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The hepatotoxicity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in rats

Ultrastructural evidence of a delayed microtubular toxicity

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Summary. A few cases of liver involvement have been reported in patients receiving treatment with the antineoplastic nitrosourea CCNU. A single oral dose of 20 or 50 mg/kg CCNU in female Wistar rats induced an important increase in transaminases between day 2 and day 6, followed by a second, moderate increase between day 21 and day 28. Alkaline phosphatases and conjugated hyperbilirubinemia (threefold-increase) were noted for the two doses and were greater for the highest dose. Histological and ultrastructural studies disclosed hepatic lesions of two types: during the first phase of transaminase increase, inflammation of the portal tracts; during the second phase marked dilation of bile canaliculi and numerous filamentous bundles distributed at random throughout the liver cell cytoplasm like normal microtubules. Thus, CCNU induced pericholangitis and intrahepatic cholestasis with microtubular abnormalities. The long-term evolution of hepatic alterations revealed that in the 3rd month after a single oral dose of 20 mg/kg CCNU, lesions were persistent but stable; no reversibility was observed in the 3rd month after 50 mg/kg CCNU, and evolution towards cholangiolysis and biliary cirrhosis was noted. We suggest that CCNU causes a bimodal hepatotoxicity in rats: an early and prolonged ductal injury and a delayed anti-liver cell microtubule toxicity.

Introduction

Many nitrosourea derivatives have been synthesized and screened for potential antineoplastic activity; only three have exhibited exceptional carcinostatic potential and are in current clinical use: 1,3-*bis* (2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(4-methyl cyclohexyl)-1-nitrosourea (MeCCNU). These drugs are highly lipid-soluble, cross the blood-brain barrier, are distributed widely to tissues, and have an extremely short plasma half-life. They decompose nonenzymatically at relatively rapid rates, and their biotransformation products are bound to macromolecules through alkylation of nucleic acids and proteins as well as through carbamylation of proteins in intact cells [30].

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CCNU is at least as or more active than BCNU against L1210 leukemia cells implanted intraperitoneally or intracerebrally in mice [7]. It is more lipid-soluble, which might enhance passage across the blood-brain barrier. In contrast to BCNU, CCNU has only a single chloroethyl group and a cyclohexyl group.

Although the clinically used nitrosoureas are not potent hepatotoxins at the therapeutic doses, the liver toxicity of these drugs is well known, particularly when BCNU is used in high doses followed by autologous bone marrow transplantation [18, 37]. Liver damage was observed in experimental animals with inoculation of a single oral dose of BCNU [38] and was confirmed in humans during phase I and II trials. In an early phase I trial of BCNU, De Vita et al. [11] reported changes in hepatic functional values occurring in up to 26% of patients. In later phase II trials [24, 27], a few patients developed hepatic abnormalities with usual doses of BCNU; these were transient in some cases, whereas in the others the damage may have contributed to death.

CCNU was first evaluated in clinical trials in the late 1960s; it has since been used on a broad spectrum of tumors, especially those of the brain (for review see [39]). Clinical tolerance is relatively good; however, toxicity is manifested as acute nausea and vomiting and as delayed, dose-limiting bone marrow suppression. In humans, CCNU is less hepatotoxic than BCNU at clinically used doses; however, cases of liver toxicity have also been reported [10, 20, 21, 28]. The hepatic toxicity of CCNU in dogs and monkeys has been noted by Carter and Newman [7], and recent studies in rats [1, 14, 22] have revealed by light and electron microscopy that CCNU causes interlobular bile duct and common bile duct injuries associated with cholestasis at early times after treatment with a single oral dose of this drug.

The mechanism of nitrosourea-induced hepatic toxicity is still not clear. The objective of the present study was to evaluate the experimental hepatotoxicity of CCNU at later stages following the inoculation of a single oral dose of CCNU.

Materials and methods

Experimental procedures. Female Wistar rats weighing approximately 200 g with free access to food and water throughout the study were given a single dose of CCNU by gastric intubation. CCNU was dissolved in 1% carboxy-

methyl cellulose solution so that the final volume given was 1 ml. Control rats ($n = 20$) received only carboxymethyl cellulose solution in an equivalent volume. A total of 216 animals were treated: 108 received 20 mg/kg CCNU (equivalent to 120 mg/m^2), and 108 received 50 mg/kg (300 mg/m^2). The animals were sacrificed at regular intervals: 2, 4, 6, 8, 15, 21, 28, and 90 days posttreatment.

The rats were placed in diuresis cages for two consecutive 24-h periods before sacrifice. The presence of bilirubin in the urine was evaluated using Multistix strips. Blood specimens were taken from the aorta at the time of sacrifice: 2–3 ml for hematologic study was collected on 0.06 ml 8.5% EDTA K3 and agitated immediately; 2–3 ml was collected in a dry tube.

Blood counts were carried out using a Coulter Electronics Counter (Inc, Hialeah, Florida, USA). The erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were determined at regular intervals between the 1st and 90th days in both groups. Assays of total and direct-reacting plasma bilirubin were carried out using a colorimetric assay with a Boehringer-Mannheim Diagnostica kit; glutamic-pyruvic transaminase activity was assayed colorimetrically with a Bio-Merieux test kit. Alkaline phosphatase activity was assayed colorimetrically with a *p*-nitrophenylphosphatase substrate [5]. Statistical analysis was carried out using Student's *t*-test for the comparison of means.

