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Studies on the mechanism of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)-induced hepatotoxicity

III. Ultrastructural characterization of bile duct injury

N. W. Kretschmer, P. J. Boor, R. A. el Azhary, A. E. Ahmed, and E. S. Reynolds*

Chemical Pathology Division, Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77550, USA

Summary. The antineoplastic nitrosourea CCNU is a known hepatotoxin which has been shown to cause hyperbilirubinemia and reduction in bile flow. We studied morphological alterations in the common bile duct and interlobular bile ducts at 6, 12, and 24 h in male rats given a single oral dose (50 mg/kg) of CCNU. The portal vein was perfused with 1.0% glutaraldehyde fixative. Portal areas and the common bile duct were selectively dissected and processed using standard methods for light and transmission electron microscopy. The epithelial cells of larger common bile duct and interlobular bile ducts showed increased rough endoplasmic reticulum, markedly increased free ribosomes, and mitochondrial degeneration at 6 and 12 h after CCNU. There was also bile imbibition and loss of microvilli, which increased in severity at 12 and 24 h. The interstitium showed infiltration by acute inflammatory cells and dilated capillaries at 6 h. By 24 h, degeneration of epithelial cells was extensive; cells became necrotic and sloughed into the duct lumen. The smaller bile ductules showed no significant degenerative changes; adjacent hepatocytes were unremarkable. Early CCNU injury appears localized in the large bile ducts and reflects inflammatory edema, bile stasis, and degeneration of epithelial cells. Our studies suggest that this ductal injury may reflect metabolism of CCNU to reactive species within the bile ducts.

Introduction

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea is an antitumor agent which has been used to treat malignant brain tumors and Hodgkin's disease [19]. Its use as a chemotherapeutic agent has been limited by its modest antitumor activity, bone marrow suppression, and renal, hepatic, and gastrointestinal toxicity [11, 19].

Nitrosoureas are known to cause delayed and cumulative dose-dependent target organ toxicities such as lung and kidney damage. CCNU has been shown in monkeys to cause interstitial nephritis [14]. Clinically, three reports exist of patients receiving high cumulative doses of CCNU resulting in fatal chronic renal failure [2, 6, 15]. Gastrointestinal toxicity in patients is manifested as nausea and vomiting 1–2 h after CCNU administration; vomiting may be so severe as to cause vertebral compression fractures and tearing of the esophageal mucosa [4, 20]. However, the mechanism of toxicity in gastrointestinal, renal, and hepatic tissue is not fully understood.

Other major side effects of CCNU include hematopoietic depression and hepatic injury [11]. Recent light-microscopic studies in our laboratory have shown that CCNU causes interlobular bile duct and common bile duct injury associated with hyperbilirubinemia and cholestasis [1]. Time-course studies showed that CCNU ductal injury progresses from initial edema and polymorphonuclear leukocyte (PMN) infiltration in portal areas at 12 h to complete sloughing of bile duct epithelium. The objective of the present study, therefore, was to investigate the hepatotoxic effect of CCNU further by examining several levels of the biliary system, as well as adjacent hepatocyte ultrastructure, at early times after treatment with a single oral dose of CCNU.

Materials and methods

Animals. Male Sprague-Dawley rats (Timco Breeding Laboratories, Houston, Tex, USA) weighing approximately 175 g were kept under standard laboratory conditions with free access to food and water. Groups of four rats were given, by gavage, 50 mg/kg CCNU (CeeNU Laboratories) in mineral oil and killed at 6, 12, and 24 h after treatment; control rats received mineral oil only.

Rats were anesthetized with sodium pentobarbital (5 mg/kg) and the portal vein was perfused briefly at low pressures [5] with 0.9% saline containing isoproterenol (0.2 mg/ml) followed by 1.0% glutaraldehyde in 0.1 *M* Pipes buffer (Sigma), pH 7.35, for 5 min. Slices of central liver lobes with portal areas containing bile ductules and interlobular bile ducts were dissected and trimmed to 3 mm × 1 mm slices and placed in 0.1 *M* Pipes buffer, pH 7.35. Common bile ducts closest to the liver were separately dissected and immersed in Karnovsky's fixative [7] for 4 h then placed into 0.1 *M* sodium cacodylate buffer, pH 7.35.

Tissue processing. All tissues were postfixed in 1% aqueous osmium tetroxide, stained in saturated uranyl acetate, dehydrated in a graded ethanol series, embedded in Epon 812, and oriented for cross-sectioning. Semithin sections (1 μm) were cut on a Sorvall MT2-B ultratome, stained with 1% toluidine blue, and observed by light microscopy.

* Professor and Chairperson, Department of Pathology, the University of Texas Medical Branch; died on November 12, 1983
Offprint requests to: Paul J. Boor

Blocks were selected for thin sectioning to include at least nine sections per rat for each of the three types of ducts examined, i.e., the largest common bile duct, the interlobular bile ducts (defined as ca. 30 epithelial cells), and the smaller bile ductules (defined as ducts containing ≤ 6 epithelial cells). All hepatocytes examined were no more than three of four cells away from the duct interstitium. Thin sections were stained with uranyl acetate and Reynold's lead citrate [13] and examined with a Philips 400 electron microscope.

