Characterization of a Primary Bile Ductular Cell Culture From the Livers of Rats During Extrahepatic Cholestasis

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The establishment of novel bile ductular cell cultures was accomplished with the use of explants of a hyperplastic bile ductular tissue preparation obtained from rat livers at 10 to 15 weeks after bile duct ligation or a bile ductular cell fraction isolated from this tissue preparation by a procedure involving Percoll density gradient centrifugation. Observations made on these primary explant and monolayer bile ductular cell cultures were limited to the first 3 days of culture where the morphologic features of the bile ductular epithelium remained fairly well preserved, while fibroblast contamination was found to be very low. These cultured cells also retained over this period a high specific activity for the bile ductular cell marker enzyme γ-glutamyl transpeptidase, as well as possessed measurable but decreasing specific activities for leucine aminopeptidase and alkaline phosphatase. Karyotypic analysis of the cultured monolayer cells further showed them to be diploid. In addition, preliminary transplantation studies demonstrated the presence of well-differentiated bile ductular-like structures following inoculation of the freshly isolated bile ductular cell fraction into the interscapular fat pads of recipient rats. (Am J Pathol 1985, 120:67-78)

IN RATS and other mammals, extensive bile ductular hyperplasia without evidence of carcinomatous change is a characteristic chronic pathologic feature of liver during experimental extrahepatic cholestasis produced by ligation of the common bile duct.[1-4] In this respect, the isolation of well-differentiated hyperplastic bile ductular tissue and cells induced by such a noncarcinogenic stimulus can provide an important approach for examining the functional and biochemical properties of this epithelial cell. In addition, it has the potential for leading to the development of a novel cell culture system for investigating various aspects of the characteristics and regulation of bile ductular cell differentiation and proliferation, as well as of cholangiolar carcinogenesis under the conditions of a highly controlled environment. Furthermore, it is apparent that studies defining the biochemical and functional properties of hyperplastic bile ductular tissues and cells resulting from bile duct ligation has a direct bearing on 1) whether there are specific lineage differences between these cells and the "bile ductular" or "oval" cells stimulated to proliferate in liver by many chemical carcinogens[5-7] and 2) what role, if any, the bile ductular or oval cell plays in chemical hepatocarcinogenesis.[8]

Surprisingly, there have been very few reports concerning the isolation and culturing of bile ductal or ductular cells from either normal or noncarcinogen-induced cholestatic rat liver. Wootten et al[9] investigated the properties of alkaline phosphatase in biliary tract tissue preparations obtained from rat liver after bile duct ligation, and Oda et al[10] described a technique utilizing discontinuous sucrose density gradient centrifugation to isolate a bile-duct-enriched fraction from normal rat liver. More recently, Grant and Billing[11] isolated a fraction rich in bile ductular cells from the livers of rats at 6-20 days after bile duct ligation by isopyknic centrifugation of a crude nonparenchymal fraction through a linear metrizamide gradient. Ledda et al[12] have also just described a similar technique to isolate a bile ductular cell-enriched population from rat liver

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11–13 days after bile duct ligation. In addition, these investigators have shown this freshly isolated cell population to contain very low, but measurable, amounts of aminopyrine-N-demethylase and aniline hydroxylase activities and to be resistant to aflatoxin B_1 cytotoxicity when exposed to this procarcinogen in primary culture for a period of 24–48 hours. However, no morphologic characterization of these cultures was performed.

Our laboratory has very recently reported the isolation of a hyperplastic, well-differentiated bile ductular tissue preparation from the livers of rats at 10–13 weeks after bile duct ligation.[13] In addition, we have described the isolation from this tissue preparation of a viable cell fraction that was highly enriched in bile ductular cells and small intact ductules.[13] We now present for the first time a combined morphologic and enzyme marker characterization of novel short-term primary bile ductular cell cultures that were established from explants of the hyperplastic bile ductular tissue preparation itself, as well as from the isolated bile ductular cell-enriched fraction. Transplantation of the freshly isolated bile ductular cell fraction into the interscapular fat pads of suitable host rats was also examined.

Materials and Methods

Animals

Fisher 344 male rats, 126–150 g, were purchased from Harlan Sprague Dawley, Madison, Wisconsin. The rats were housed 2–3 per cage and maintained under a 12-hour light/dark illumination schedule. Food and water were given *ad libitum*. Bile duct ligation was performed by double ligating of the common bile duct, followed by resectioning of the intervening segment.[14]

Bile Ductular Cell Culture

The hyperplastic bile ductular tissue preparation was obtained from the cholestatic rat livers at 10–15 weeks after bile duct ligation by a slight modification of our previously described procedure.[13] The isolation of the bile-ductular-cell–enriched fraction from this tissue preparation involving density gradient centrifugation in Percoll was also done as previously described.[13] Primary explant cultures were initiated with bile ductular tissue fragments obtained on a Nitex Swiss nylon monofilament screen (TETKO, Elmford, NY) with a mesh pore diameter of 100 μ. Washed tissue fragments were then suspended in plating medium and plated into 35-mm or 60-mm collagen-coated tissue culture plates[15] at protein amounts ranging between 1 and 4 mg/plate. Primary monolayer cell cultures were established with the isolated bile-ductular-cell–enriched fraction. Following one or two washings in plating medium, this population of cells, having in each experiment a viability of $\geqslant 95\%$ as determined by trypan blue dye exclusion, was plated at a density of $2\text{–}4 \times 10^6$ cells/sq cm in collagen-coated tissue culture plates 35 or 60 mm in diameter. The tissue culture medium used for cell plating and for the subsequent maintenance of both the primary explant and monolayer cell cultures was composed of Leibovitz (L-15) medium (K.C. Biological, Inc., Lenexa, Kans), pH 7.4, supplemented with HEPES (18 mM), albumin (2 mg/ml), penicillin (100 μg/ml), streptomycin (100 μg/ml), glucose (1.5 mg/ml), insulin (0.5 μg/ml), and 10% fetal calf serum. In some cases, the medium also contained 10^{-6} M dexamethasone. Medium changes were made every 24 hours after the initial cell plating.