Morphological studies. Histological and ultrastructural studies were carried out with groups of 12 animals, 4 of which were chosen at random for sacrifice at the times indicated above. Two of these animals were used for light microscopic study and the other two for electron microscopic study.

For light microscopy, the animals were perfused with 100 m*M* phosphate buffer (PBS) (pH 7.4) and liver specimens were fixed in MFA solution (methanol, 85 ml; formol, 10 ml; acetic acid, 5 ml/100 ml) for 3–4 h. After dehydration in graded methanol solutions, specimens were embedded in paraffin and 3-μm sections were stained with hematoxylin-eosin, periodic acid-Schiff, and Masson trichromic stain.

For electron microscopy, the animals were perfused with PBS solution, followed by 2.5% glutaraldehyde solution in 0.1 *M* cacodylate buffer (saccharose 5%, pH 7.3). Fixation was continued for 1 h after the dissection of 1-mm^3 blocks. Postfixation was carried out with 2% osmium tetroxide for 1 h. After dehydration in a graded ethanol series and embedding in epoxy resin (glycidether 100, Merck, Darmstadt, FRG), ultrathin sections were cut on a Reichert OM U-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips CM 10 transmission electron microscope.

Results

No death was seen with any of the treatments.

Changes in the blood count

There was no significant change in the erythrocyte count, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, or mean hemoglobin concentration in groups receiving 20 or 50 mg/kg CCNU. There was a significant decrease in hematocrit in animals receiving 20 mg/kg in comparison with controls (39.3 ± 2.7% vs 42.9 ± 1.7% in controls $P < 0.05$).

Specific liver function tests

(a) Hepatic transaminases (Fig. 1). At a dose of 20 mg/kg, treated animals showed a biphasic rise in transaminase activity (SGPT). There was a clear increase between the 2nd and 6th days, reaching a value of 403 ± 68 IU/l on the 4th day (48 ± 24 IU/l in the controls), with a return to normal between the 8th and 15th days. There was a second rise between the 21st and 28th days (215 ± 32 IU/l).

At a dose of 50 mg/kg, a major increase in SGPT activity (809 ± 80 IU/l) was seen beginning on the 2nd day of treatment. This sudden rise was followed by a rapid decrease, however, renormalization was not attained (167 ± 74 IU/l). A second rise was noted on the 15th day (400 ± 20 IU/l), followed by a new decrease, at no time returning to normal values.

(b) Serum alkaline phosphatase (Fig. 2). At a dose of 20 mg/kg CCNU, there was a two-fold rise in serum alkaline phosphatase between the 4th and 8th days. The greatest increase was seen on the 6th day (111.2 ± 20.7 IU/l in treated animals vs 61.1 ± 11.3 IU/l in controls). Between the 8th and 28th days alkaline phosphatase decreased, returning to normal in this group.

At a dose of 50 mg/kg CCNU, serum alkaline phosphatase showed an increase beginning on the 2nd day of treatment and remained significantly elevated beyond the 28th day.

(c) Serum bilirubin. A slight elevation in serum bilirubin levels developed between the 6th and 8th days of treatment with 20 mg/kg CCNU; there was no bilirubinuria.

At a dose of 50 mg/kg CCNU (Fig. 3), conjugated hyperbilirubinemia was noted beginning on the 2nd day and reaching a maximum on the 8th day (43 ± 18 mg/l vs 2.6 ± 1.0 mg/l in controls). Although there was a subsequent decrease in values, they remained very high between the 15th and 28th days. A parallel but lower rise in free bi-

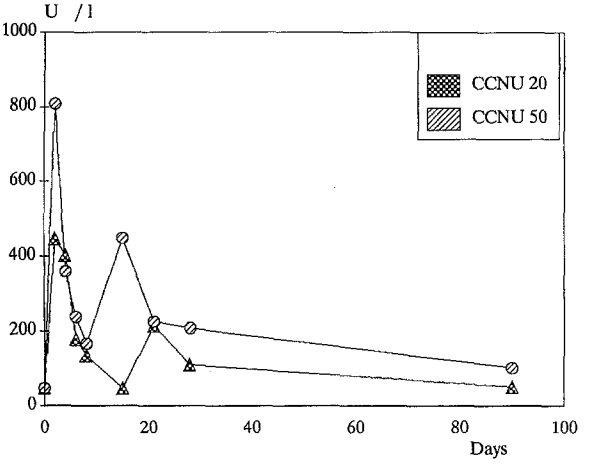

Fig. 1. Changes in serum transaminase activity (SGPT) with doses of 20 or 50 mg/kg p.o. CCNU as a function of time (mean for 6 experimental points)

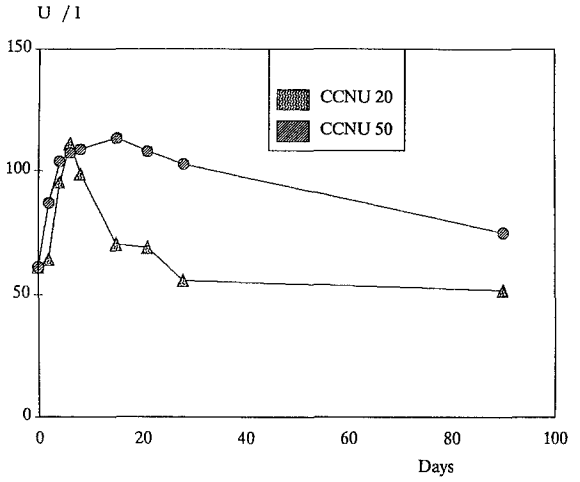

Fig. 2. Changes in serum alkaline phosphatase activity with 20 or 50 mg/kg p.o. CCNU on days 0–90 (mean for 6 experimental points)

lirubin was noted on and after the 2nd day. A maximum was noted on the 21st day (11.5 ± 4.6 mg/l vs 1.0 ± 0.3 mg/l in controls), and this value remained abnormally high throughout the period of treatment. A very marked bilirubinuria appeared on the 4th day in all rats treated with 50 mg/kg CCNU.

