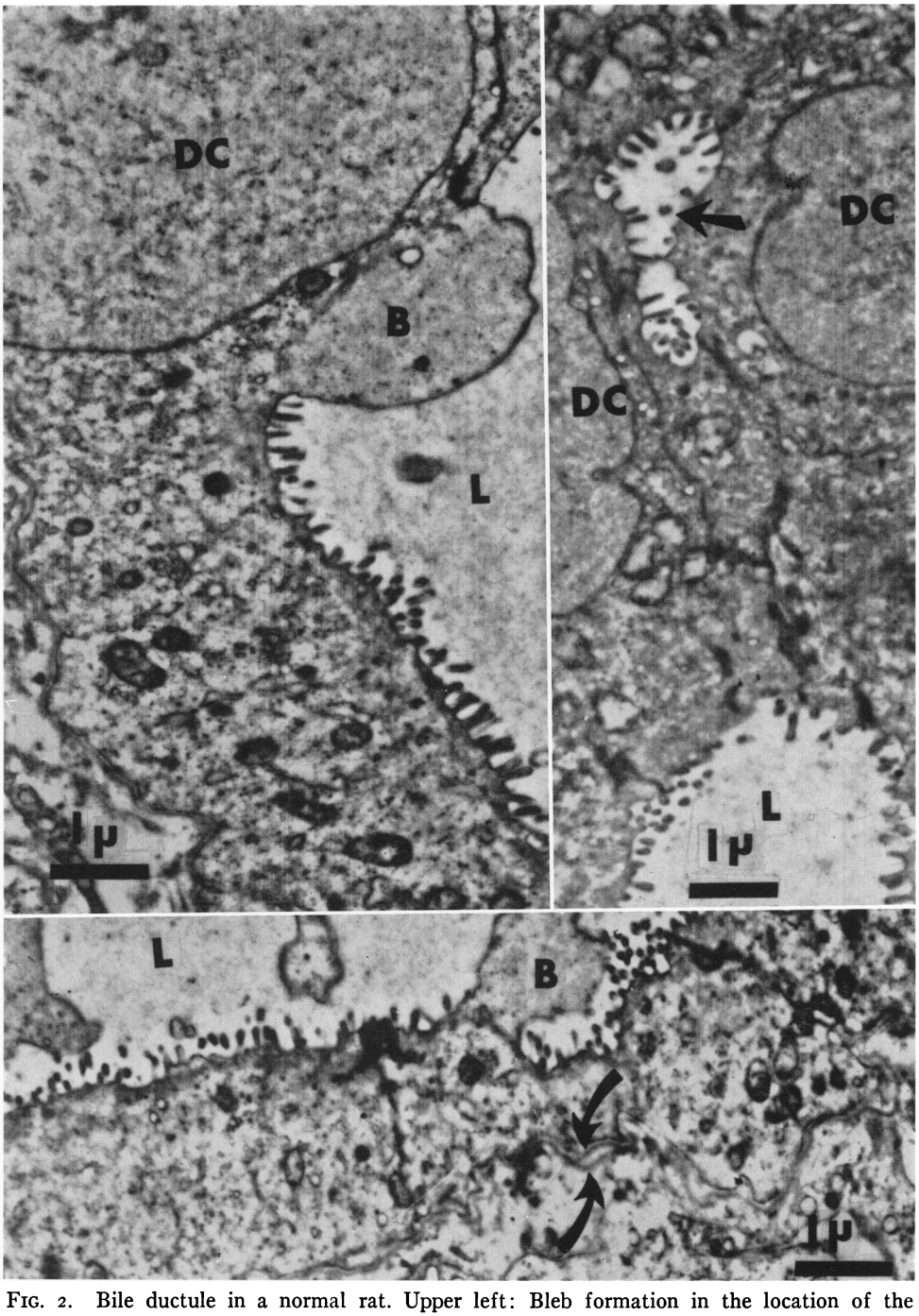


FIG. 2. Bile ductule in a normal rat. Upper left: Bleb formation in the location of the lumen surface. Upper right: Canaliculus (arrow) between neighboring parenchymal cells. Lower half: Duplication of the basement membrane (arrows). L, lumen; DC, ductular epithelium; B, bleb. × 13,000.

