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The nature and significance of liver cell vacuolation following hepatocellular injury – an analysis based on observations on rats rendered tolerant to hepatotoxic damage

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Abstract Swelling with nonlipid cytoplasmic vacuolation of diffusely distributed hepatocytes is seen consistently after mild acute and subacute liver injury. Several lines of evidence point to the possibility that this change may reflect a cellular adaptation beneficial to the host, rather than a degenerative change. The nature and significance of this morphological manifestation were tested in batches of albino rats given small doses of a variety of hepatotoxins, some of which were subsequently challenged with a large highly necrogenic dose of carbon tetrachloride (CCl_4). Morphological and biochemical investigations showed that cytoplasmic vacuolation of liver cells following low doses of toxins was due to excess accumulation of glycogen, predominantly of the monoparticulate form. These cells lacked features of degeneration or regeneration and were much less susceptible to injury by the large dose CCl_4, as assessed by structural and serum enzyme analyses. This tolerance to toxic damage seemed to be associated with excess accumulation of intracellular glycogen. We conclude from these and other observations on animal and human livers that many of the vacuolated hepatocytes seen in liver injury are cells adaptively altered to resist further insult rather than cells undergoing hydropic degeneration, as is commonly believed.

Key words Hepatocyte cytoplasmic vacuolation · Glycogen accumulation · Tolerance to toxic injury · Adaptation versus degeneration

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Introduction

Swelling of hepatocytes with nonlipid vacuolation of the cytoplasm is frequently seen in acute liver injury induced by hepatitis viruses and a variety of hepatotoxic agents. This morphological change has conventionally been attributed to alterations in the injured cell as a result of hydropic change. Carbon tetrachloride (CCl_4) has been used for years as a prototype agent for studying cell damage; it consistently produces morphologically detectable degeneration and necrosis of liver cells within 12–24 h of intoxication. Injury induced by this chemical is most marked in the central part of the hepatic lobule, the damage becoming progressively less marked toward the peripheral lobular area [29]. This gradient is directly related to the intracellular concentration of the enzyme cytochrome P-450, which splits CCl_4 to its actively toxic derivative CCl_3 [14, 15, 30]. In a normal animal, therefore, the extent of damage in the hepatic lobule is determined by the dose of CCl_4, with peripheral lobular cells escaping injury with a low to medium dose of the chemical. In an earlier study on rats given a small dose of CCl_4, we observed necrosis and fatty change affecting only a few rows of centrilobular hepatocytes, while the majority of other cells in the lobules outside the area of injury and therefore likely to be undamaged by the toxin were somewhat swollen with nonlipid vacuolation of the cytoplasm [10]. These vacuolated cells were most prominent at 48–72 h after intoxication. Unlike similarly located normal hepatocytes of untreated control animals, the vacuolated cells resisted injury by a subsequent large, otherwise lethal, dose of the same chemical. A centrilobular distribution of injury is also induced by some other toxic agents, while, with still others, such as aflatoxin B_1 (AFB_1) and allyl formate (AlFo) these geographical patterns of injury and noninjury are reversed [26]. Necrosis caused by AFB_1 is probably dependent on the formation of AFB_1-dihydrodiol and subsequent rapid adduct formation, while that caused by AlFo probably requires its conversion to the toxic metabolite, acrolein, by alcohol dehydrogenase. In an experiment carried out to examine

the variability of acute hepatotoxic effects of AFB_1 among rats of various strains and ages [26], we again encountered similar vacuolation in liver cells following periportal necrosis induced by the agent, but this time in the surviving centrilobular area.

Meanwhile, during investigations on the clinicopathological features of subacute hepatic failure in humans, we noticed that patients whose liver biopsies contained swollen, vacuolated hepatocytes frequently recovered from coma, while those whose biopsies lacked such vacuolated cells generally succumbed to the disease [27, 28]. Also, it is well known that in the early post-acute phase of non-fatal viral hepatitis in humans (by the time the patient reports to hospital the acute phase is in decline and recovery has started), the most striking hepatocytic change is a non-lipid vacuolated swelling of the cytoplasm ascribed to degenerative changes. Interestingly, in spite of such widespread "degenerative changes", these patients always recover spontaneously.