Enzyme, Chemical, and Histochemical Assay Procedures

All enzyme and chemical procedures were performed with whole cell homogenates prepared in 0.001 M Tris-HCl buffer, pH 7.0. The activities of γ-glutamyl transpeptidase and alkaline phosphatase were determined as previously described.[13,15,16] Leucine aminopeptidase activity was assayed according to a slight modification of the procedure of Goldbarg and Rutenburg.[17] Protein was measured by the method Lowry et al[18] with bovine serum albumin as the standard, and inorganic phosphate was assayed by the method of Ames.[19] Histochemical detection of the activity of γ-glutamyl transpeptidase was performed on cultured bile ductular cells as previously described.[13] Histochemical demonstration of leucine aminopeptidase was done according to the procedure of Nachlas et al.[20]

Electron Microscopy

The hyperplastic bile ductular tissue preparations were cut into 1 mm cubes and fixed for 2 hours at room temperature in 3.0% gultaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were then postfixed for 1.5 hour on ice in 1.0% OsO_4 in 0.1 M cacodylate buffer, pH 7.4, dehydrated, and embedded in Epon–Araldite. Freshly isolated bile ductular cells were pelleted and then fixed for 30 minutes at room temperature in 3.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The cell pellet was washed in buffer and embedded in 7.0% agar before postfixation for 1 hour on ice with 1.0% OsO_4 in 0.1 M cacodylate buffer. Following dehydration with alcohols, the fixed cell pellets were embedded in Epon–Araldite. Bile ductular cells in short-term primary explant and monolayer cell culture were fixed in tissue culture dishes for 45 minutes at room tempera-

ture with 3.0% cacodylate-buffered glutaraldehyde. The cultured cells were then washed in buffer and postfixed for 45 min on ice in 2.0% OsO_4. After dehydration through 90% ethanol, the cultured cells were infiltrated with 90% through 97% hydroxypropyl methacrylate (Electron Microscopy Sciences, Fort Washington, Pa) according to the procedure of Brinkley et al.[21] The cells were then embedded in Luft's Epon 812, and the resulting polymerized resin was immersed in liquid nitrogen in order to separate the embedded cells from the tissue culture dish. Small areas of the cell monolayer were then cut out and glued onto BEEM capsule studs. All thin sections were made with a Sorvall MT-2 ultramicrotome equipped with a diamond knife and stained with uranyl acetate and lead citrate. All sections were examined at 75 kv with a Hitachi H-500 electron microscope.

Karyotyping of Cultured Bile Ductular Cells

Bile ductular cells in 2-day-old primary monolayer cell culture were incubated overnight in 10^{-7} M Colcemid (Sigma Chemical Co., St. Louis, Mo). The medium was then removed, and the cultures were harvested by incubation for 10 minutes at 37 C with 0.25% trypsin (K.C. Biological) in phosphate-buffered saline, pH 7.4. Harvested cells were pelleted by centrifugation at 2000 rpm for 5 minutes at 4 C, and cell pellet was resuspended in 5 ml of 0.075 M KCl and incubated for 20 minutes at 37 C. An equal volume of ice-cold ethanol/acetic acid (3:1) was then added to the cell suspension, and the cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 4 C. After a single washing in ethanol/acetic acid, cells from a single culture were resuspended in 0.2 ml ethanol/acetic acid, and drops of the cell suspension were then individually pipetted onto clear ice-cold glass microscope slides. After air-drying of the slides, they were stained for 10 minutes with 2.0% Giemsa stain. Photomicrographs of the metaphase chromosome spreads were then taken and evaluated according to an established karyotyping technique.[22]

Bile Ductular Cell Transplantation

Cell transplantation of freshly isolated hyperplastic bile ductular cells was performed essentially according to the interscapular fat pad cellular transplantation procedure of Jirtle and Michalopoulos.[23,24] Bile ductular cells from a rat at 15 weeks after bile duct ligation were suspended on ice in a solution of 0.26% rat tail collagen in L-15 medium, pH 7.0 (R. Jirtle and G. Michalopoulos, personal communication). The cells were then inoculated at a concentration of approximately 4 to 6 $\times$ 10^6 cells in a 0.2–0.3-ml volume into the intrascapular fat pads of 6–7-week-old Fisher 344 male rats that were subjected 2 hours earlier to a two-thirds hepatectomy.[24] After 3 weeks, the rats were sacrificed, and their fat pads were removed and then fixed in 10% neutral buffered formalin. Following appropriate processing, the fat pad tissue samples were sectioned, stained with hematoxylin and eosin (H&E), and examined by light microscopy for the presence of formed bile ductules.

Results

Light- and electron-microscopic examinations of the isolated hyperplastic bile ductular tissue preparation from the livers of the bile-duct–ligated rats revealed them to be composed almost entirely of well-differentiated bile ductules supported by a fine fibrotic structure. In this regard, the ultrastructural features of the hyperplastic bile ductular cells in the preparation (Figure 1) were consistent with those already reported for proliferated bile ductular cells as observed in the intact livers of rats that had been previously subjected to noncarcinogenic cholestatic stimuli, such as bile duct ligation or chronic intoxication with α-naphthyl isothiocyanate.[2,4]

In short-term culture (Days 1 to 3), primary explants of the bile ductular tissue preparation retained many of the morphologic characteristics of the freshly isolated samples. As shown in Figure 2a, the bile ductular epithelial cells in these short-term primary explant cultures exhibited a certain degree of polarity with respect to maintaining their organization around distinct lumens. These lumens, in turn, were limited by well-developed junctional complexes located between adjacent epithelial cells. In addition, the lumens were readily observed to be lined by small microvilli, and on occasion, a single cilium could be seen projecting into some of the lumens (data not shown).

The bile ductular epithelial cells in the short-term primary explants closely resembled those *in vivo*, as well as those in the freshly isolated tissue preparations, in terms of their round or ovoid indented nucleus with single nucleolus, their high nuclear/cytoplasmic ratio, and their sparse cytoplasmic complement of small mitochondria and profiles of rough endoplasmic reticulum. Desmosomes were also readily observed, and cell shape varied from being somewhat cuboidal to oblong. In addition, these cells were found to possess a mature Golgi apparatus and to contain small lipid droplets in their cytoplasm.