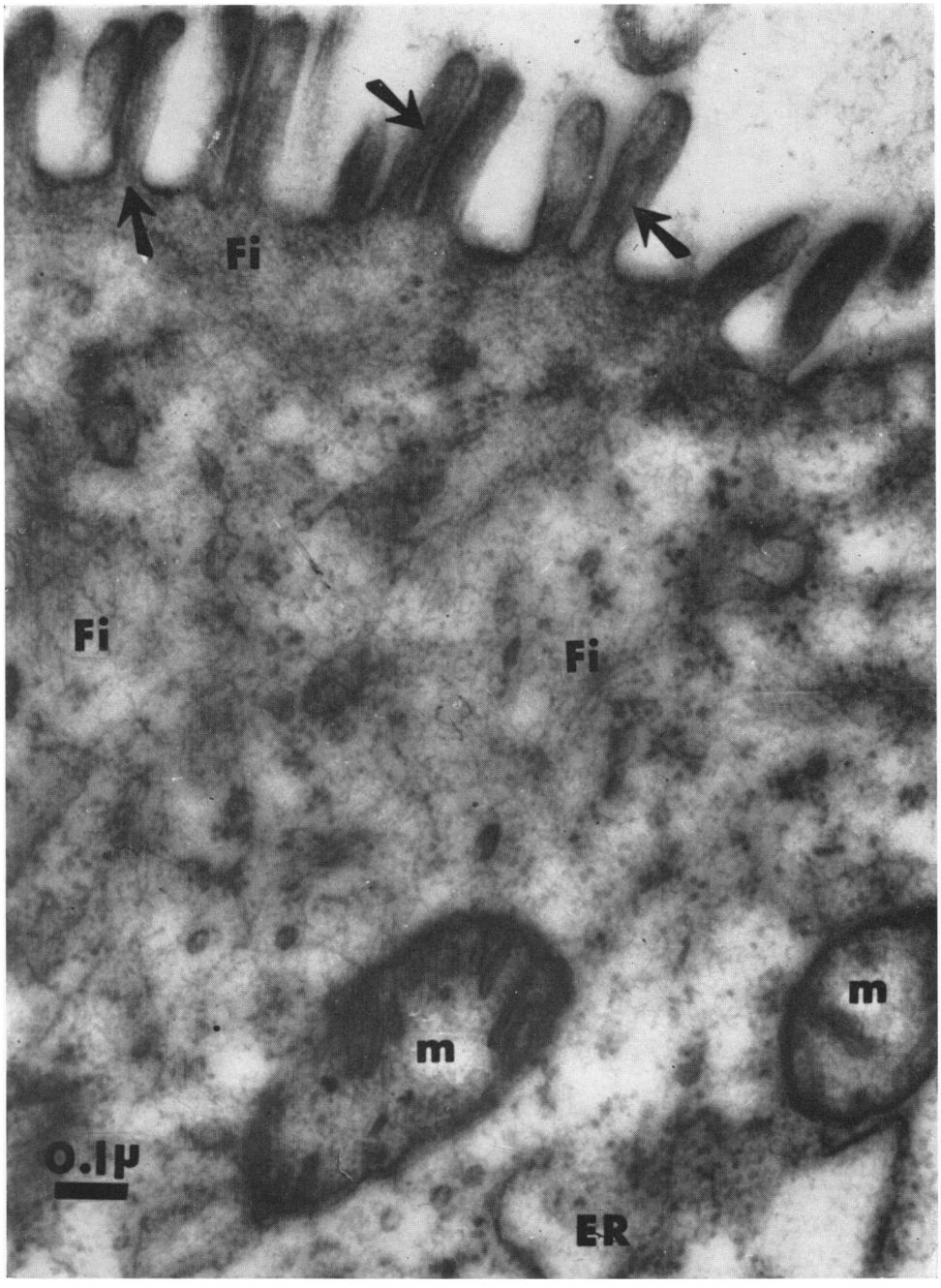


FIG. 3. Detail of lumen border of a ductular cell, showing fine filaments (arrows) in microvilli which appear to be connected to a fine cytoplasmic network (Fi). The latter is thinner than the endoplasmic reticulum (ER) and is not related to mitochondria (m). × 95,000.