A sum total of these findings in experimental and human liver injury raises an important question –whether such vacuolar swelling of liver cells occurring under these circumstances is a manifestation of retrogessive alterations due to cell damage or of protective adaptation by the cell. The present experiments were designed to address some aspects of this issue by exploring, first, the nature of the liver cell vacuolation occurring after mild injury induced with low doses of hepatotoxic agents and, secondly, the relevance of this morphological change to the tolerance acquired by the cells to subsequent injury, and particularly to injury from CCl_4, a consistently hepatocytotoxic chemical. Findings in these studies indicate that certain morphological alterations of hepatocytes, which occur soon after liver injury and are currently believed to be due to degenerative hydropic change, actually largely represent cellular adaptation of a protective nature.

Materials and methods

Animals and experimental procedures

Male albino rats of the Wistar and Holzman strains and weighing 100–150 g each were obtained from the animal facilities of our institutions. They were housed in cages in pairs and, except where indicated, allowed unrestricted access to standard rat cube diet and water. Groups of rats, with a minimum of 6 per group, were subjected to various experimental regimens as shown in Fig. 1 and Tables 1 and 2. Three hepatotoxins, CCl_4, AFB_1 and AlFo, were used to induce liver injury, CCL_4 as the standard necrogenic agent for cells in the more centrilobular zone but sparing the outer lobular cells and AFB_1 and AlFo for the opposite effects, with necrosis of periportal cells and sparing of the central lobular cells. Holzman strain rats were used for AlFo intoxication, as our previous experience [26] had shown them to be more susceptible to this agent.

To examine whether the hepatocellular vacuolation was modified or halted by fasting, food was withdrawn for the last 24 h before sacrifice or for 24 h immediately after CCl_4 dosing in the animals in groups 1b and 1c, respectively (Fig. 1, Table 1). Animals in group 4 (4a and 4b, controls) received injections of equivalent volumes of vehicle only. In separate sets of animals in groups 5 (a, b), 6 and 7 (experiment II), the relationship of the presence or absence of vacuolation in hepatocytes surviving after priming with a small dose of one of the different toxins to the necrogenic effect of a large dose of CCl_4 was tested (Fig. 1, Table 2). The latter was

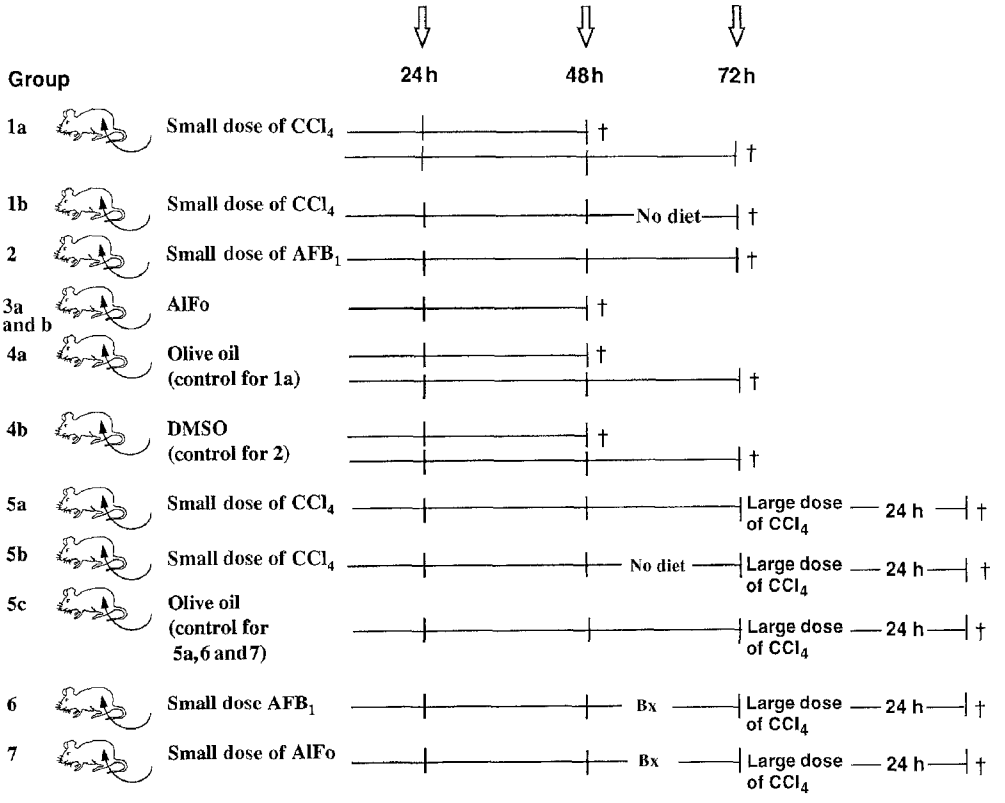

Fig. 1 Experimental design in various animal groups (*mouse* i.p. injection, *continuous line* unrestricted diet, *double arrows* times after initial injection, B_x liver biopsy, *death dagger* sacrifice)