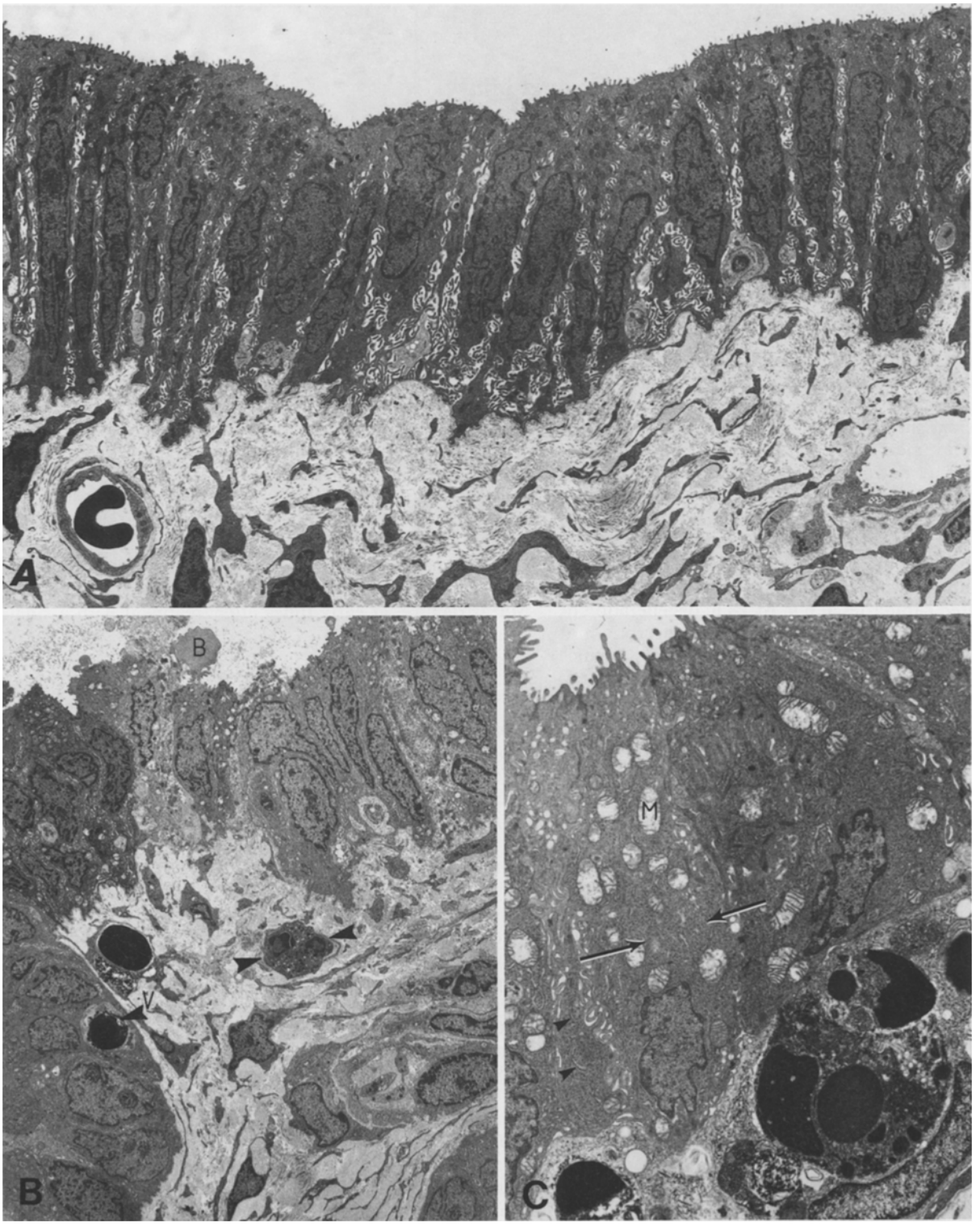

Fig. 1. Electron micrographs of common bile ducts **A** Control. ×3375. **B** Edema begins 6 h after CCNU treatment and is accompanied by PMN infiltration of interstitium *(arrowheads)*, some loss of microvilli, and bleb formation *(B)*. Note bile contained within vacuoles *(V)*. ×2100. **C** At 12 h after CCNU there is mitochondrial degeneration *(M)* with abundant free ribosomes *(arrows)* and RER *(arrowheads)*. *Lower right corner*, bile contained in vacuole. ×7300

Results

Common bile duct

At 6, 12, and 24 h after CCNU, epithelial cells showed increasing amounts of rough endoplasmic reticulum (RER), free ribosomes, and mitochondrial degeneration consisting of swollen mitochondria with decreased numbers of cristae (Table 1, Figs. 1C, 2). Bile imbibition was seen at 6 h and was increasingly severe at 12 and 24 h, as evidenced by dilated vacuoles containing electron-dense and/or electron-