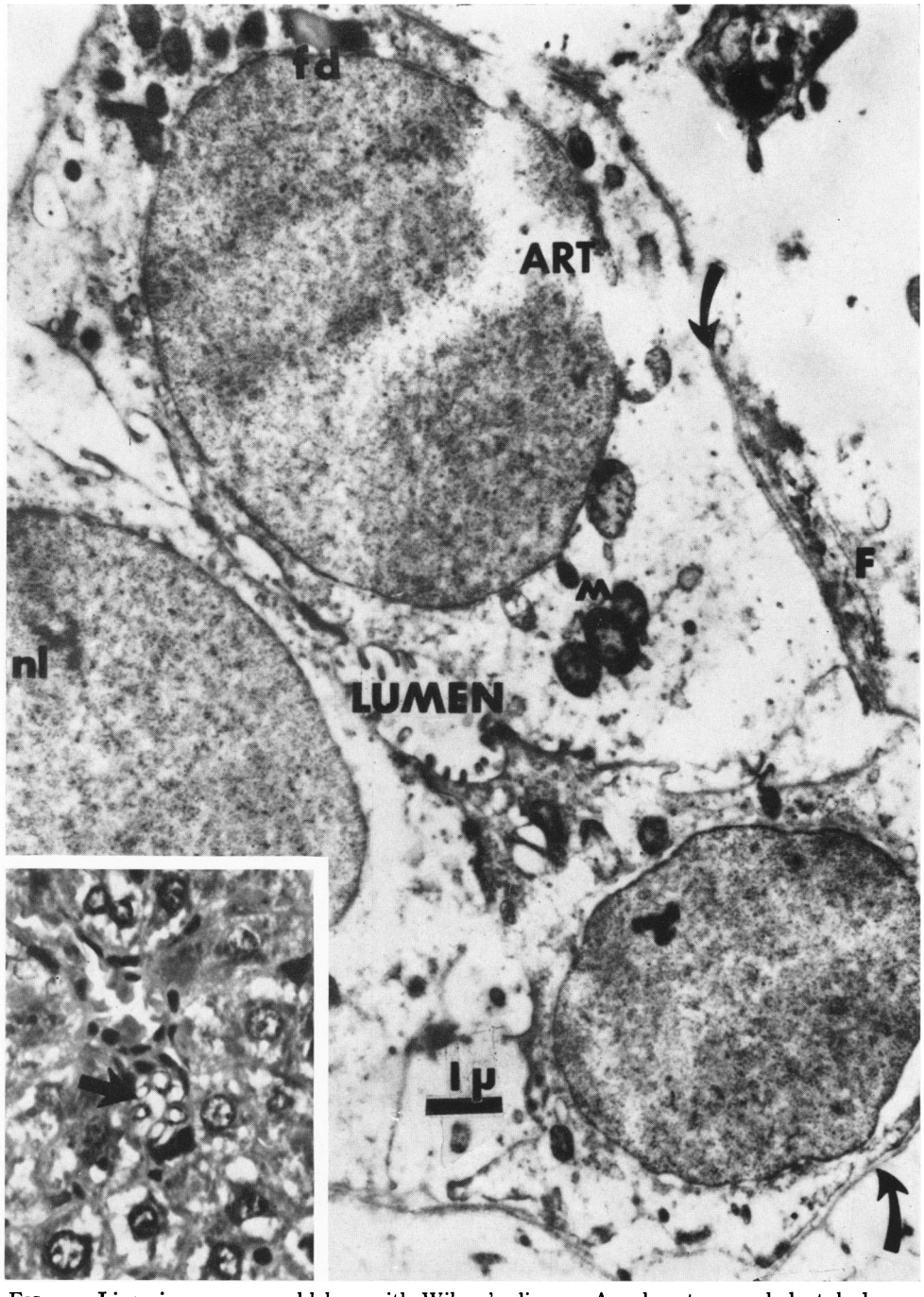


FIG. 4. Liver in a 12-year-old boy with Wilson's disease. An almost normal ductule has a lumen about 2 μ in diameter. There are apparently 4 to 8 microvilli per cell. One of the nuclei has a small nucleolus (nl). The mitochondria in the ductular cells are small, and a fat droplet (fd) is seen in one. The basement membrane (arrows) is surrounded by a few reticulum fibers (F). The borders between ductular cells are more irregular than is usual, and the cells are separated from one another. An artifact (ART) is present in the collodion grid. × 10,000. Insert: Conventional microscopic section in the same specimen, showing a ductule (arrow) in the parenchyma. Hematoxylin and eosin stain. × 400.