Table 1 Experiment I. Induction of hepatocytic change following small doses of hepatotoxins

Group	Animal		Hepatotoxin/ vehicle	Dose per 100 g body wt.	Diet	No. sacrificed at	
	Strain	Number				48 h	72 h
Experimental							
1a	Wistar	50	CCl_4	25 µl	Unrestricted	20	30
1b	Wistar	24	CCl_4	25 µl	None for last 24 h	12	12
1c	Wistar	10	CCl_4	25 µl	None for first 24 h	10	–
2	Holzman	15	AFB_1	0.25 mg	Unrestricted	–	15
3a	Holzman	20	AlFo	6 µl	Unrestricted	20	–
3b	Wistar	20	AlFo	6 µl	Unrestricted	20	–
Control							
4a	Wistar	20	Olive oil	Vol.=in 1a	Unrestricted	5	15
4b	Holzman	8	DMSO	Vol.=in 2	Unrestricted	4	4

Table 2 Experiment II. Effect of large dose of CCl_4 on hepatocytes with or without changes following priming doses of toxins/vehicle

Group	No. of animals	Priming toxin/vehicle	Diet	Liver biopsy	Timing of large CCl_4 dose (h after priming)
5a	12	CCl_4	Unrestricted	No	72
5b	12	CCl_4	None for 24 h before large dose of CCl_4	No	72
5c	6	Olive oil	Unrestricted	No	72
6	10	AFB_1	Unrestricted	Yes	72
7	15	AlFo	Unrestricted	Yes	48

administered at 72 h in animals primed with CCl_4 or AFB_1, but at 48 h in those primed with AlFo (Fig. 1, Table 2), since the priming-induced vacuolation in apparently uninjured hepatocytes was best manifested after these intervals with the different agents. Group 5c animals served as controls for effects of the large dose of CCl_4. Because of the relative inconsistency in toxic effects of AFB_1 and AlFo, animals in groups 6 and 7 were subjected to open-wedge biopsy of the liver between 48 and 60 h after priming, to check for the occurrence of vacuolation in hepatocytes in the nontarget zonal area.

All toxins were administered by i.p. injection in appropriate vehicles. CCl_4 (BDH, England) was prepared as a 5% or a 50% solution in olive oil. For the small dose of CCl_4, the 5% solution was injected in volumes calculated to deliver 25 µl CCl_4/100 g body weight, where as for the large dose of CCl_4 the 50% solution was used to deliver 250 µl CCl_4/100 g body weight. AFB_1 (Makor Chemicals, Jerusalem) was administered as a 0.01% solution in dimethylsulphoxide (DMSO; BDH, India) at a dose of 0.25 mg/100 g body weight. Allyl formate (Schuchardt, Germany) was given as a solution in physiological saline in a dose of 6 µl/100 g body weight.

Open-wedge biopsy of the liver (Table 2, groups 6 and 7) was performed 48 h after toxin priming (Fig. 1), through a small midline abdominal incision under ether anaesthesia and with standard aseptic procedures. Animals in experiment I were killed at 48 or 72 h after dosing with toxin, whereas all animals in experiment II were killed 24 h after the large necrogenic dose of CCl_4 (Fig. 1, Tables 1, 2). Animals were killed after light ether anaesthesia, by opening the chest and rapid exsanguination by withdrawal of blood by cardiac puncture and subsequent cutting of the heart. From each animal the entire liver was quickly removed and sliced at 2– to 3-mm intervals. Blood was collected in plain sterile tubes and serum was separated soon after complete clotting. Approximately 10 g of liver was frozen at –70° C and stored at this temperature for estimation of glycogen.

Examination of tissues

Fresh tissues were snap-frozen in isopentane (Fluka, Switzerland), cooled to –150° C by liquid nitrogen. These were stored at –80° C and 6– to 8-µm-thick sections were subsequently cut in a cryostat. For paraffin sections, fresh liver slices were fixed in 10% buffered formol alcohol (10% formalin in absolute alcohol), processed and sectioned according to routine procedures. Cryostat and paraffin sections were stained by haematoxylin and eosin (HE) and by periodic acid-Schiff (PAS) stain with and without prior diastase digestion. Frozen sections were also stained for lipid by oil red O and for glucose-6-phosphatase (G-6-Pase) by the technique of Wachstein and Meisel [38]. Methyl green pyronine (MGP) stain for ribonucleoprotein was done on paraffin sections.