By Day 3 of culture, bile ductular epithelial cells were frequently seen extending in the form of monolayer outgrowths from the primary tissue explants (Figure 2b). These outgrowths were readily recognized at the nonluminal edges of the explant and were distinguished in

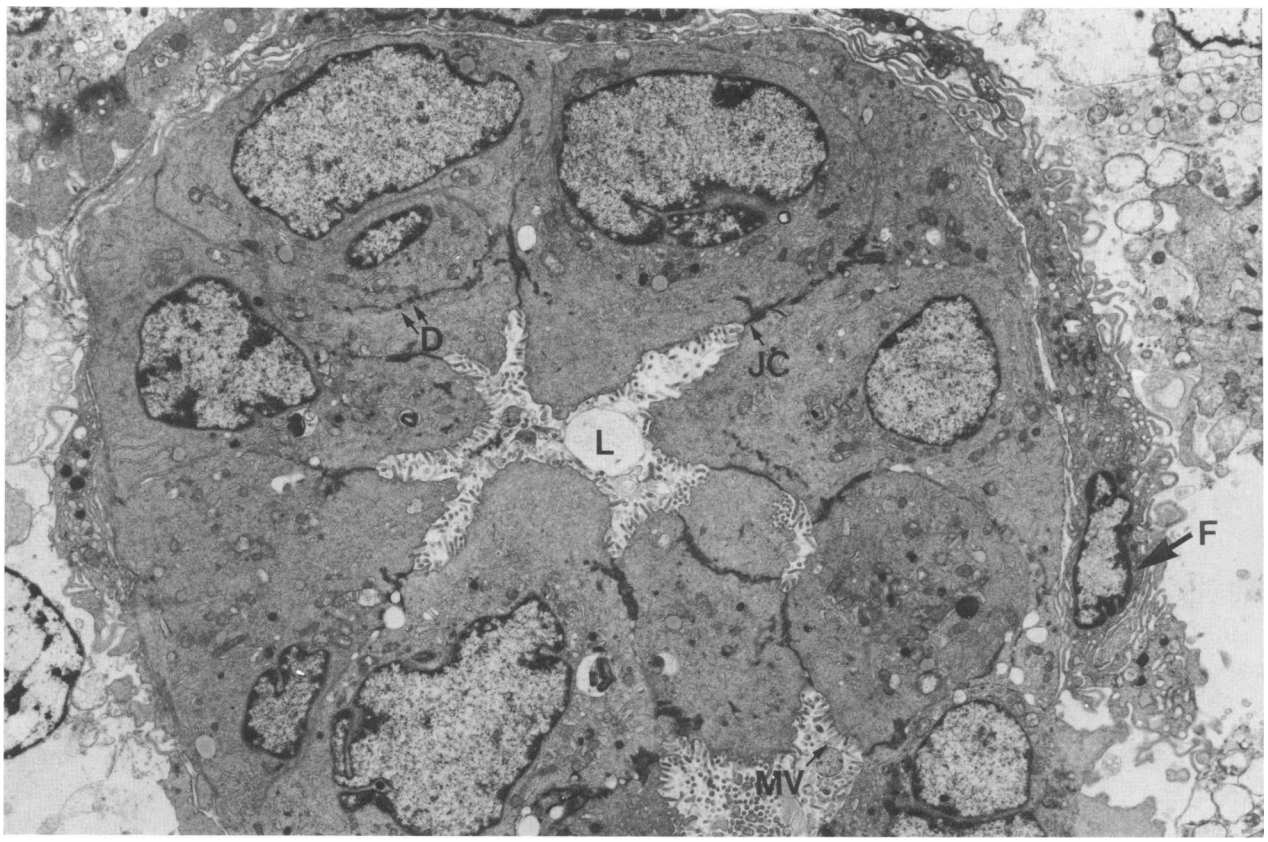


Figure 1—Electron micrograph of a bile ductule within a hyperplastic bile ductular tissue preparation isolated from rat liver at ~12 weeks after bile duct ligation. *L*, lumen lined by numerous projecting microvilli (*MV*); *JC*, junctional complex; *D*, desmosomes; *F*, periductular fibroblast. (×3900)

part from the explant by their lack of definite lumens. Small microvilli and junctional complexes were found at cell surfaces located at the periphery of the outgrowths. Furthermore, distinctly organized basement membrane components were not observed to be associated with the cellular outgrowths. Nevertheless, the epithelial cells comprising the monolayer outgrowths possessed morphologic features which closely resembled those of the bile ductular cell.

Electron-microscopic examination of the isolated bile-ductular-cell–enriched population from the hyperplastic bile ductular tissue preparation showed it to be comprised for the most part of single bile ductular cells and some small intact bile ductules (Figures 3c). In addition, histochemical enzyme determinations made on cell smears demonstrated that between 70% and 90% of the cells in this isolated population were positive for the marker enzyme activities of γ-glutamyl transpeptidase and leucine aminopeptidase (Figure 3a and b). Contamination of the total peak bile-ductular-cell–enriched population by hepatocytes, as determined with cell smears stained with H&E or for glucose-6-phosphatase activity, was found to be <0.2%, while Kupffer cells (macrophages) stained for peroxidase activity made up approximately 2.0% of this isolated cell population.[13] Cell smears stained with Wright's stain also indicated a <1.0% contamination of the bile-ductular-cell–enriched population by the various leukocyte types and by red blood cells (data not shown). A small number of "dark" or electron-dense cells, morphologically similar to the bile ductular cells, were also noted by electron microscopy in the isolated bile ductular cell population (data not shown).