Histopathology

CCNU 20 mg/kg. Microscopic examination disclosed edema and inflammation of the portal tracts starting on the 2nd day of treatment. The portal tracts were slightly enlarged and contained a diffuse accumulation of lymphocytes, plasma cells, and eosinophils. There were no vascular changes or lesions in the bile ductules. The remainder of the liver appeared normal.

On the 8th and 15th days posttreatment, these changes were associated with focal parenchymatous lesions: the accumulation of inflammatory cells (lymphocytes, histocytes, granulocytes) and the presence of a few necrotic liver cells. From the 28th day to the 3rd month posttreatment, liver damage was not severe. Edema and moderate inflammation of the portal tracts were associated with a few modifications in the bile ductules' epithelial cells. Consequently, this liver injury was not an active one; however, it gave evidence of the persistence in liver abnormalities after a single oral dose of 20 mg/kg CCNU in rats.

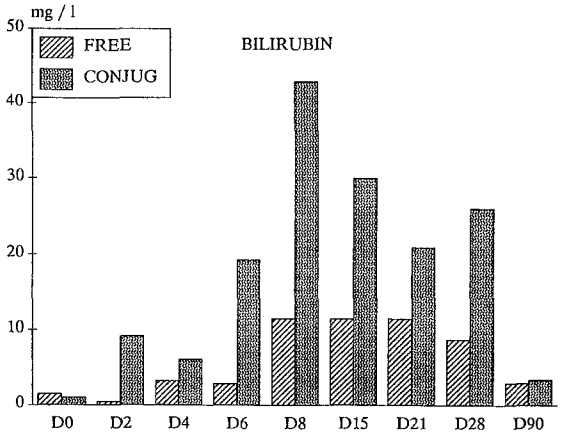

Fig. 3. Pattern for elevation in free and conjugated serum bilirubin at different days after a single p.o. dose of 50 mg/kg CCNU (mean for 6 experimental points)

CCNU 50 mg/kg (Fig. 4). Edema and portal inflammation were both noted beginning on the 2nd day after treatment; however, the magnitude of the effect was greater and there were some necrotic liver cells.

Between the 4th and the 21st days, portal tracts still had an inflammatory aspect, with lymphocytes and histiocytes surrounding the bile ducts. There was minimal steatosis, and numerous necrotic hepatocytes surrounded by inflammatory cells were seen.

On the 28th day, injury was only seen in portal tracts. Ductular epithelial cells were less numerous and they showed lytic alterations, with reduced cytoplasm and hyperchromatic nucleus (Fig. 4B). Bile ducts and ductules still had a perceptible lumen. Most infiltrating inflammatory cells in the portal regions were lymphocytes and plasma cells admixing with numerous mastocytes.

In the 3rd month, portal bile ductules were surrounded by and embedded in a dense fibrous tissue. The ductular epithelium tended to disappear and only a few remaining degenerative and necrotic cells were seen. In some portal areas bile ductules totally disappeared (Fig. 4C), whereas in others ductular proliferation was found. Changes in liver trabeculae were also observed: in the periportal area liver cells were large (ten fold greater than a normal hepatocyte), with hypertrophic nucleus and acidophilic cytoplasm; in the centrolobular area hepatocytes were small and regular similar to normal cells (Fig. 4A). This lobular disarray was associated with a widening of portal tracts and a mild perilobular fibrosis. Such alterations closely resembled those observed in the advanced precirrhotic stage of biliary cirrhosis.

Ultrastructural study

In control rats, parenchymal liver cells were moderately rich in glycogen. Cytoplasmic organelles were normal and some lipid droplets were seen. Bile canaliculi were of normal size, with no reduction of canalicular microvilli. No modification in the pericanalicular ectoplasm was observed, and microtubules were seen at random in the cytoplasm. The sinusoids were slightly dilated, with a few circulating lymphocytes.

CCNU 20 mg/kg (Fig. 5). Hepatocytes were not markedly altered, whatever the delay posttreatment. Nuclei and cell organelles were normal. Minor lipid droplets appeared on the 15th day and were still present on the 28th day. No bile pigment overload of liver cells was seen before the 8th day, and this remained inconspicuous from the 15th to the 90th day posttreatment.