For electron microscopy, small pieces (1-mm cube) of freshly obtained tissue were fixed in 3% glutaraldehyde in Millonig phosphate buffer (pH 7.2), then post-fixed in 1% osmium tetroxide (pH 7.2) and embedded in Araldite. Semithin sections were cut on LKB ultramicrotomes and stained with toluidine blue. Representative blocks were then selected for subsequent semithin and ultrathin sections. After removal of the araldite with sodium ethoxide [21] semithin sections were also stained with PAS and MGP. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed and photographed in Jeol 100 Cx and 1200 Ex electron microscopes.

In order to evaluate the regenerative status of hepatocytes of different morphology and location, cells preparing for division were identified by immunostaining for proliferating cell nuclear antigen (PCNA). Deparaffinised sections were incubated overnight at 4° C with a monoclonal antibody (DAKO, Glostrup, Denmark), and following treatment with peroxidase-labelled avidine–biotine complex, colour was developed with diaminobenzidine as substrate. Light haematoxylin was used for counter-staining. With each batch of staining a section of human vermiform appendix was included as a positive control, and replacement of primary an-

tibody with normal mouse serum during the procedure on such a section served as a negative control.

Morphometry

Estimations of the proportion of lobular area undergoing necrosis after the large dose of CCl_4 and the size of the vacuolated hepatocyte relative to that of the normal one were obtained by morphometric measurements in a CAS 200 image analyser (Becton Dickinson) using a micrometer programme. In case of the former, distances from the central vein to edge of the portal area and to the outer edge of the necrotic area were measured in ten lobules of at least 6 animals in groups 5a, 5b and 5c. A total of 500 vacuolated and normal hepatocytes were measured in animals in groups 1a and 4a, respectively.

Chemical determinations

For the estimation of glycogen, frozen liver tissues from 10 animals each in groups 1a and 4a sacrificed 72 h after the small dose of CCl_4 and olive oil (control) respectively, and 6 animals each in groups 5a and 5c sacrificed 24 h after the large dose of CCl_4 administered 72 h after the small dose of CCl_4 or olive oil respectively were examined within 24 h of collection by the method of Keppler and Decker [20]. Briefly, liver homogenate prepared in 0.6 M perchloric acid was hydrolysed at 37° C for 2 h by amyloglucosidase (1,4-*a*-D-glucan glucohydrolase EC 3.2.1.3) obtained from Sigma Chemicals (St. Louis, Mo.). The glucose obtained from this hydrolysis reaction was determined by an enzymatic spectrophotometric glucose/peroxidase technique with chromogen (glucose GOD-PAP reagent, obtained from Boehringer Mannheim, Germany) Sys 2,BM/Hitachi 717/911. Standard concentrations of glycogen (obtained from Boehringer Mannheim, Germany) were treated in the same way. Results were expressed as milligrams per gram of liver (wet weight).

Serum ALT and AST were estimated in 15 animals each in groups 1a and 4a sacrificed at 72 h and 6 animals each in groups 5a and 5c by standard methods in a Hitachi 911 autoanalyser.

Statistical analysis of values for morphometric measurements, glycogen and transaminases for differences among various groups of animals was carried out by the Mann–Whitney test.