Short-term primary epithelial cell cultures (Days 1–3) could readily be established by plating the isolated bile-ductular-cell–enriched population. The plating efficiency for these cells was routinely found to be about 50% when they were seeded in the presence of 10% fetal calf serum on collagen-coated plastic culture dishes. Under the maintenance conditions used in this study, distinct monolayer colonies of γ-glutamyl-transpeptidase–positive epithelial cells were observed by Days 2 and 3 of primary culture (Figure 4a). At this time, little fibroblast contamination of the cultures was noted, and karyotypic analysis of 12 random metaphase spreads, as exemplified in Figure 4b, demonstrated only cells with

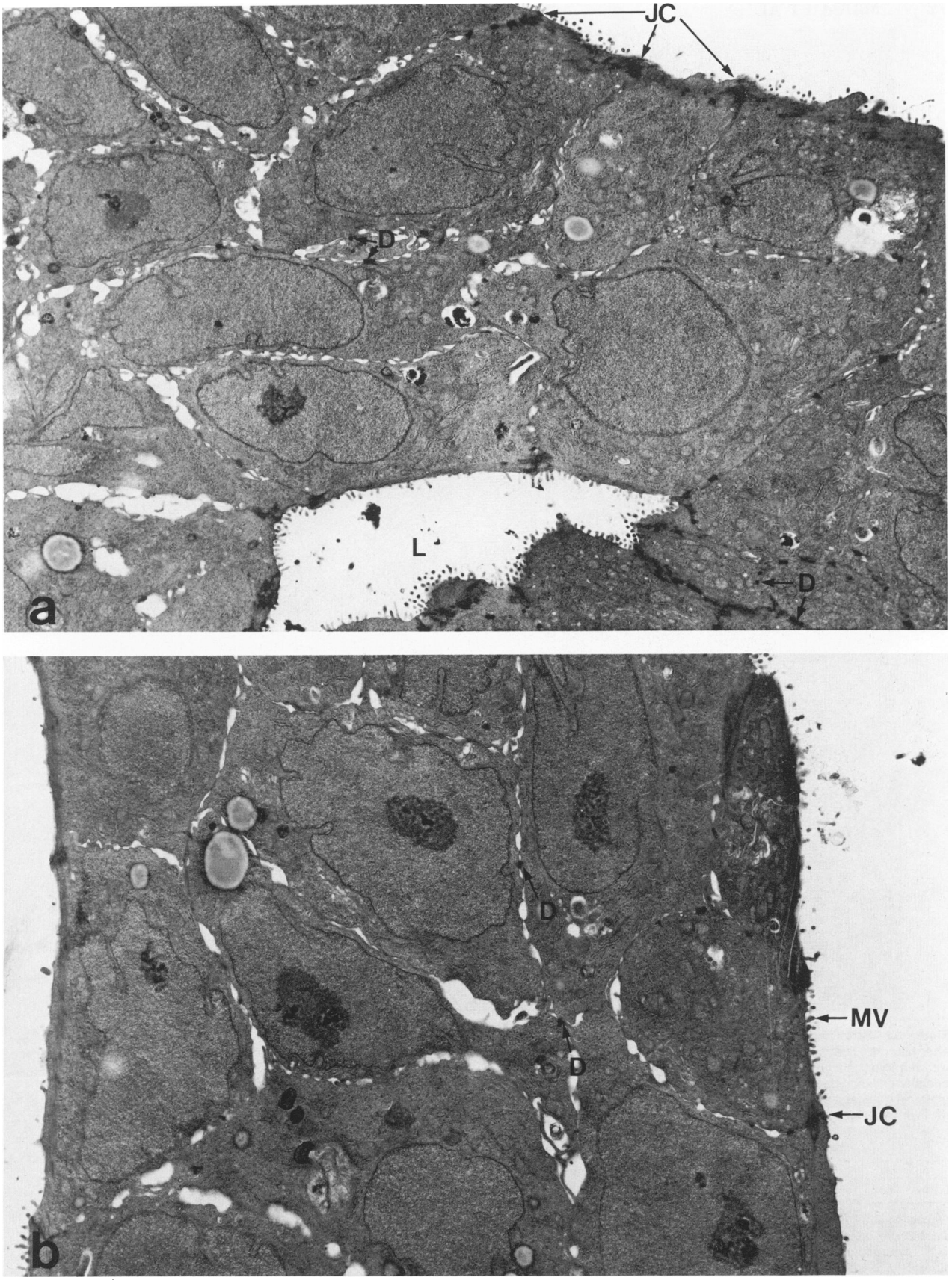


Figure 2a—Electron micrograph of a 3-day-old primary explant culture derived from a hyperplastic bile ductular tissue preparation isolated from rat liver at ~12 weeks after bile duct ligation. Culture medium contained 10^{-6} M dexamethasone. *L*, lumen; *D*, desmosomes; *JC*, junctional complexes. (×4900) **b**—Electron micrograph of a bile ductular cell outgrowth from the 3-day-old primary explant culture described above. *MV*, microvilli; *JC*, junctional complex; *D*, desmosomes. (×5950)