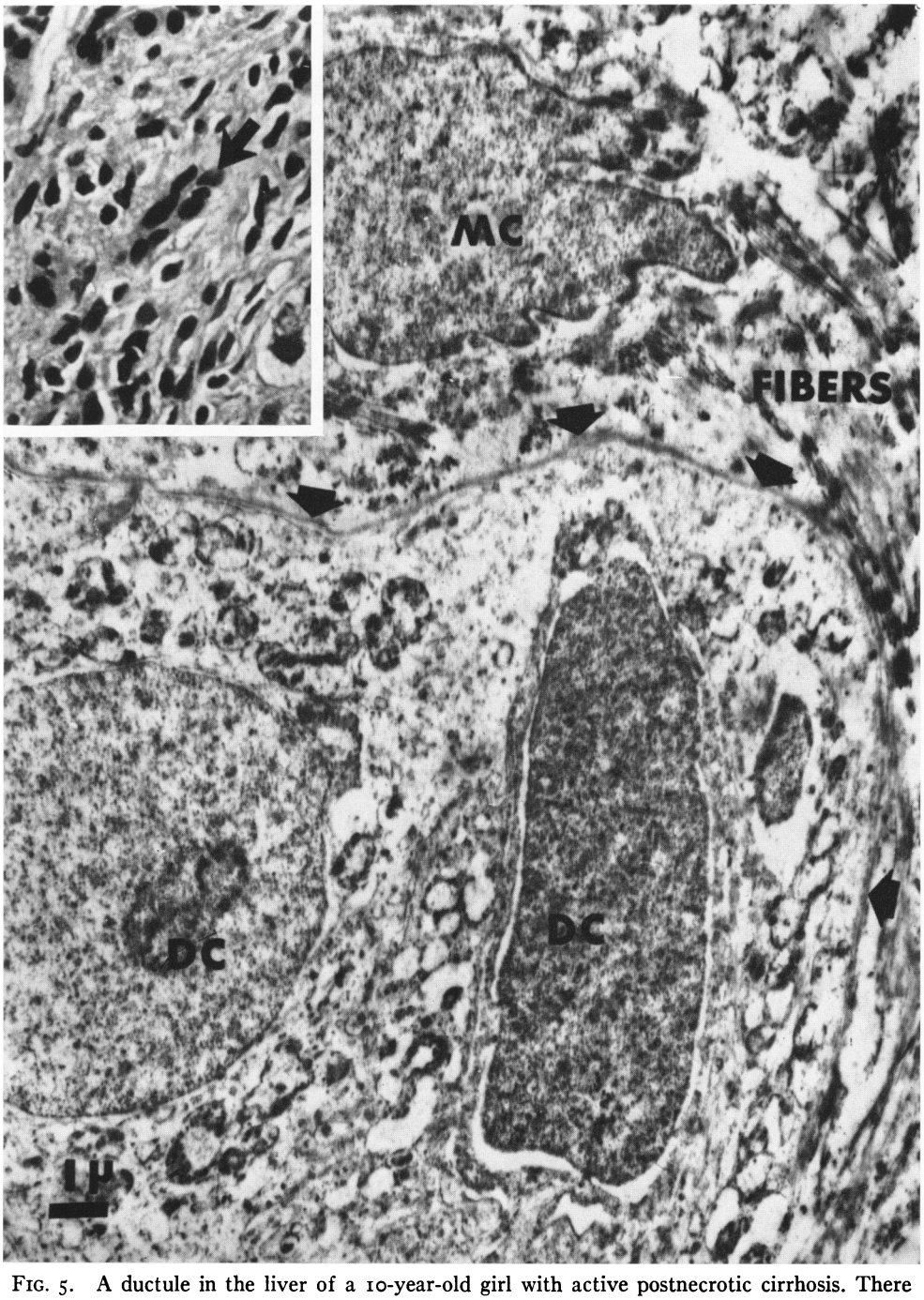


FIG. 5. A ductule in the liver of a 10-year-old girl with active postnecrotic cirrhosis. There is variation in ductular cell nuclei (DC). Fibers appear to be coming from a mesenchymal cell (MC) outside the basement membrane (arrows). × 8000. Insert: A proliferated ductule (arrow) in the same specimen. Hematoxylin and eosin stain. × 400.