Two main alterations of parenchymal cells occurred after the 6th day: pathologic dilation of the bile canaliculi and modification of the cytoskeletal components. Bile canaliculi were abnormally and irregularly dilated, with a decrease in the number of microvilli (Fig. 5A). Numerous short bundles of filaments appeared. These bundles were dense, short, and broad, with frayed ends occasionally continuing with normal microtubules. They were seen at

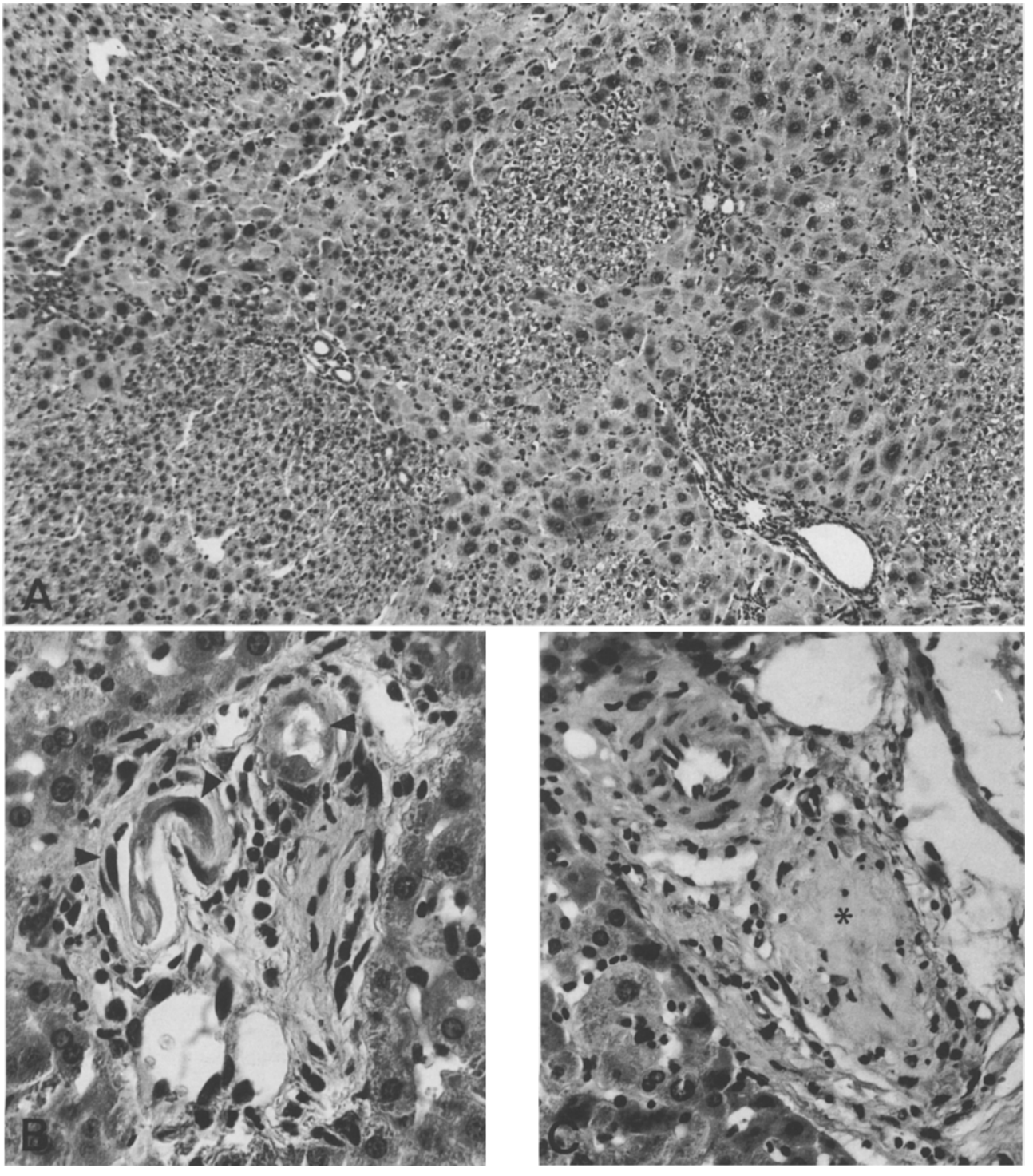

Fig. 4. Microscopic sections taken from the liver of rats after 50 mg/kg CCNU. **A:** 3rd month posttreatment: Lobular disorganization with hypertrophic periportal liver cells when centrolobular hepatocytes are of normal size (H & E stain. ×80). **B:** Day 28 posttreatment: Portal tract alterations with degenerative and necrotic epithelial cells in bile ductules (*arrowheads*) and mild interstitial inflammatory reaction (H & E stain. ×250). **C:** 3rd month posttreatment: Bile ductules totally disappearing in scarring fibrosis (*asterisk*) (H & E stain. ×200)

random in the cytoplasm but they were more numerous in the Golgi area, near plasma membranes, and near bile canaliculi. They were obviously distinct from the microfilamentous pericanalicular web, the morphologic structure of which was preserved, and they were reminiscent of altered microtubules (Fig. 5B); simultaneously, normal microtubules were distinctly reduced in number. These changes were very marked between the 6th and 15th days and affected all liver cells and bile canaliculi; they were more attenuated on the 28th day; only mild focal alterations persisted into the 3rd month, while normal microtubules reappeared.