Table 1. Fine structural changes in bile ducts 6, 12 and 24 h after CCNU

Findings	Common bile duct			Interlobular bile duct			Bile ductule		
	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
I. Epithelial cells									
Increase in RER	±	+	++	±	+	+	±	±	+
Increase in free ribosomes	+	++	++	±	+	++	±	±	+
Increase in SER	+	++	++	±	±	++	±	±	±
Mitochondrial degeneration	±	++	++	0	±	+	0	0	0
Bile imbibition in dilated vacuoles	±	++	++	±	+	++	0	0	0
Widened intercellular spaces	0	±	++	±	+	++	0	0	±
Necrosis	±	±	++	0	0	++	0	0	0
Microvillar changes	±	+	++	±	±	++	0	0	±
Lumen dilation	±	+	++	±	+	++	0	0	0
II. Interstitium									
Edema	±	+	++	0	±	++	0	±	±
Inflammatory cells	±	+	++	±	+	++	0	±	±
Infiltrating epithelial cells	±	+	++	0	±	++	0	0	0
Extravascular fibrin	0	0	0	0	±	+	0	0	0

++, severe; +, moderate; ±, mild; 0, not seen

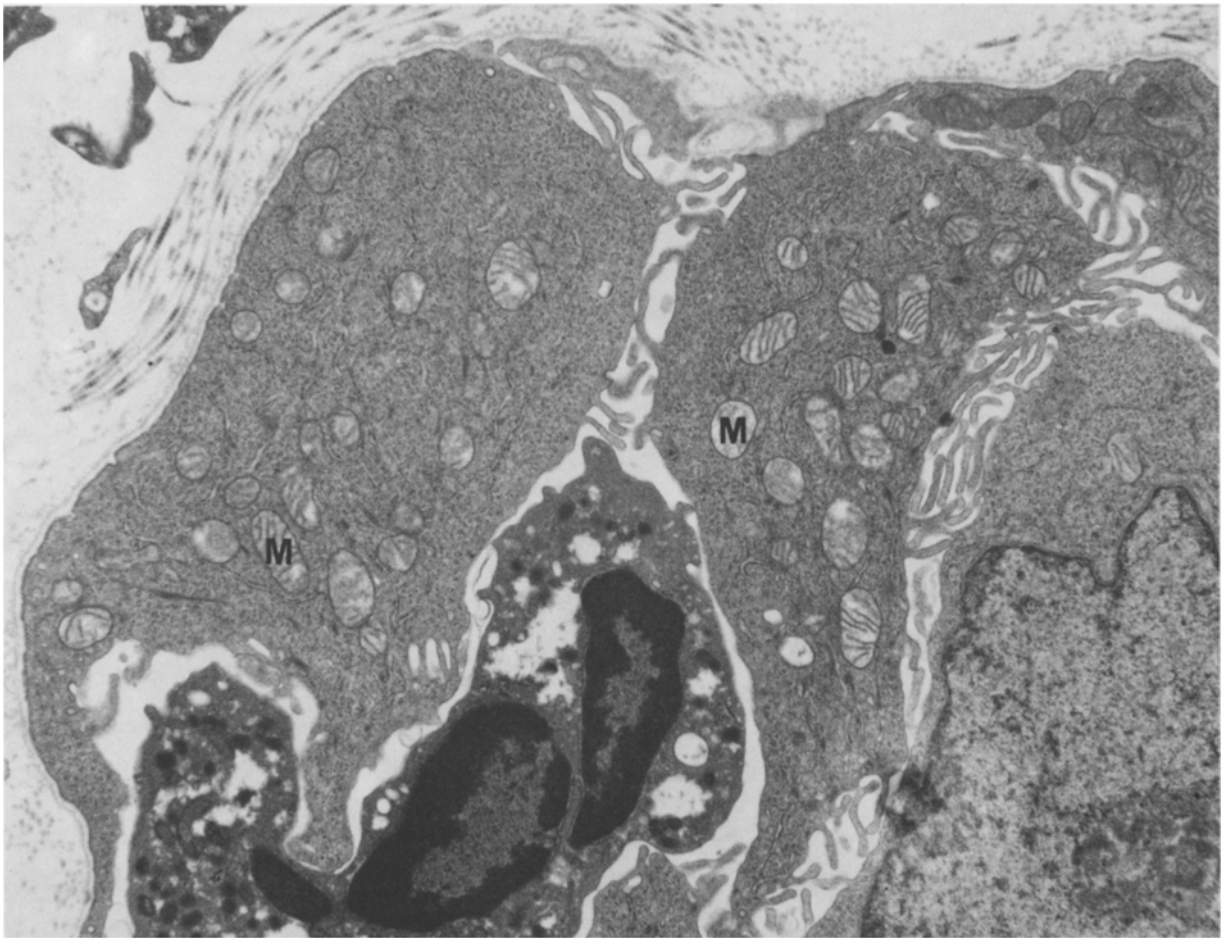

Fig. 2. Higher power electron micrograph:common bile duct at 24 h at the base of the epithelium. There is marked degeneration of mitochondria *(M)* with swelling and loss of cristae, and accumulation of free ribosomes in cytoplasm. Note PMN infiltrating within epithelium. ×12900

lucent material (Fig. 1B, C). Dilated capillaries were also seen at 6 h. The interdigitated membranes at the basal portion of the epithelial cells were separated by widened intercellular spaces at 24 h. Microvillar changes consisting of loss of microvilli and bleb formation appeared at 6 h and worsened by 12 and 24 h (Fig. 1B). The duct lumen also became increasingly dilated in conjunction with edema of the interstitium. Necrotic and sloughing cells were seen by 24 h (Fig. 7). Inflammatory cells (consisting predominantly of PMNs) with a few macrophages were infiltrating the periductal interstitium at 6 h and were seen within the epithelial cells at 24 h (Figs. 1B, 2).