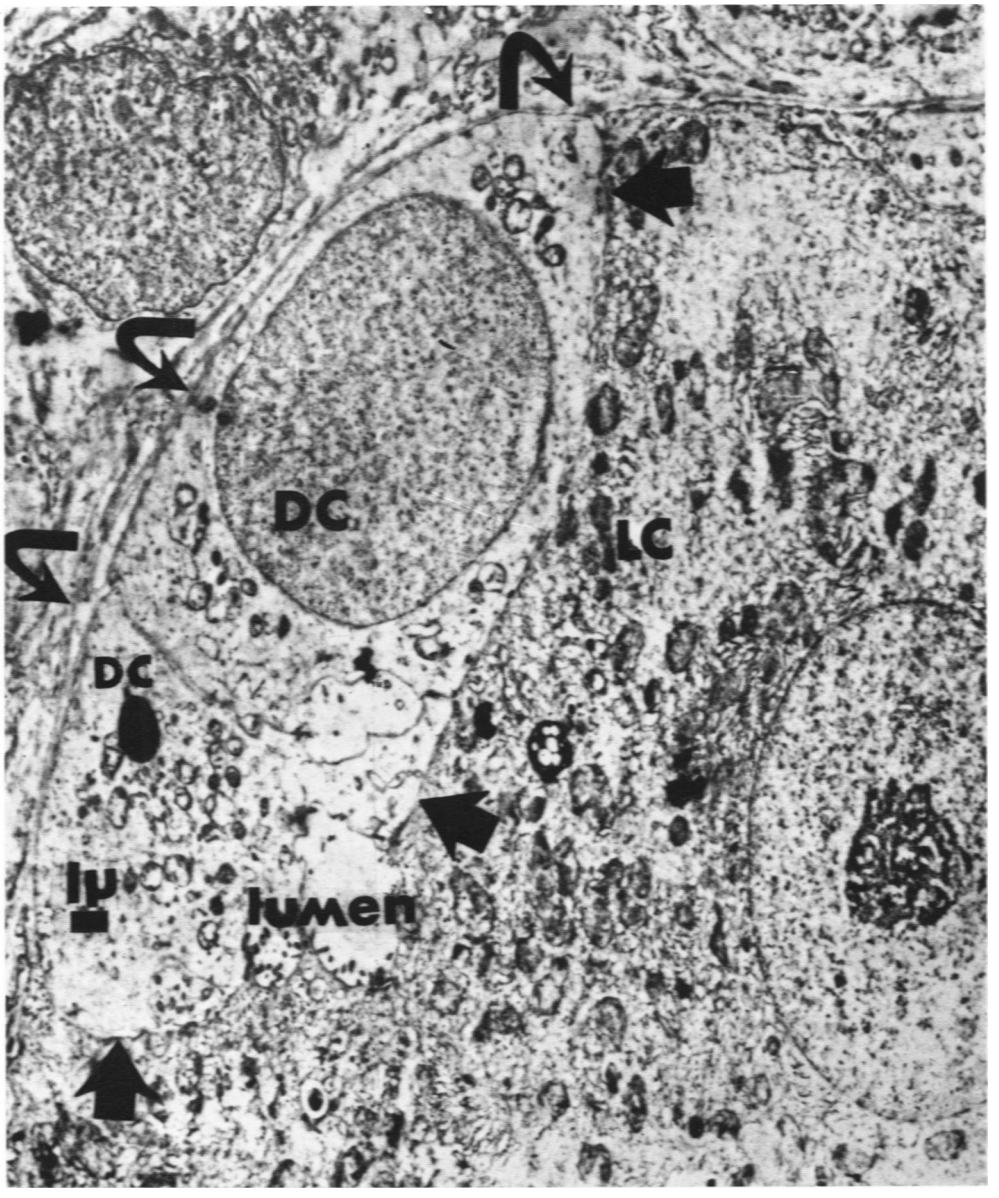