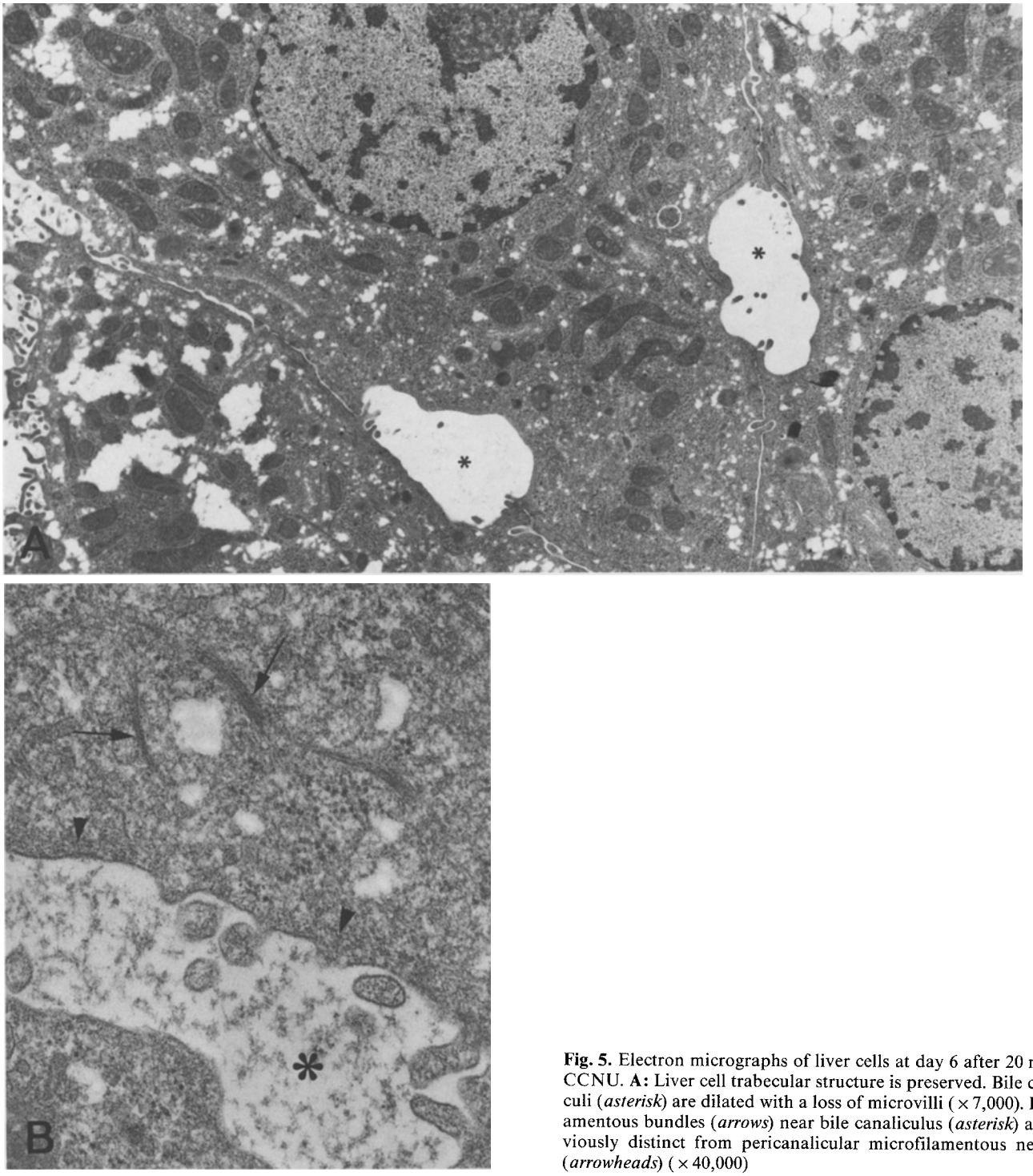

Fig. 5. Electron micrographs of liver cells at day 6 after 20 mg/kg CCNU. **A:** Liver cell trabecular structure is preserved. Bile canaliculi (*asterisk*) are dilated with a loss of microvilli (×7,000). **B:** Filamentous bundles (*arrows*) near bile canaliculus (*asterisk*) are obviously distinct from pericanalicular microfilamentous network (*arrowheads*) (×40,000)

The sinusoids were moderately dilated, containing a few lymphocytes and plasma cells. Endothelial and Kupffer cells, perisinusoidal space, and fat-storing cells were normal.

CCNU 50 mg/kg (Fig. 6). Lesions were more severe and more variously distributed. Swelling of a few hepatocytes was seen on the 6th day. On the 15th day, liver cell trabeculae were atrophic, with increased electron density of the cell cytoplasm. Some hepatocytes were necrotic, surrounded by neutrophilic granulocytes and macrophages. Kupffer cells were packed with large phagolysosomes.

There were alterations as previously described in bile canaliculi and cytoskeletal abnormalities, beginning very early (by the 4th day). The dilation of bile canaliculi, the reduction of canalicular microvilli, and the increased number of pericanalicular vesicles were quite severe. There were numerous, diffuse filamentous bundles, and this lesion was unchanged in the 3rd month (Fig. 6B and C). There was a distinct intracytoplasmic biliary pigment overload in these animals.