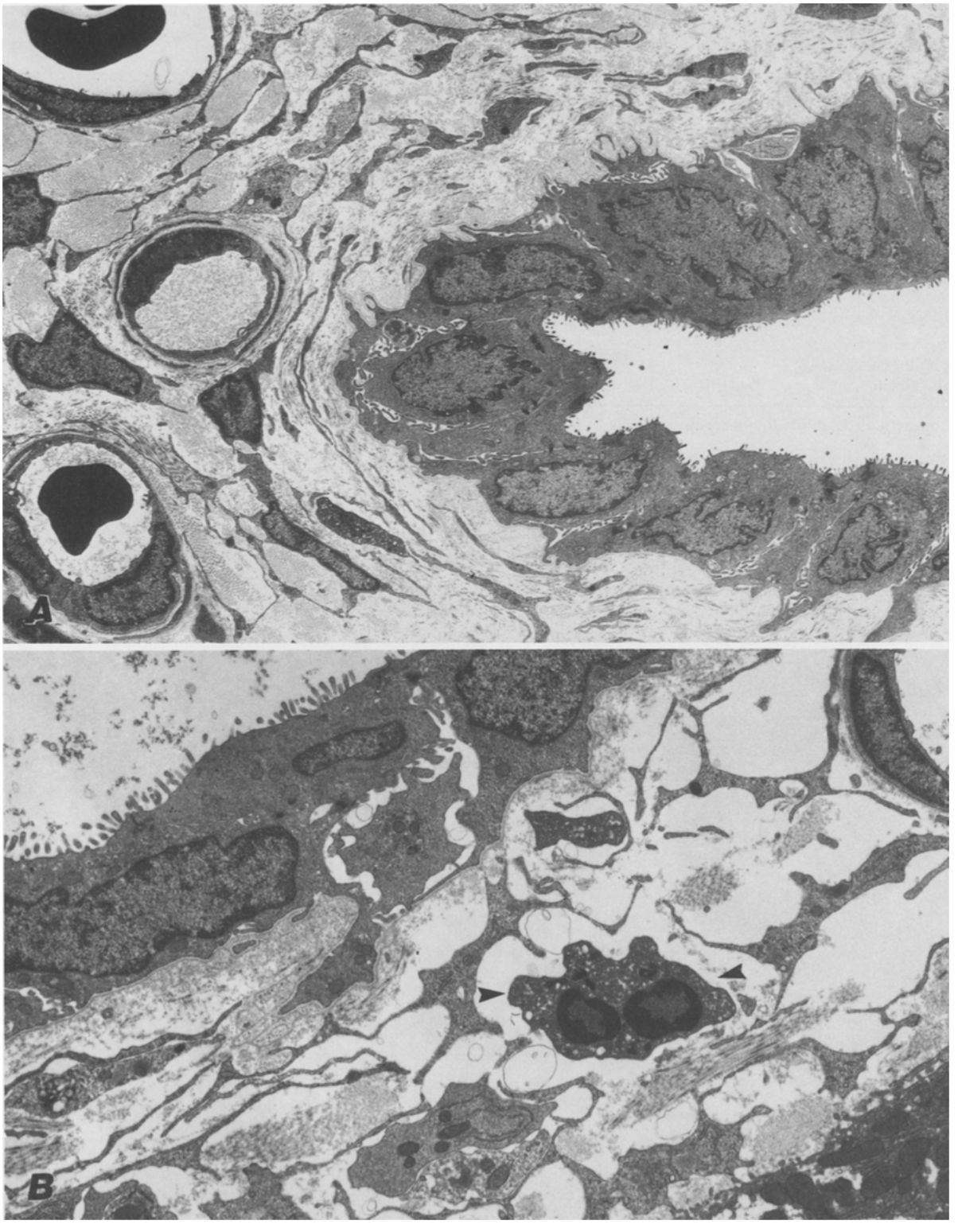

Fig. 3. Interlobular bile ducts. **A** Control. ×4250. **B** Slight dema is seen 6 h after CCNU with infiltrating PMN *(arrowheads)*. Note that the epithelial cells are normal. ×6944

Interlobular bile duct

At 6 and 12 h the RER was prominent, whereas free ribosomes were significantly increased at 24 h in the epithelial cells (Table 1). At 6 and 12 h mitochondrial degeneration was noted; at 24 h the degenerative changes were more severe. Bile imbibition in dilated vacuoles was also most abundant at 24 h (Fig. 4). The widened intercellular spaces at the basal membranes of epithelial cells were markedly widened at both 12 and 24 h (Fig.4). Dilated capillaries were also demonstrated at 24 h. Necrotic and sloughed epithelial cells were occasionally seen at 24 h (Fig. 7). Thinned epithelial cells, with a loss of microvilli and bleb formation due to ductal dilation, were seen beginning at 12 h; complete loss of microvilli occurred by 24 h (Fig. 4). At 12 and 24 h, small amounts of extravascular fibrin with a characteristic cross-banding pattern (Fig. 4) were seen in the interstitium near the epithelial basement membrane. Macrophages and PMNs started infiltrating the interstitium at 6 h and progressed into the epithelial cells by 12 h. Necrotic PMNs were noted in the duct lumen at 24 h (Figs. 3B, 4).

