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ULTRASTRUCTURAL CHANGES IN ISOLATED RAT HEPATOCYTES EXPOSED TO DIFFERENT CCl_4 CONCENTRATIONS

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SUMMARY: Ultrastructural data are presented on time-course changes in isolated rat hepatocyte suspensions exposed either to 1.2 or 1.8 mM CCl_4 for up to 1 h. The subcellular changes at the lower concentration, but not the higher, are shown to closely parallel those reported to occur in rat hepatocytes following ingestion of CCl_4.

Isolated hepatocyte systems are now being used frequently as models for studying the mechanism of chemical-induced cytotoxicity, but discrepancies between experimental results in them and in vivo response have been reported (1). For example, ultrastructural studies have indicated that the time-course morphological changes elicited by CCl_4 in isolated hepatocytes under conditions analogous to those employed by most investigators in the field are not identical to results in animals treated with this hepatotoxin, in that the plasma membrane and not the ER is structurally altered initially (2,3). Recent biochemical studies (4), however, suggest that this inconsistency may derive from too high a CCl_4 concentration in the reaction medium, resulting in massive intracellular enzyme release from nonspecific solvation effects rather than cytochrome P-450 activation of CCl_4, the generally accepted mechanism of action. At lower concentrations, microsomal uptake of Ca^{2+} is impaired before loss of cytoplasmic enzymes, as occurs in vivo (4).

In the present study, we document that the ultrastructural sequelae observed in response to CCl_4 in vitro are concentration-dependent and define experimental conditions for subsequent biochemical investigations on the

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Abbreviations: ER, endoplasmic reticulum; GHO, glutaraldehyde; LDH, lactate dehydrogenase

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cytotoxic mechanism. To our knowledge, this is the first report showing that sequential structural changes can be produced in isolated hepatocytes exposed to a hepatotoxin similar to those that occur in the cells *in vivo*.

MATERIALS AND METHODS

Hepatocytes were isolated from unfasted rats by *in situ* perfusion of the liver with collagenase and suspended in hormone-supplemented Waymouth's 752/1 medium at 1.5 x 10^6 cells/ml, as described in (5). After transfer of 4.0 ml to 25-ml Erlenmeyer flasks, either 6 or 9 µl of CCl_4 was added to the center well, which was covered with a glass disc, and the flasks were gassed with O_2:CO_2 (95:5) for 30 sec. The flasks were stoppered, the glass discs dislodged to initiate CCl_4 exposure, and the flasks incubated for 1 h at 37°C in a shaking water bath as described in (6). Equilibration of CCl_4 between headspace and solution was found to be >95% complete within 5 min. At 5, 15, 30, and 60 min, 0.4-ml aliquots were taken from the flask side arm and diluted immediately in 3% GHO in 0.1 M phosphate buffer, pH 7.4, for electron microscopy. Additional 0.3- and 0.1-ml aliquots were taken from the flasks after 1 h for measurement of LDH release, an indicator of plasma membrane integrity, and gas chromatographic (gc) analysis of CCl_4, respectively, by the analytical procedures described previously (6).

Cells fixed in 3% GHO were washed 3 times in phosphate buffer by lightly pelleting the cells, decanting the buffer solutions and resuspending them at 30-min intervals. The final pellet was made in a 1% agar solution and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 45 min. The cells were then dehydrated in a graded series of ethanol solutions and passed through 100% propylene oxide prior to infiltration with Araldite. The agar pellets containing the hepatocytes were cut in appropriate size blocks, embedded in Araldite, and polymerized at 37°C for 24 h, then at 60°C for 24 h.