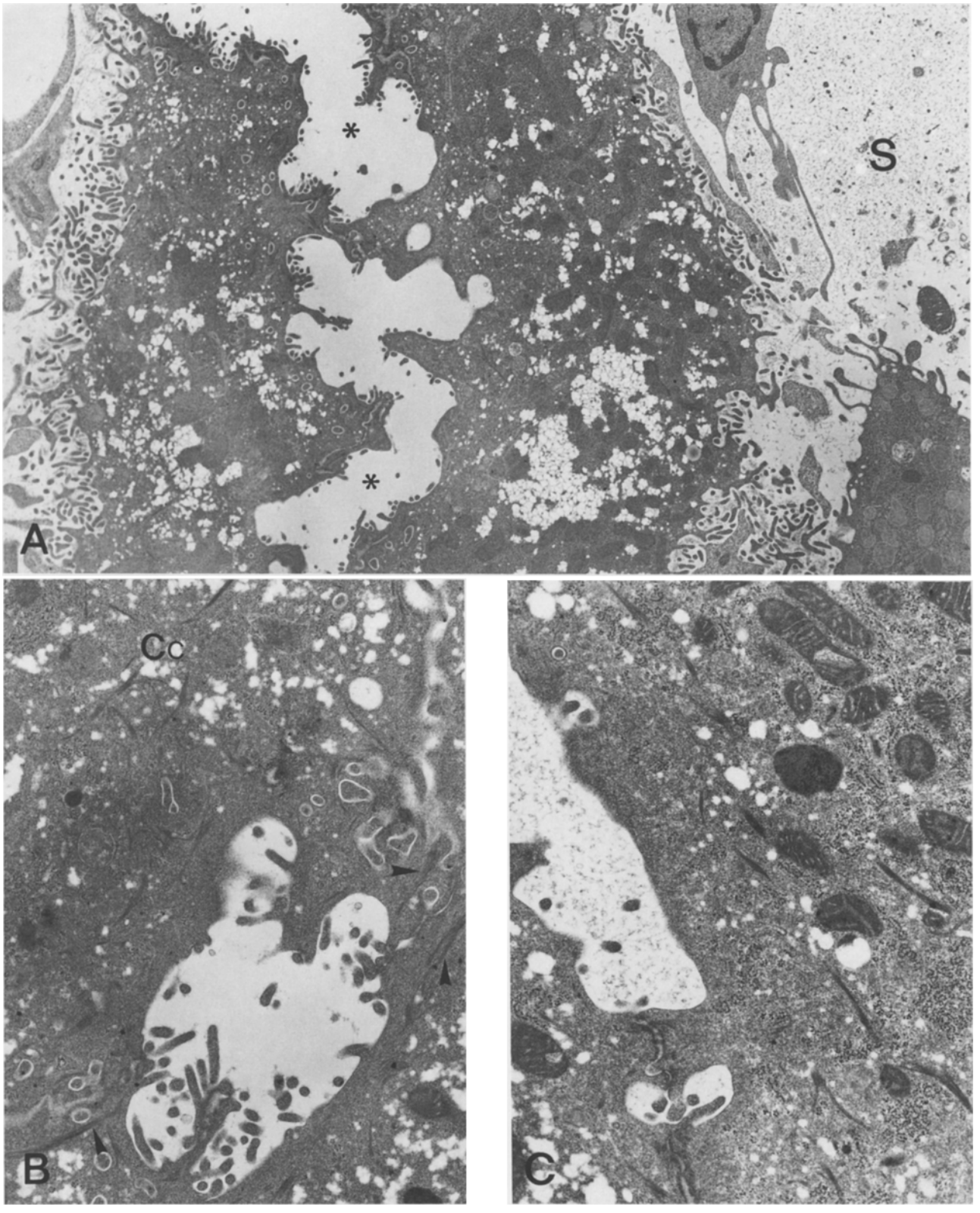

Fig. 6. Electron micrographs of liver cells at day 28 after 50 mg/kg CCNU. **A:** liver cell trabeculae are atrophic. Bile canaliculi (*asterisks*) are dilated. *S*, sinusoidal lumen (×6,400). **B:** filamentous bundles nearing the plasma membranes (*arrowheads*) and the cytocenter (*Cc*) (×16,000). **C:** filamentous bundles nearing dilated bile canaliculus (×16,000)