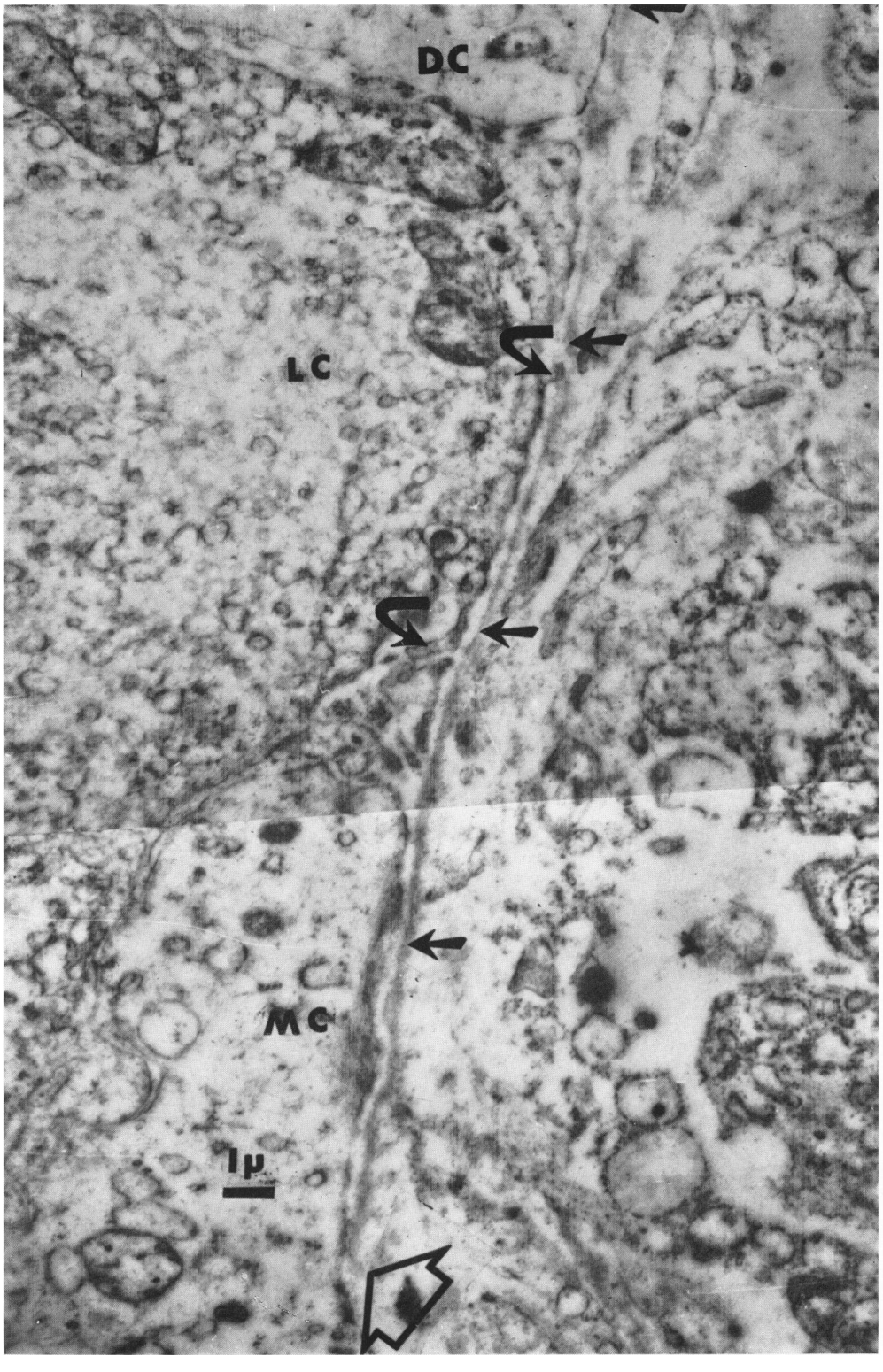