The bile canaliculi were usually dilated with a loss of microvilli at 12 h. Intracanalicular bile thrombi were also present at 12 h. Bile thrombi appeared to be mostly laminated, curled, and polymorphous.

Bile ductule

Bile ductules showed normal epithelial cells at all times examined (Table 1). The most significant change noted was macrophage infiltration near the base of the epithelial cells at 24 h (Fig. 5B). The interstitium appeared only slightly edematous, even at 24 h, although capillary dilation was present by 6 h.

Canaliculi and hepatocytes

The hepatocytes adjacent to ducts examined remained essentially normal. At 6 h, the hepatic bile canaliculi showed dilation, loss of microvilli, and intracanalicular bile thrombi (Fig. 6).

Discussion

The antineoplastic nitrosourea CCNU causes a unique hepatic injury which is localized in the larger common bile duct and interlobular bile duct with relative sparing of the smaller bile ductule. The major initial damage by CCNU begins 6 h after administration with interstitial inflammatory cell infiltration, edema, and duct dilation. Injury of duct epithelial cells occurs later (12–24 h) and is evidenced by bile imbibition in dilated vacuoles and widened intracellular spaces. Early organelle proliferation (RER) and damage (especially of mitochondria) is also noted, and at later times the epithelial cells become necrotic and slought into the duct lumen. The hepatocytes, however, remain essentially normal in the early times examined in this study, although bile canaliculi become dilated and contain bile thrombi. Hepatocyte damage occurring subsequent to the bile duct injury is localized near portal triads, suggesting that hepatocyte injury may be due to diffusion of toxic metabolites out of portal areas or to the bile stasis itself.

In this study, localized inflammatory edema rapidly accumulated in the interstitium surrounding bile ducts. This edema probably resulted from increased endothelial

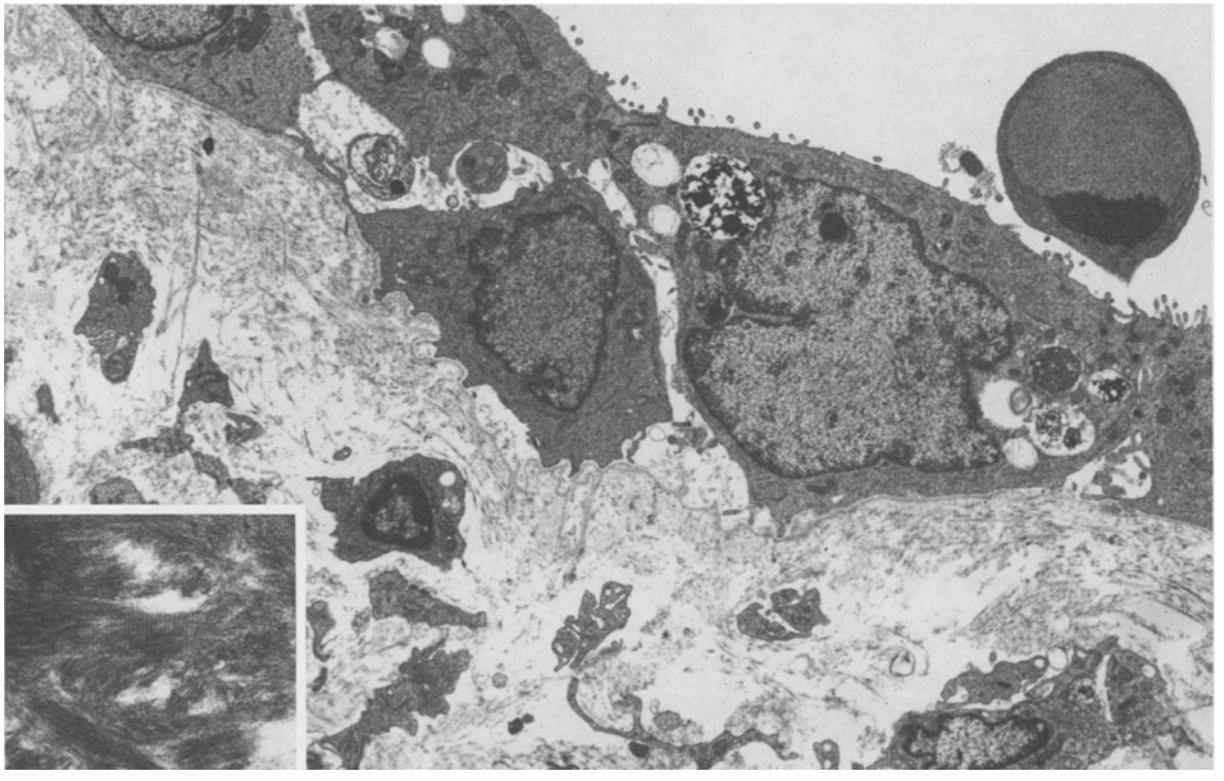

Fig. 4. Interlobular bile duct 24 h after CCNU. The epithelial cells have lost microvilli; cellular debris is now present in lumen. Note bile within vacuoles and widened intercellular spaces. × 5940. The edematous interstitium also contains scattered fibrin *(inset* × 29750)