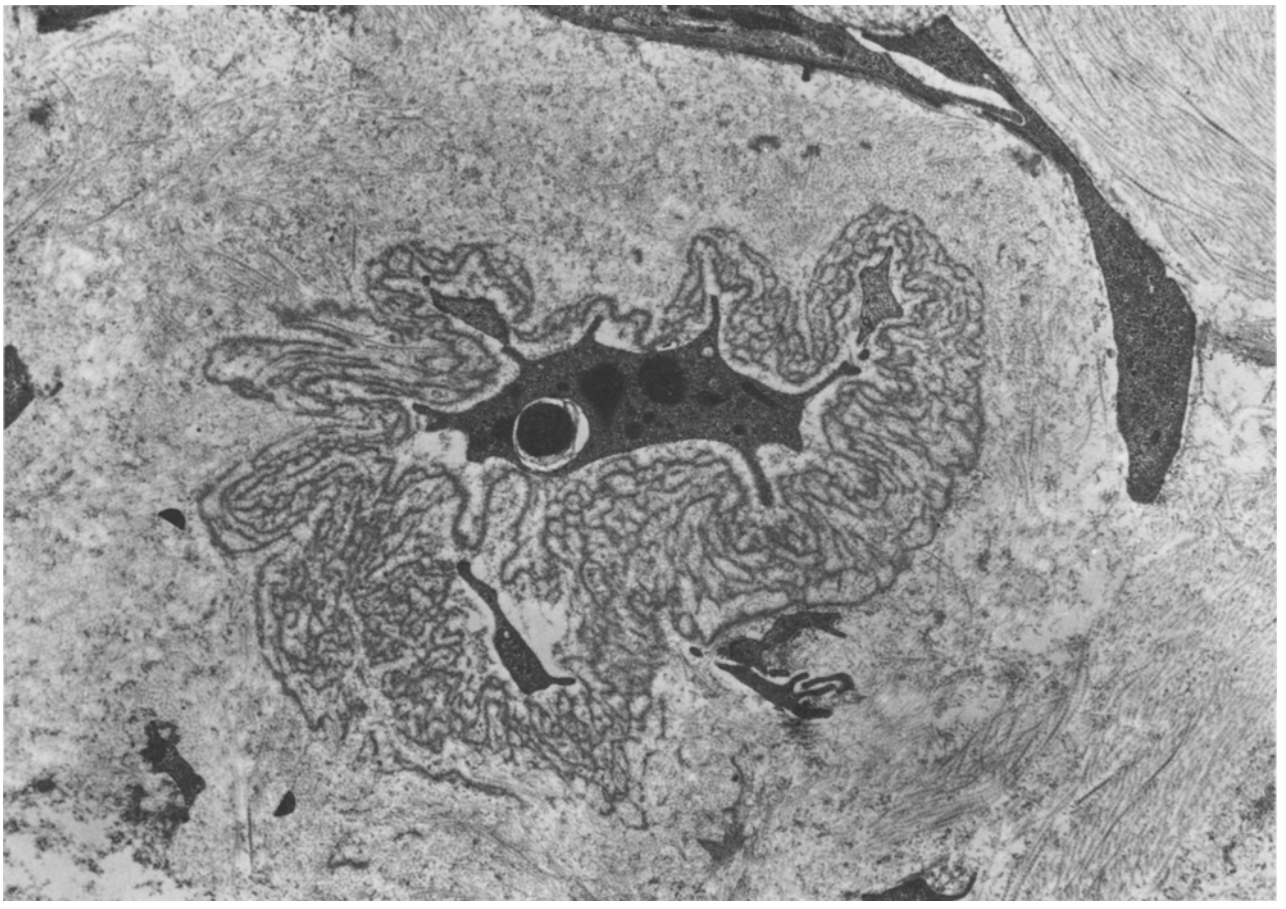

Fig. 7. Electron micrograph of bile ductule in the 3rd month after 50 mg/kg CCNU. A few cytoplasmic residues with degenerative changes are surrounded by multistratified basal laminae and are embedded in a dense collagen fibrosis (×9500)

In the portal areas, the interstitium showed infiltration by inflammatory cells (macrophages and neutrophilic granulocytes), along with major edema and mild fibrosis. On the 15th day, alterations in the bile ductules were seen; there was a slight ductular dilation with microvillar alterations, a widening of intercellular spaces, an increased amount of filaments in biliary epithelium, a stratification of basement membrane, and a periductal fibrosis. In the 3rd month, several bile ductules were almost completely destroyed (Fig. 7); only a few cellular residues without central lumen, surrounded by thickened and multistratified basement membrane were found.

Discussion

CCNU is a nitrosourea that has been used to treat brain tumors, Hodgkin's disease, non-Hodgkin's lymphomas, melanoma, lung cancer, and other solid tumors [39]. The clinical and biological tolerance for this cytostatic agent appears to be good at the therapeutic doses, and there have been few reports of hepatic toxicity after CCNU treatment in man. Nevertheless, Hoogstraten et al. [20, 21] and Moertel et al. [28] have mentioned a delayed elevation in hepatic enzyme activity starting 2–4 weeks after the first dose (130 mg/m^2), occasionally resolving 14 days later. De Labarthe et al. [10] have reported one case of hepatic toxicity in a woman who developed jaundice 4 months after the beginning of CCNU (total dose, 840 mg); clinical and laboratory signs of hepatopathy resolved on the 40th day.

In our study, a single oral dose of CCNU in rats caused a delayed and persistent hepatopathy. This effect was dose-dependent: the rise in alkaline phosphatase was transient at a dose of 20 mg/kg CCNU and constant between the 4th and 28th day at a dose of 50 mg/kg. In view of the dilated bile canaliculi, reduction of canalicular microvilli, and portal inflammation, the liver damage could be classed as cholestatic, with mixed portal and canalicular changes [33]. The early and major rise in transaminases coincided with the development of an inflammatory reaction in the portal tracts. The late and lesser increase in transaminases between the 15th and 20th days is concurrent with established canalicular dilation.

After giving rats 30 or 75 mg/kg BCNU, Thompson and Larson [38] have also noted the development of intrahepatic cholestasis. Conjugated hyperbilirubinemia was maximal at the 7th day but later shifted to free hyperbilirubinemia. A number of factors could be implicated in this elevation of unconjugated serum bilirubin [38]: the inhibition of glucuronyl transferase or deficiency of its UDPG substrate, the osmotic fragility of erythrocytes promoting hemolysis. In the present study, the hyperbilirubinemia seen with high-dose CCNU was the glucuroconjugated form, with a maximum on the 8th day. While in agreement with Thompson and Larson, we find a rise in free bilirubin

between the 8th and 28th days of high-dose treatment, it is difficult to attribute this to direct erythrocytic toxicity of CCNU since our hematologic results do not show any significant change.

In our animal study, the final evolution of hepatic damage was dose-dependent. At a dose of 20 mg/kg, liver function and hepatic morphology never completely returned to normal but did not progress from the 1st to the 3rd month. At a dose of 50 mg/kg, liver damage evolved irreversibly in spite of a single initial dose of CCNU. Indeed, in the 3rd month histopathologic study revealed true ductular cholangiolysis, focal ductular proliferation, and lobular disarray resembling scarring or the precirrhotic stage of biliary cirrhosis. The delayed cholangiolytic injury we observed in bile ductules is identical with that previously reported by Ahmed et al. [1] and Kretschmer et al. [22], who described the same lesions soon after treatment with a single oral dose of CCNU. This epithelial toxicity initially involving the larger bile ductules and bile ducts thus seems to reach eventually the whole biliary tree.