FIG. 6. A liver specimen from a 54-year-old man with inactive postnecrotic cirrhosis. Two ductular cells (DC) border a parenchymal cell (LC) and share a common lumen with it. The ductular-parenchymal cell border (straight arrows) is straight, and no basement membrane is present although one is seen on the outside border of the ductular cells (curved arrows). × 3500.

FIG. 7. Detail of the right upper portion of Figure 6. There is extension of the basement membrane (solid arrows) along the surfaces of the ductular cell (DC), the parenchymal cell (LC) and the mesenchymal cell (MC). Microvilli may be seen on the surface of the parenchymal cell beneath the basement membrane extension (curved arrows). The membrane terminates after appearing to fray slightly (open arrow). × 8000.



7

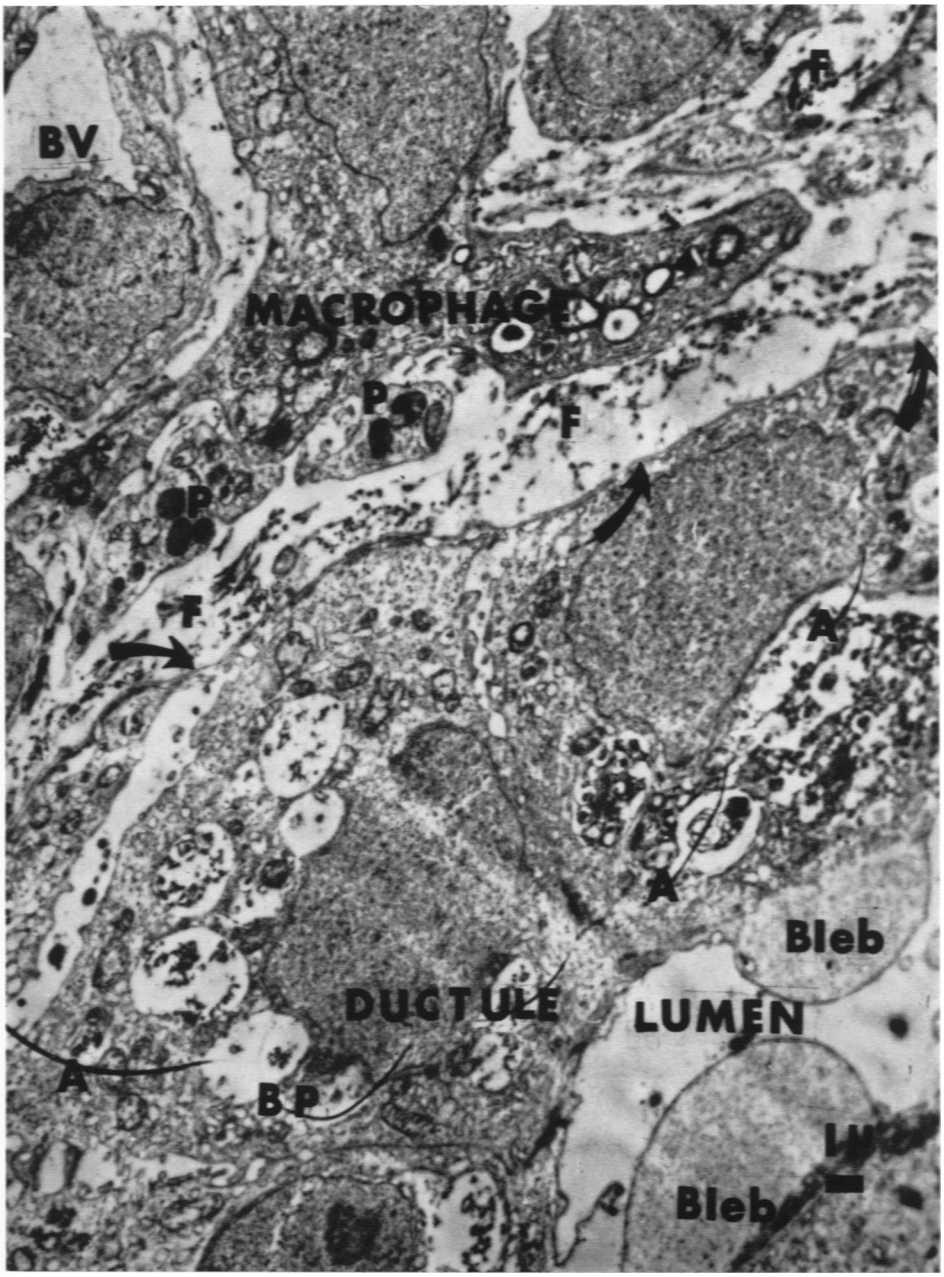


FIG. 8. A liver specimen from a patient with subacute cholangiolitis. A ductule is surrounded by a basement membrane (arrows). Fibrils (F), a macrophage containing pigment (P), and a blood vessel (BV) are evident near by. Vessels in ductular cells contain osmiophilic material that may be bile pigment (BP). The lumen shows two large blebs. Artifacts (A) are in the collodion grid. × 5000.