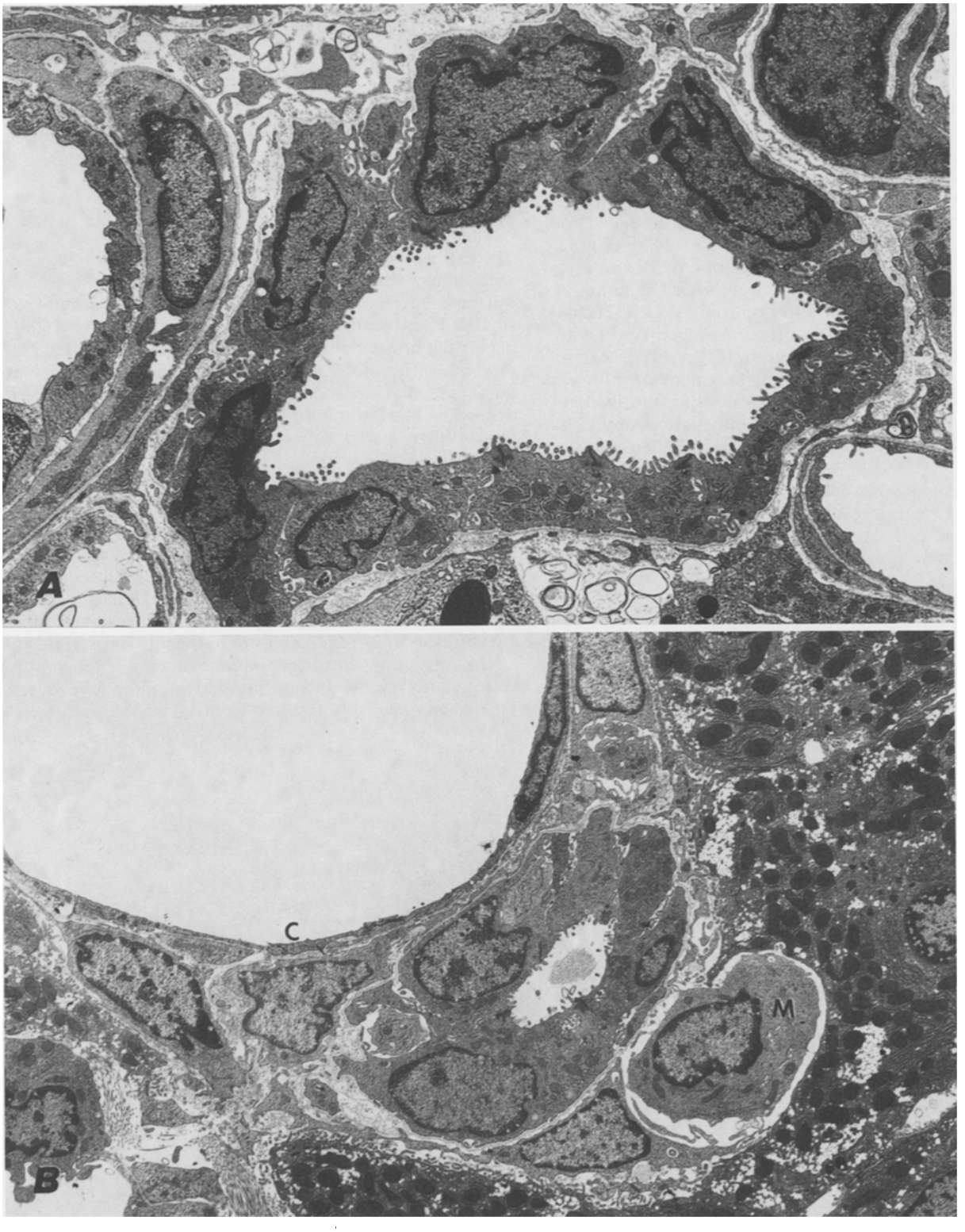

Fig. 5. Bile ductules. **A** Control. ×7000. **B** 24 h after CCNU the epithelial cells are normal with a few macrophages infiltrating *(M)*. Note dilated capillary *(C)* ×4675

cell permeability as evidenced morphologically by dilated capillaries in the interstitium. The proposed mechanism of in inflammatory edema is that vasodilation and increased endothelial cell permeability are mediated by the release of histamine from tissue mast cells and the activation of kinins [9]. Both cause vascular dilation (which was evidenced at early times in this study) and migration of PMNs and macrophages into surrounding connective tissue (which was also prominent at the later times in our study). The presence of fibrin in the ductular interstitium at later time-points is also an indication of capillary cell injury.