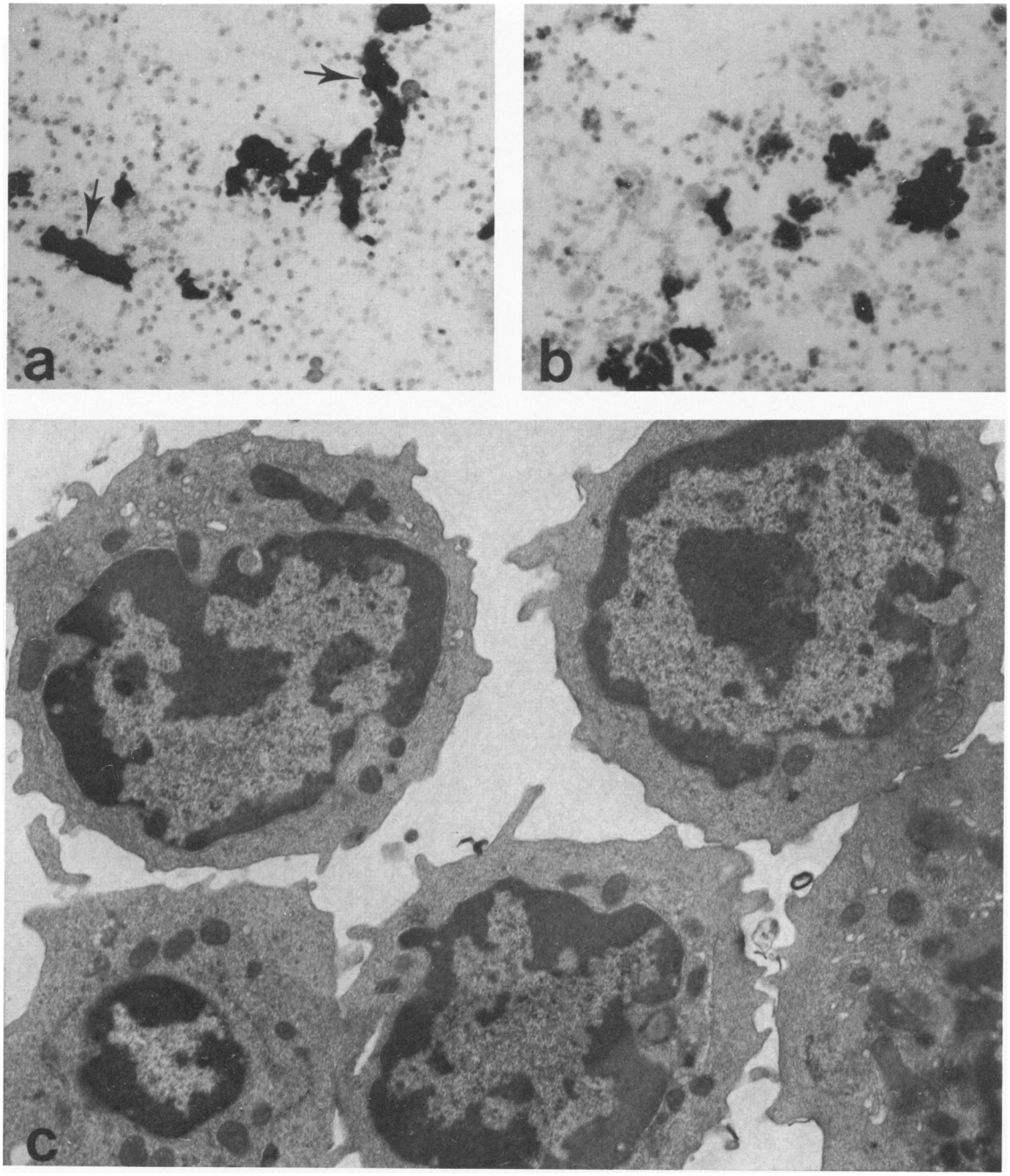
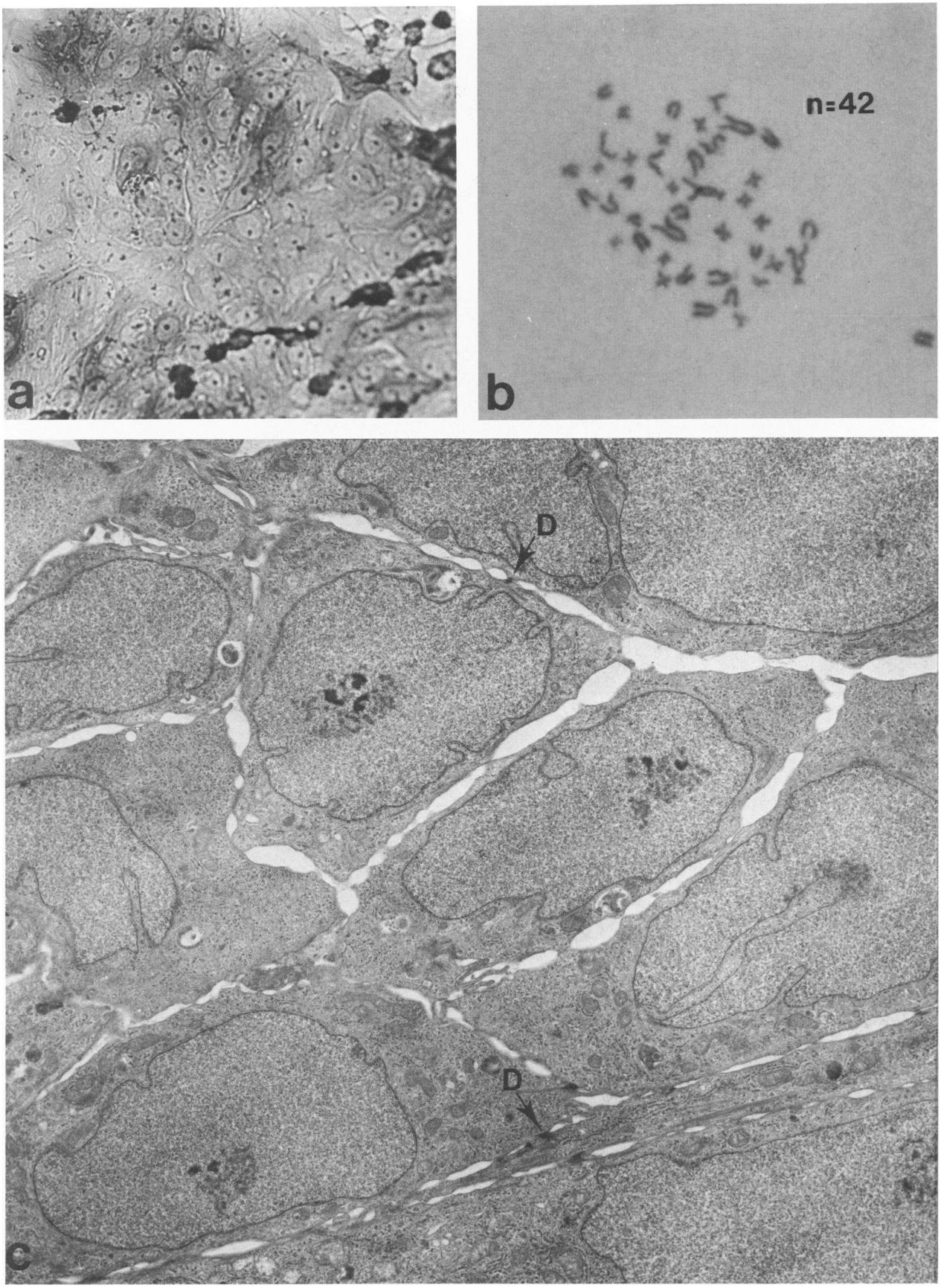