The histopathologic changes we described in the liver of CCNU-treated rats correspond quite closely with those that have been observed after single oral doses of BCNU in rats [38]. Furthermore, they also have some similarities to those produced by α-naphtylisothiocyanate [4]. These observations provide tempting speculation regarding the identity of the offending chemical substance, since it has been postulated that one of the breakdown products of BCNU [29] and of CCNU [34, 35] is an isocyanate derivative.

Pharmacologic disposition studies of CCNU indicate very rapid absorption, distribution, and metabolism. Oliviero et al. [30, 31] and Lee et al. [23] have demonstrated that only metabolites and degradation products could be identified in plasma within 1–6 h following oral drug treatment in animals and humans. The plasma levels of CCNU degradation products were prolonged; the primary excretory route of these products was through the kidneys, with biliary secretion and reabsorption from the gastrointestinal tract playing a predominant role [31]. Thus, the liver is exposed to relatively high concentrations of metabolic degradation products that might affect cellular function later.

The biotransformation products of CCNU have been reported to bind to macromolecules through the alkylation of nucleic acids and proteins as well as through the carbamylation of proteins in intact cells [8, 9]. Alkylation of proteins by CCNU occurs nonselectively compared with highly specific carbamylation by an active site-directed mechanism [2, 3]. Upon degradation CCNU has been reported to provide a 2-chloroethyl alkylating intermediate, possibly 2-chloroethyl carbonium ion [34, 35]. Such a biotransformation reportedly occurs extensively in the bile ducts and might then damage the most adjacent cells, resulting in the observed injury to the bile duct epithelium [22]; concurrently CCNU is rapidly hydroxylated by rat liver microsomes in the cyclohexyl moiety, and this hydroxylation is cytochrome P450-dependent [19, 26]. The various cyclohexyl isocyanates formed from hydroxy CCNU metabolites are apparently liable for carbamylation [26]. It is as yet uncertain what role carbamylation has in the antitumor effect of CCNU, and it has been suggested that carbamylation plays, at best, a minor part in either antitumor activity or normal tissue toxicity [39].

Brodie et al. [6] have explored the question as to whether drug-microtubule interactions may participate to some extent in the antitumor activity of nitrosoureas. They found that nitrosoureas that degrade to form isocyanates (CCNU, BCNU, MeCCNU) inhibit the polymerization of purified brain tubulin in a dose-dependent manner. The stoichiometry of the inhibition was related to the nitrosourea-derived isocyanates, and the ^{14}C-labelled-cyclohexyl moiety derived from CCNU appeared to bind covalently to the α and β tubulin monomers. Since in cultured human lymphoma cells an increased sensitivity to CCNU was observed in the early S phase of the cell cycle [12], this could reflect in part the disturbance by this drug of the degradation of tubulin occurring at this time [15]. The inhibition of tubulin polymerization or degradation by nitrosourea-derived isocyanates might thus participate in antitumor activity or tissue toxicity. Such a microtubular inhibition could act by alteration of the mitotic apparatus and cell division or by disturbance of the cell secretory functions such as bile secretion.

The evidence as to the role of microtubules in bile secretion is still conflicting. Some investigators have failed to show any effect of microtubule inhibitors on bile lipid secretion [16, 36], whereas others have provided evidence that the hepatocytic microtubular network participates in the translocation of lipids from microsomes to the canalicular membrane [17]. However, recent works have cast significant light on this controversy [13, 32]. They have found that microtubule inhibitors did not affect the basal bile flow and bile canalicular contractions but did block the expected increase in bile flow or contractions when choleretic bile acids were used; this suggests a permissive role for microtubules in bile flow and bile canalicular contractions [13, 32].

The antimitotic drugs colchicine and vinblastine, which have been studied for bile secretion inhibition, are characterized by a specific binding to tubulin. Colchicine, which inhibits the polymerization of tubulin into microtubules, has been reported to cause the almost complete disappearance of microtubules in the hepatocytes [13], and vinblastine reportedly leads to a conversion of microtubules to paracrystals [25]. In our experimental study, electron microscopy revealed abnormal, dense, filamentous bundles that were obviously distinct from the microfilamentous pericanalicular web. Their distribution throughout the hepatocyte cytoplasm was identical to that described for normal microtubules, which were simultaneously reduced in number; this phenomenon appears to parallel the degree of bile canaliculi dilation and is consistent with the aberration in microtubular structure. These observations suggest that microtubular alteration occurs in vivo in the liver cells of CCNU-treated rats and could explain the intrahepatic cholestasis induced by this drug.

The results of the present study indicate that CCNU causes a bimodal liver toxicity in rats. A ductal injury occurs soon after treatment and, with high-dose CCNU, progresses from initial inflammation of the portal tracts to the subtotal destruction of epithelial cells in the whole biliary tree, leading to final lesions resembling biliary cirrhosis. This could be attributed to the toxic effects that have been reported for 2-chloroethyl alkylating metabolites [22]. Concurrently, a delayed liver cell toxicity occurs in association with a worsening of cholestasis. Electron microscopic study of the hepatocyte cytoskeleton discloses at that

point a unique injury of the microtubular alteration type. This could be attributed to the toxic effects of carbamylating cyclohexyl isocyanates formed during the chemical degradation of CCNU [6]. Whether isocyanate derivatives act by inhibiting polymerization or by preventing depolymerization of liver cell microtubules is still to be determined.

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