Following the formation of periductal inflammatory

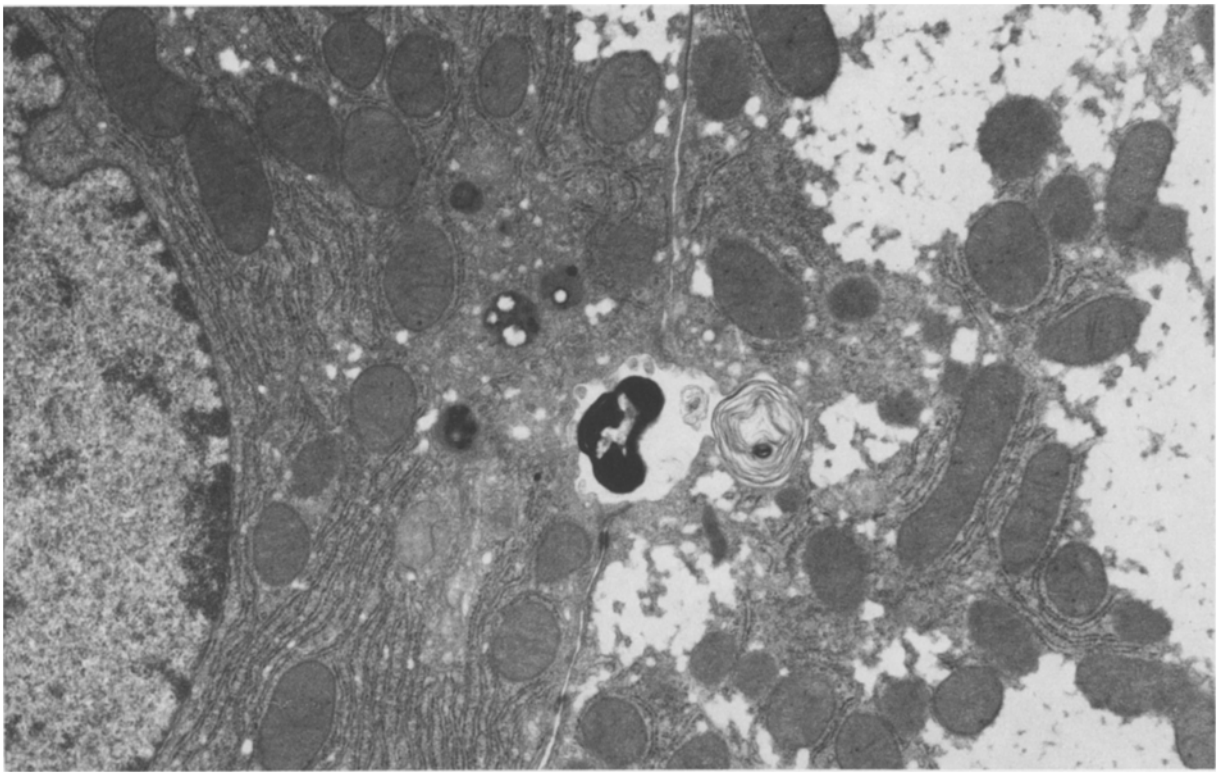

Fig. 6. At 12 h after CCNU the hepatocanaliculi contain bile thrombi consisting of lamellar membranous whorls. Canaliculi are dilated and have lost microvilli. × 15000

edema, duct epithelial cells exhibited a variety of degenerative changes in this study. Initially, free cytoplasmic ribosomes and highly developed RER within the epithelial cells were markedly increased in number, indicating increased protein metabolism, which might conceivably be caused by CCNU metabolites secreted or absorbed into the cells. This phenomenon has been previously observed in bile ducts of rats fed δ-naphthyl isothiocyanate [1, 3].

In previous biochemical studies of CCNU toxicity, it has been shown that serum conjugated bilirubin levels increase dramatically over time, beginning at 24 h [1, 3]. Hyperbilirubinemia associated with bile stasis may reflect regurgitation of bile into the bloodstream. Tanikawa [18] demonstrated ultrastructurally at least three main routes of bile regurgitation into the bloodstream: (1) transhepatocytic; (2) through communication of the bile canaliculus with the space of Disse; and (3) through the bile ductule. In cholestasis, the bile canaliculus is dilated with loss or stunting of its microvilli and widening of the intercellular space. Microvilli develop along the lateral cell border [12, 17, 21]. Bile regurgitation through the bile ductule is presumably due to enhancement of the absorptive function of the ductule epithelial cells during cholestasis. Morphologically, due to increased absorption, bile precipitation or bile imbibition (such as noted in the present study) occurs within the epithelial cells and widened intercellular spaces are also noted.

Following the toxic injury of bile duct epithelium observed in the present study, bile stasis most likely results in bilirubin or bile acids regurgitating through the epithelial cell into the portal tissue space and draining either into the lymph vessels or directly into the sinusoidal bloodstream, as has been shown in previous experimental models of bile stasis [10].