RESULTS AND DISCUSSION

The ultrastructural characteristics of hepatocytes freshly isolated by collagenase perfusion are very similar to those frequently reported for normal liver. Narrow cisternae of rough ER are observed in parallel arrays within the cytoplasm. Ribosomes are attached to the membranes or are present in the cytoplasmic matrix in various aggregates or linear arrays. The mitochondria, Golgi complex, microbodies, and lysosomes all appear within the normal range of numbers and structural characteristics of those *in vivo*. Numerous microvilli appear on the cell surface similar to those observed extending into the space of Disse in intact tissue (Fig. 1). Polarization of the organelles within the cytoplasm and the flat lateral plasma membranes between adjacent hepatocytes in *in vivo* preparations are not present in the *in vitro* cell suspensions. However, this does not appear to interfere seriously with their capabilities for responding to CCl_4 similarly to cells *in vivo*, as will be

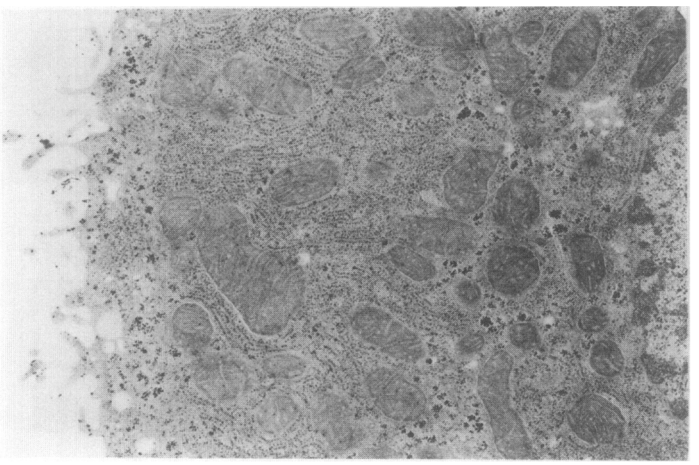


Fig. 1 Control isolated hepatocytes with characteristic microvilli protruding from the plasma membrane, beds of rough ER, and mitochondria in coupled configuration. Glycogen is scattered in the cytoplasm. x11,830.

seen below. Rapid fixation that takes place in the in vitro environment that permits the fixative solution to surround and quickly penetrate the cells frequently preserves the mitochondria in a highly coupled state compared with those usually seen in liver taken from an animal.

Sequential sampling over a 1 hour period of isolated hepatocytes prepared by collagenase perfusion and exposed to 1.2 mM of CCl_4 results in the following ultrastructural alterations. Initially, the number of normal microvilli (Fig. 1) covering the surface of the isolated cells is substantially reduced (Fig. 2) and their normal morphology often becomes pleomorphic. With time, changes in the plasma membrane tend to return to a more normal condition, but complete recovery was not obtained.

Other changes in the cytoplasm that occur at this level of exposure include dilation of the rough ER (Figs. 2-4), followed by loss of the ribosomes from its surface (degranulation) and eventual vesiculation of the cisternae (Figs. 3-4). The smooth ER condenses and becomes a tubular aggregate (Fig. 4). The Golgi complex appears to be reduced in activity with the Golgi vacuoles containing an abnormal flocculent material. Mitochondria retain their normal coupled configuration (Figs. 2-4). At this concentration, net LDH release induced by the CCl_4 (LDH release in CCl_4 flasks less control

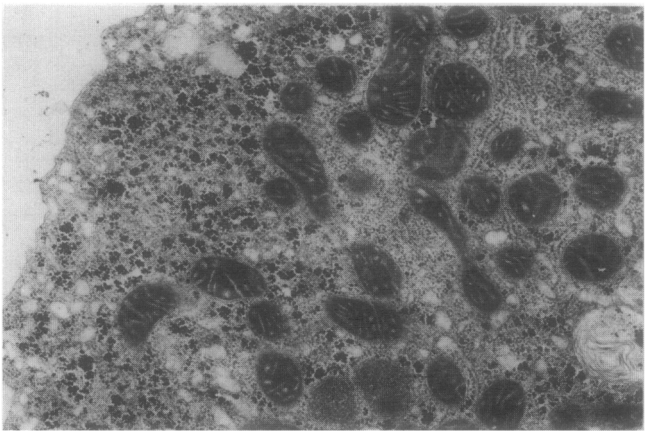
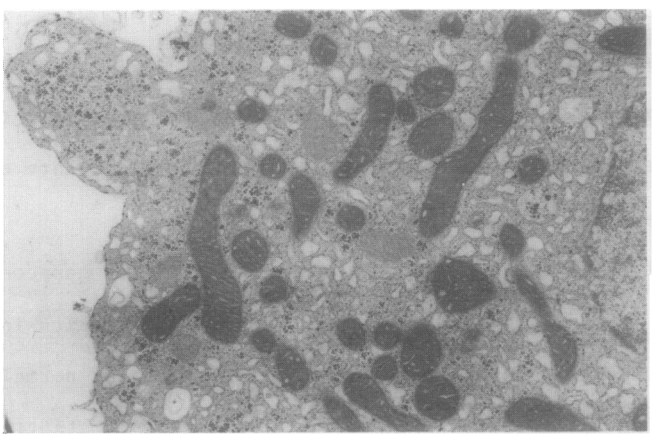