Figure 3a—Histochemical staining for γ-glutamyl transpeptidase activity in a bile-ductular-cell–enriched population isolated from rat liver at 12 weeks after bile duct ligation. Both the single cells and small, intact bile ductules (*arrows*) in this preparation are strongly positive for this enzyme activity, with the intact ductules showing the more intense activity. (×40) **b**—Histochemical staining for leucine aminopeptidase activity in the same bile-ductular-cell–enriched population. Note the marked similarity in cellular staining patterns obtained for both leucine aminopeptidase activity and for γ-glutamyl transpeptidase activity. (×40) **c**—Electron micrograph of single bile ductular cells in the bile-ductular-cell–enriched population isolated from rat liver at 12 weeks after bile duct ligation. (×12,500)

Figure 4a—A 3-day-old primary epithelial monolayer culture derived from the total peak bile-ductular-cell–enriched population isolated in Percoll gradients from rat liver at 13 weeks after bile duct ligation and stained for γ-glutamyl transpeptidase activity. (×200). Note that all of the epithelial cells in this figure show a positive cytoplasmic staining for γ-glutamyl transpeptidase activity, with some cells showing a more intense staining reaction than others. **b**—A representative metaphase spread obtained from a 3-day-old primary epithelial culture from a bile-ductular-cell–enriched population isolated from rat liver at 10 weeks after bile duct ligation. (Giemsa, ×1000) **c**—Electron micrograph of a 3-day-old primary epithelial monolayer culture from the bile-ductular-cell–enriched population isolated at 13 weeks after bile duct ligation. Section was made parallel to the substratum surface. *D*, desmosomes. (×6860)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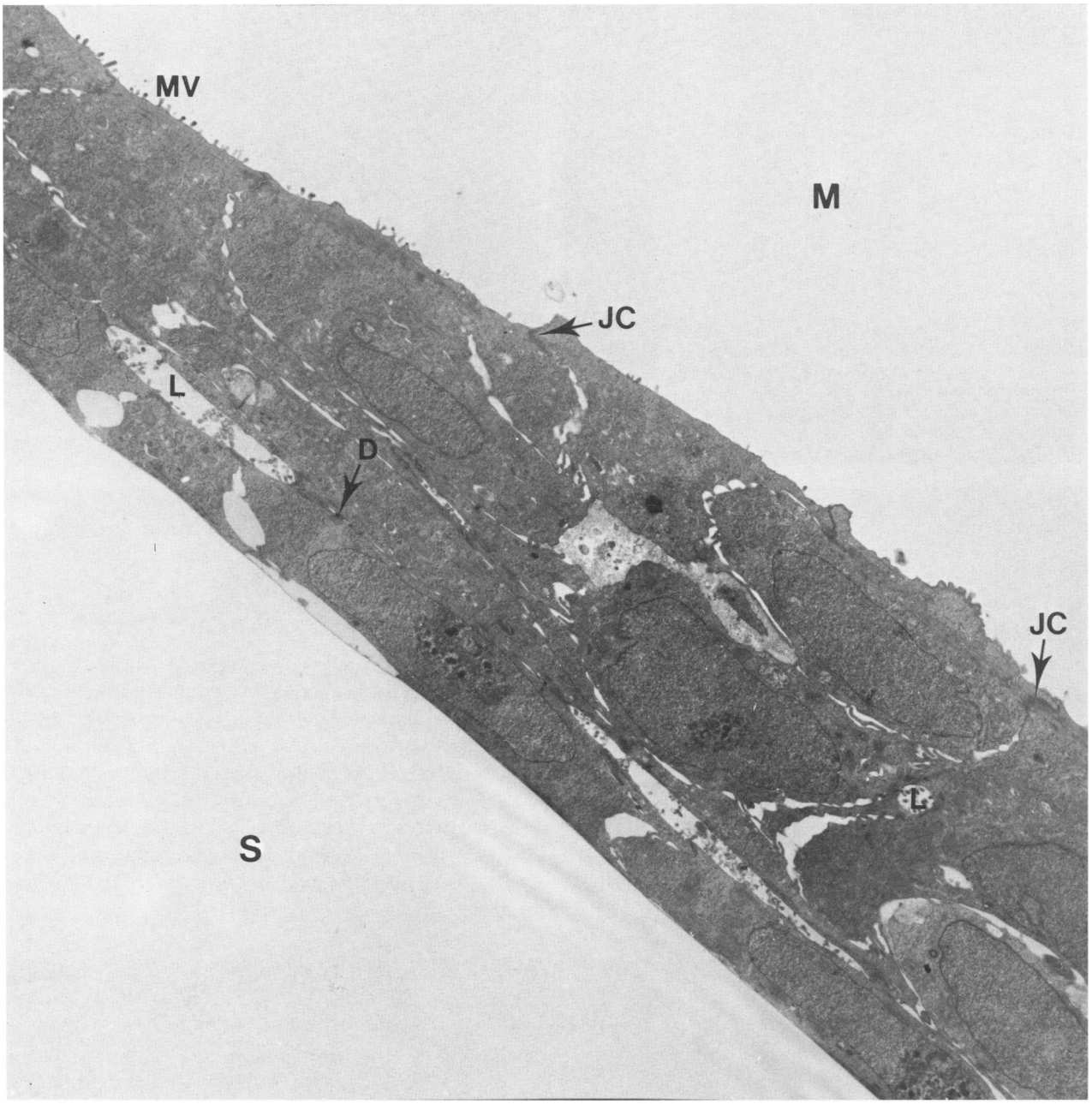

Figure 5—Electron micrograph of a 3-day-old primary epithelial monolayer culture from the bile-ductular-cell–enriched population isolated at 13 weeks after bile duct ligation. Section was made perpendicular to the substratum surface. *L*, lumen; *MV*, microvilli; *JC*, junctional complex; *D*, desmosome; *M*, medium-facing surface; *S*, substratum-facing surface. (×4880)

a normal diploid chromosome content. In addition, these cultured epithelial cells were demonstrated by electron microscopy to exhibit ultrastructural features which were also quite compatible with those of the bile ductular epithelial cell (Figures 4c and 5). Moreover, in sections cut perpendicular to the substratum surface, discreet cell-bounded lumens limited by tight junctions and containing small projecting microvilli were evident in some regions of the culture. Small microvilli were also observed at the medium-facing cell surface of the cultured epithelial cells (Figure 5).