Studies in our laboratory and others [8] have indicated extensive uptake of ^{14}C-labeled CCNU by the liver. Autoradiographic studies in our laboratory (unpublished results) have shown that bile ducts accumulate ^{14}C-CCNU more than any other area of the liver. CCNU is known to form a highly reactive and alkylating moiety, a chloroethyl species, and a carbomylating moiety. The cytotoxic action of the latter is negligible. The chloroethyl species, however, is a highly reactive cytotoxic agent. It is our hypothesis that the biotransformation of CCNU to these active moieties occurs extensively in the bile duct. Once these reactive metabolites form in the bile duct they may damage the most adjacent cells, resulting in the observed injury to bile duct epithelium. The mechanism directly involved needs to be studied and represents the current line of investigation of CCNU toxicity in our laboratory.

In summary, CCNU causes a unique injury in the common and interlobular bile ducts. These ultrastructural time-course studies show that CCNU hepatotoxicity is first observed as interstitial edema associated with inflammatory cell infiltrates, progressing to degeneration of duct epithelial cells. Within the time studied, the hepatocytes remain essentially normal.

The injury which we have described is consistent with drug-induced cholestasis, and we propose that bile ducts are the initial target in CCNU-induced hepatotoxicity. Previous studies of drug-induced cholestasis have usually focused on hepatocellular and canalicular changes as the major injuries, and have usually ignored the bile ducts. We suggest a new area (i.e., bile ducts) for the study of choles-

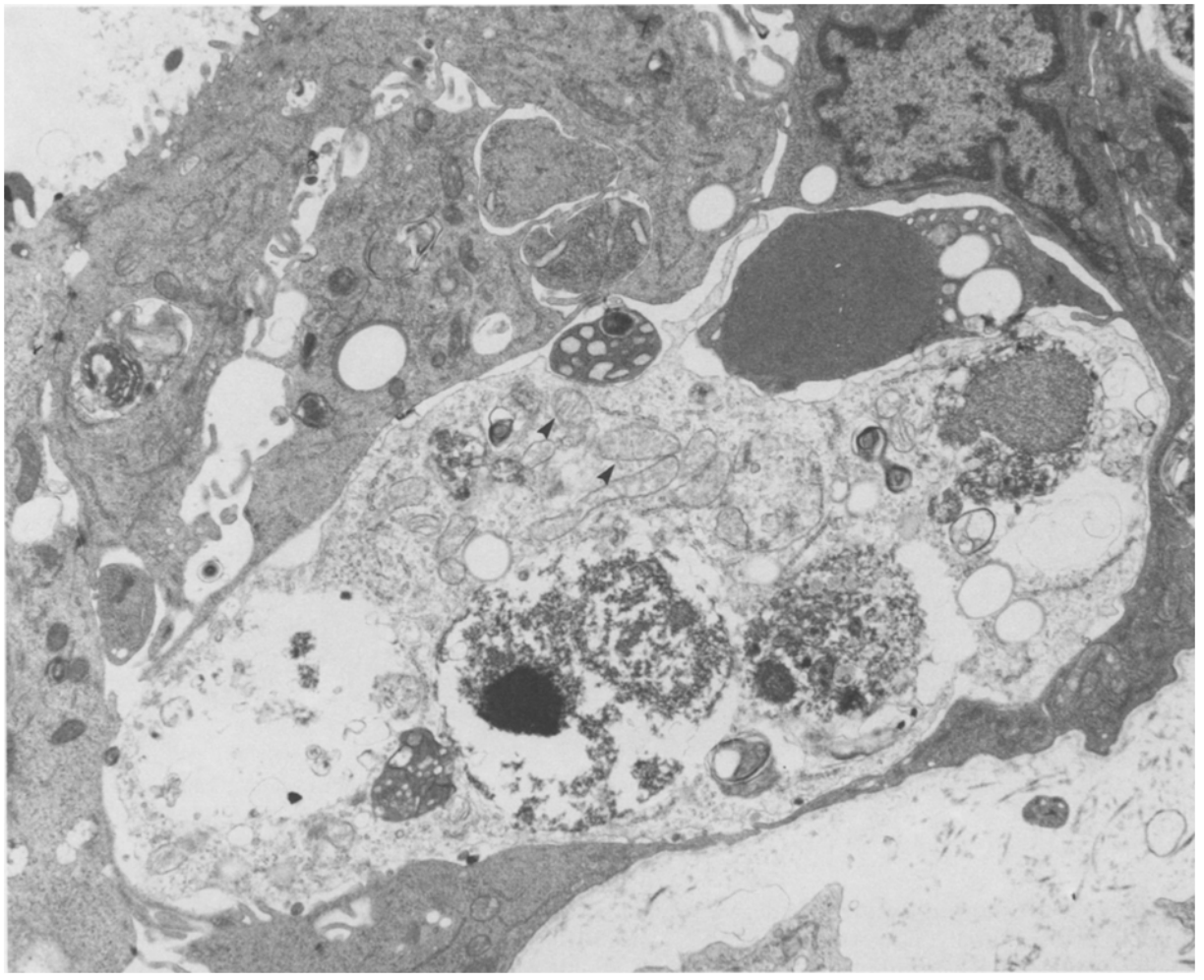

Fig. 7. Necrotic epithelial cell of common bile duct 24 h after CCNU shows extensive necrosis of epithelial cells with an invading macrophage which contains normal mitochondria *(arrowheads). L*, lumen. ×13100

tasis, and we propose an alternative mechanism of cholestatic injury by chemicals.

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