Fig. 2 Hepatocytes after 5-min exposure to 1.2 mM CCl_4. Dilation, degranulation, and vesiculation of rough ER have begun. Surface microvilli are greatly reduced although mitochondria retain the characteristic coupled configuration. x14,742.



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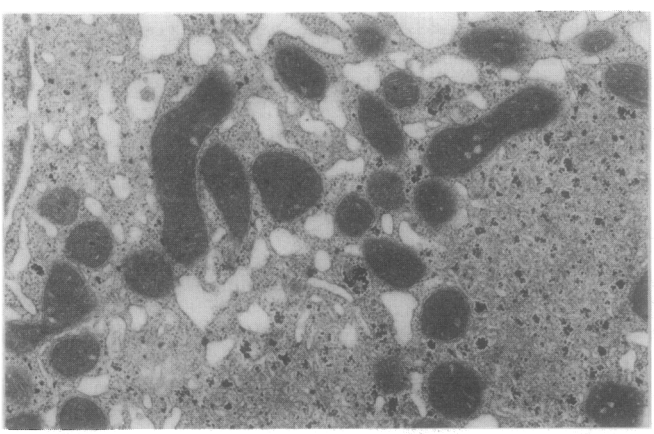

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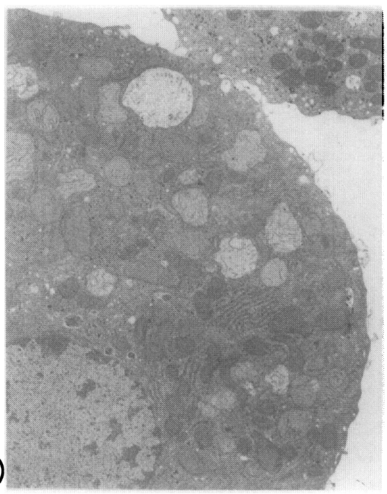
Figs. 3 and 4 After 1-h exposure to 1.2 mM CCl_4, changes in rough ER are more extensive. Vesiculation and degranulation are widespread (Fig. 3) and condensation of tubular or smooth ER is evident (Fig. 4). Mitochondria appear normal. Glycogen is observed within the condensed smooth ER. Fig. 3, x11,466. Fig. 4, x14,196.



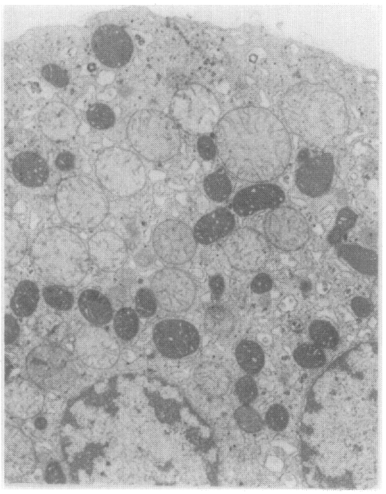


Fig. 5 Exposure to 1.8 mM of CCl_4 for 5 min results in a striking structural change in the mitochondria. Their electron density becomes varied, indicating an increasing amount of swelling. Rough ER remains intact without severe loss of ribosomes. Cell surface is devoid of microvilli. x3,780.