Table 1 shows the stability over time in culture of various marker-enzyme–specific activities characterizing both the primary explant culture and the primary monolayer cell culture that was established with the isolated bile-ductular-cell–enriched population. Here it can be seen that, in each case, the γ-glutamyl-transpeptidase–specific activity of the cultured cells remained rel-

Table 1—Enzyme Marker Specific Activities of Primary Explant and Cell Monolayer Cultures of Hyperplastic Bile Ductular Epithelium From Bile-Duct–Ligated Rat Liver

Culture	Days in culture	Specific Activity§ γ-Glutamyl transpeptidase	Leucine aminopeptidase	Alkaline phosphatase
Primary explant*	Freshly isolated	4.23 ± 0.29	0.65 ± 0.07	1.34 ± 0.09
	1	3.76 ± 0.18	0.55 ± 0.03	1.28 ± 0.07
	3	5.30 ± 0.86	0.25 ± 0.04	0.30 ± 0.05
Primary cell monolayer†	Freshly isolated	1.21 ± 0.07	0.43 ± 0.05	1.60 ± 0.11
	1	1.27 ± 0.09	0.55 ± 0.06	1.21 ± 0.12
	2	1.31 ± 0.15	0.36 ± 0.06	0.25 ± 0.03
Primary cell monolayer‡	Freshly isolated	2.79 ± 0.17	0.57 ± 0.04	4.40 ± 0.28
	1	2.16 ± 0.12	0.58 ± 0.05	4.41 ± 0.37
	2	1.90 ± 0.22	0.36 ± 0.04	2.10 ± 0.30
	3	3.04 ± 0.57	0.38 ± 0.07	1.39 ± 0.27

* Established from a hyperplastic bile ductular tissue preparation isolated from rat liver at 10 weeks after bile duct ligation.
† Established from a hyperplastic bile ductular cell-enriched fraction isolated from rat liver at 13 weeks after bile duct ligation.
‡ Established from a hyperplastic bile ductular cell-enriched fraction isolated from rat liver at 10 weeks after bile duct ligation.
§ Each value represents the mean ± SD determined from measurements made in triplicate: γ-glutamyl transpeptidase specific activity, micromoles *p*-nitroaniline/milligram protein/hour; leucine aminopeptidase specific activity, micromoles β-naphthylamide/milligram protein/hour; alkaline phosphatase specific activity, micromoles phosphate/milligram protein/hour.

atively constant over a 3-day culture period with respect to the values obtained for this enzyme with fresh tissue and cell isolates. In comparison, these cultures exhibited measurable but decreasing specific activities of the marker enzymes leucine aminopeptidase and alkaline phosphatase over the same period of time.

Preliminary transplantation results with the isolated bile-ductular-cell–enriched population further support its potential usefulness. In this regard, organized foci of bile-ductular–like structures were detected in the scapular fat pads of appropriate recipient rats at 3 weeks after inoculation of the isolated bile ductular peak gradient population into this tissue site (Figure 6). Only formed ductules were observed within the fat pads, and there was no evidence for the presence of hepatocytes in this tissue after transplantation.

Discussion

In the present study we have employed both morphologic and biochemical criteria to demonstrate in an unequivocal manner the establishment of primary explant and cell monolayer cultures of hyperplastic bile ductular epithelial cells from the livers of rats at 10–15 weeks after bile duct ligation. The 10–15-week period after bile duct ligation was chosen as the time point for isolating the hyperplastic bile ductular tissue preparation because by this time the bile ductular cell proliferation *in vivo*

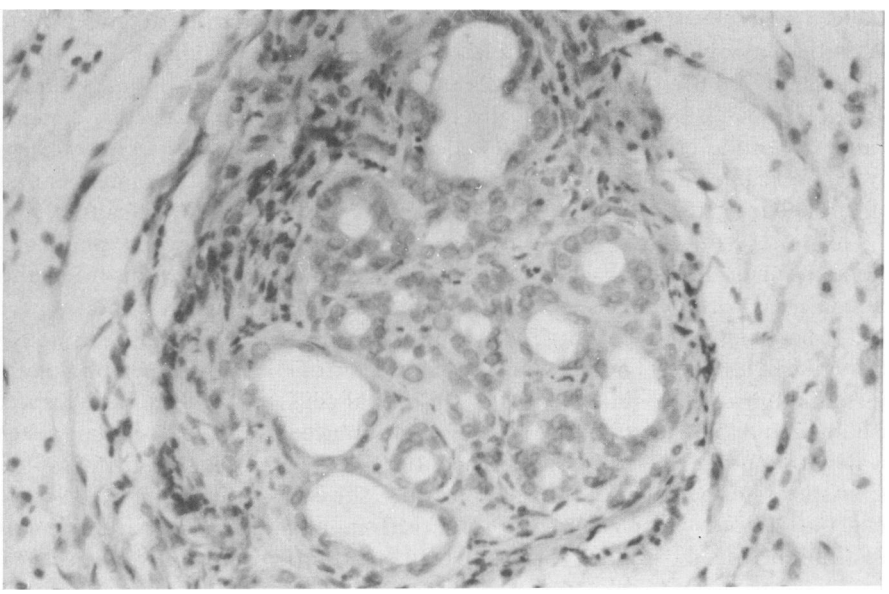

Figure 6—Presence of well-differentiated ductular tissue in the interscapular fat pad of an isologous recipient rat at 3 weeks after transplantation of ~4 × 10^6 cells from the bile-ductular-cell–enriched peak fraction obtained from rat liver at 15 weeks after bile duct ligation. (H&E, ×200)

has been shown to become stabilized at a relatively low rate, with much of the liver mass (ie, >50%) now found to be occupied by well-differentiated hyperplastic bile ductular tissue.[4,13]