Fig. 6 Many of the cells exposed to 1.8 mM CCl_4 for 1 h possess mitochondria in either coupled or swollen, uncoupled states. Dense inclusions of swirled membranes are evident in the cisternae of the rough ER. Degranulation and dilation of rough ER are prevalent. x4,704.

cell LDH release ÷ total intracellular LDH content determined for the same number of cells separately less control cell release) progressively increased from 5.7% at 5 min to 11.6% after 1 h.

At 1.8 mM of CCl_4 and either a 5-min or 60-min exposure, the mitochondrial changes are pronounced, as shown in in Figs. 5-7, respectively. The electron density and the degree of swelling of the mitochondria changes from one to another within the cytoplasm of the same cell (Figs. 5-7). Some mitochondria appear coupled, while others are swollen and uncoupled. Upon extended exposure, some mitochondria develop vacuoles and swirls of lamellar membranes within their matrices (Fig. 7). Surprisingly, the rough ER in these cells frequently appears intact and distributed between the mitochondria (Fig. 7). At this higher CCl_4 concentration, net LDH release was 70% after 1 h.

Comparison of the results at 1.2 mM CCl_4 with ultrastructural studies on the livers of CCl_4-treated rats (7) indicates clearly that the susceptible hepatocyte populations in either environment respond in a closely parallel manner. At the higher CCl_4 concentration, the results are similar to those

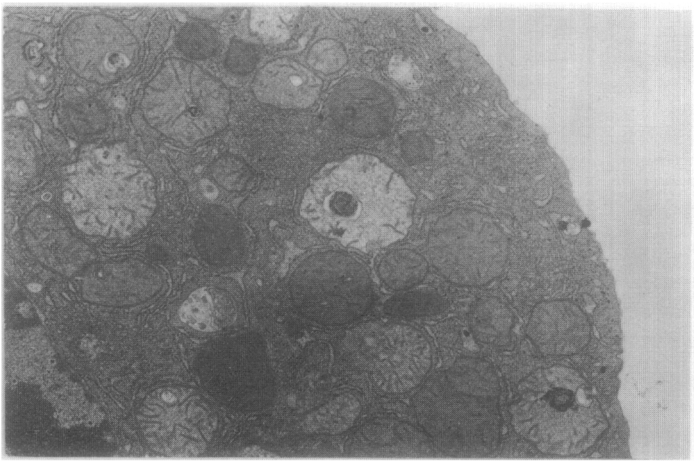


Fig. 7 Higher magnification of cells exposed to 1.8 mM CCl_4 for 1 h, showing dense membranous inclusion within mitochondria. Swelling, resulting in a gradation of electron density, is also apparent. Condensation of smooth ER is evident, while the rough ER appears to maintain a relatively normal appearance. x17,290.

obtained by previous investigators (2,3), but the relevance of the changes seen to the pathological sequelae occurring in hepatocytes *in vivo* is open to question. The two concentrations used here span the EC50 value of 1.65 mM for LDH release for this hepatotoxin (CCl_4 concentration that induces 50% release of the intracellular enzyme after a 2-h incubation determined previously for these experimental conditions) (6). Thus, as contended in (4), lower CCl_4 concentrations that produce subcellular changes without considerable intracellular enzyme release within 1 h are likely to result in more valid data for *in vivo* extrapolations than higher concentrations. The transition in responses recorded with CCl_4 is seen here to occur over a rather narrow concentration range, and the actual CCl_4 concentrations for eliciting these changes depend somewhat on the experimental conditions used by investigators. For example, the lower CCl_4 concentration used here (1.2 mM) is the same as the high concentration used in (4), which, under their conditions, released almost 100% of the cytoplasmic enzyme within 1 h. In our system, the 1.2 mM concentration is appropriate for studying events subsequent to disruption of the ER; much lower concentrations and possibly longer interaction times are indicated for studies to delineate biochemical alterations leading

up to this effect. The present results illustrate the value of adjunct ultrastructural data in selecting experimental conditions for mechanistic studies in isolated hepatocytes relating to *in vivo* hepatotoxicity.

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