A major advantage of the isolation procedure used in this study is that it allows for the simultaneous culturing of a tissue preparation and a cell isolate, both of which are highly enriched in viable bile ductular epithelial cells. In this regard, a good correlation was established between the ultrastructural characteristics shown by the bile ductular epithelial cells in primary monolayer cell culture versus those in primary tissue explant culture. This was particularly evident in terms of comparing the ultrastructural features of the primary monolayer cells derived from the isolated bile ductular cell-enriched population with that of the epithelial cell outgrowths from the bile ductular tissue explant cultures. Furthermore, while most of the bile ductular epithelial cells from the isolated cell fraction were in cell monolayer in primary culture, it was also observed that in some regions of this culture distinct lumens were present as well. Here, it is most probable that this organization of the bile ductular cells around lumens reflects the presence of the small intact ductules found in the cultured cell isolate and not a *de novo* formation in culture of ductular structures from single cells or small groups of cells. However, further studies are needed to completely rule out the latter possibility.

Both the primary explant and cell monolayer cultures of the hyperplastic bile ductular cells were quite comparable with respect to their profiles and stability over time in culture of the marker enzyme activities of γ-glutamyl transpeptidase, leucine aminopeptidase, and alkaline phosphatase. Each of these enzymes, in turn, have been shown to be characteristic of non-carcinogen-induced hyperplastic bile ductular cells *in vivo*.[3,9,11,25-27] In particular, γ-glutamyl transpeptidase is perhaps the most useful of the enzyme markers. We have previously shown it to be histochemically localized exclusively to the bile ductular epithelium of the well-differentiated hyperplastic bile ductular tissue isolated from the livers of bile-duct-ligated rats.[13]

The short-term primary bile ductular cell explant and monolayer cultures established in this study should not be confused with oval cell cultures derived from the livers of carcinogen-treated rats[8,28] nor with most of the liver epithelial-like cell lines previously established from normal adult or fetal rat livers.[29-31] Transitional cells usually found to be associated with carcinogen-induced oval cell proliferation, and having morphologic characteristics intermediate between the bile ductular cell and the hepatocyte[32,33] were not observed in our electron-microscopic studies. This is also consistent with our previous findings demonstrating that, unlike carcinogen-induced oval cell populations, the bile duct hyperplasia resulting from bile duct ligation does not show any indication of a nonhepatocytic cellular expression of albumin production, glucose-6-phosphatase activity, or glycogen.[13] Hayner et al[34] have also recently reported that a freshly isolated biliary epithelial cell fraction from normal rat liver possessed a selected isozyme profile that was distinct from that of the hepatocyte. In contrast, they found that an oval cell fraction isolated from the livers of carcinogen-treated rats exhibited a hybrid pattern of isozymes, which implied the presence of some cells in this population that were in intermediate states of differentiation between the biliary epithelial cell and the hepatocyte. In this regard, these authors also demonstrated by morphologic examination the presence of transitional cells in their "oval" cell isolate, although it is not certain as yet whether these cells are the ones which contained the hybrid isozymes.

The freshly isolated and cultured bile ductular cells from the bile duct-ligated rats possessed a considerably higher γ-glutamyl-transpeptidase-specific activity than that which was measured by us in an oval cell population isolated from the livers of rats fed the carcinogen DL-ethionine in a choline-deficient diet.[13] Likewise, the high γ-glutamyl-transpeptidase-specific activity of these short-term bile ductular cell cultures distinguishes them from the majority of the established rat liver epithelial-like cell lines, which are for the most part negative for this particular enzyme marker activity.[35,36] However, Tsao et al[37] have recently described the establishment of a diploid rat liver epithelial cell line from normal adult rat liver which exhibits a weakly positive histochemical reaction for γ-glutamyl transpeptidase, as well as possesses a number of other phenotypic properties described for oval cells. On the other hand, the origin of this cell line as being derived unequivocally from bile ductular epithelium still remains to be demonstrated.

At present, we have limited most of our observations of the primary epithelial monolayer cultures to the first 3 days of culture. In this regard, these primary bile ductular cell explant and monolayer cell cultures exhibited during this brief culture period, and under the present culture conditions, a very low level of fibroblast contamination, as well as a more differentiated bile ductular cell morphology than was observed in older primary cultures. With respect to the bile ductular tissue explant itself, cellular degeneration was found to be quite marked by the ninth day of culture. However, in preliminary studies in our laboratory (H. P. Cihla and A. E. Sirica, unpublished observations), we have also demonstrated the feasibility of establishing a propaga-

ble epithelial cell line derived from the primary monolayer bile ductular cell culture and found to be positive by histochemistry for γ-glutamyl transpeptidase activity. By comparison, Fausto has recently been cited in a published personal communication[37] as indicating that the γ-glutamyl transpeptidase of carcinogen-induced oval cells markedly declines in cell culture.

Our initial transplantation studies with the isolated bile-ductular-cell-enriched population obtained from the bile-duct-ligated rats have also been encouraging in that only formed, well-differentiated bile-ductular-like structures were observed within the interscapular fat pads of the recipient normal host rats. This, in turn, suggests the possibility of establishing an in culture/*in vivo* transplantation model with these cells for the purpose of studying cholangiolar carcinogenesis and the regulation of bile ductular cell functions, proliferation, and modulation under more controlled experimental conditions. Further efforts are now being made to define the optimal conditions for maintaining the hyperplastic bile ductular cells from bile-duct-ligated rats in cell culture, along with further developing conditions for their use in an *in vivo* transplantation model.

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