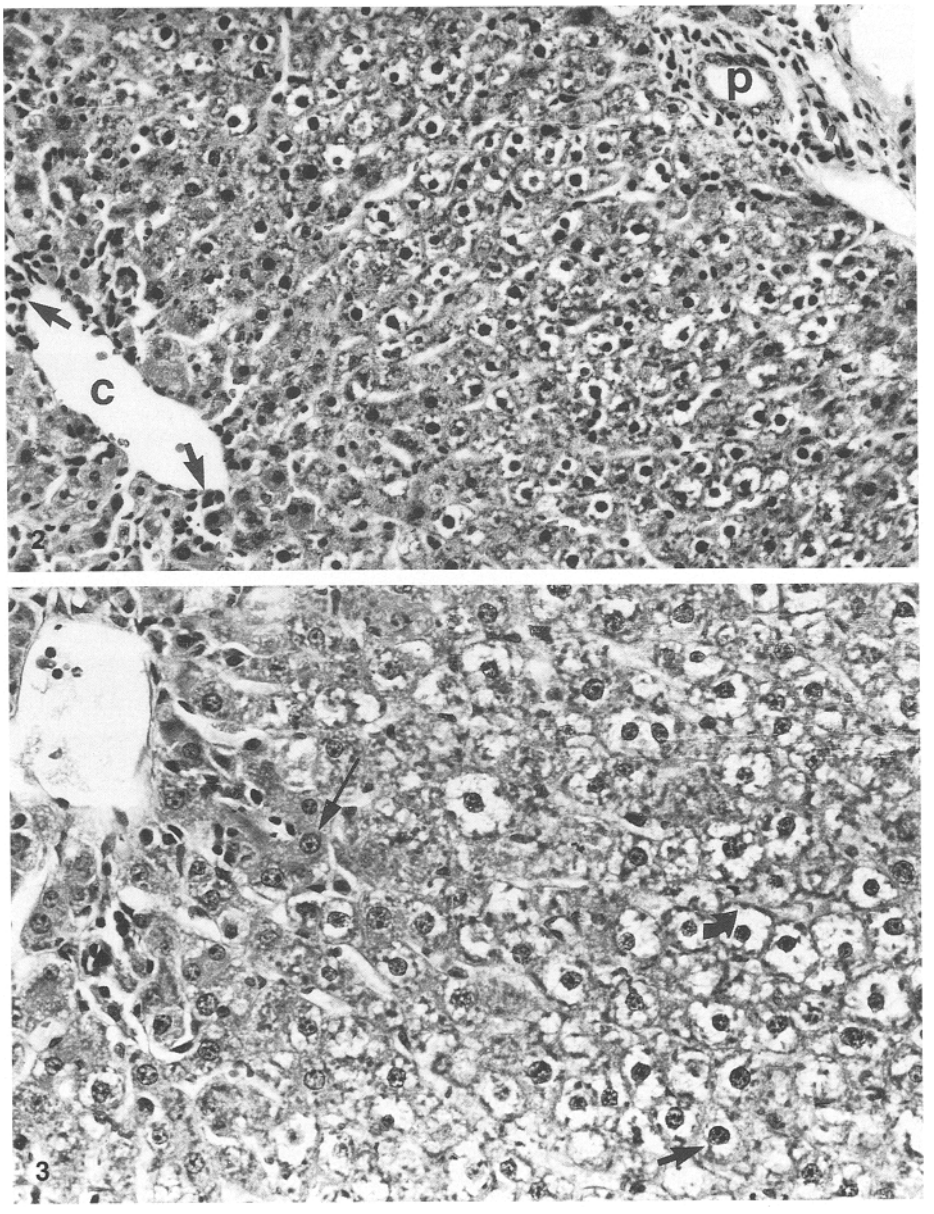

Fig. 2 Cytoplasmic vacuolation of hepatocytes around portal tract (*p*) 48 h after a small dose of CCl_4. The nuclei appear suspended in the centre of somewhat swollen cells with clearing of cytoplasm. There is a narrow zone of necrosis (*arrows*) around the central vein (*c*). Hepatocytes in the transition zone between this and the vacuolated cells show irregular vacuoles. HE, formalin-fixed tissue section, ×620

Fig. 3 Vacuolated hepatocytes show narrow irregular strands of cytoplasm compressed against cell membranes (*curved arrow*), clumped outside the nucleus or extending as bridges between the two (*thick arrow*). Note that vacuolation is unlike that for lipid. Some hepatocytes between the periportal vacuolated and pericentral necrotic ones have solid amphophilic cytoplasm (*thin arrow*). HE, formalin-fixed tissue section, ×1200

Results

Morphologic features

Small-dose CCl_4 intoxication with unrestricted diet

Animals in group 1a sacrificed 48 h after administration of the small dose of CCl_4 and with an unrestricted diet consistently showed coagulative necrosis and degeneration characteristic of CCl_4 toxicity [9] involving 10–20% of each hepatic lobule in the central zone. Necrotic cells were variously deformed with dense eosinophilic cytoplasm and pyknotic or fragmented nuclei. Degeneration was observed in the form of mild fatty change and hydropic change in a few balloon cells with cytoplasm that appeared almost empty and small, rather condensed, nuclei. These latter cells were often seen delineating the damaged from the uninjured lobular parenchyma. A few or moderate numbers of mitotic figures were seen in hepatocytes immediately outside the necrotic zone. Except in the first one or two layers around the portal tracts, the periportal hepatocytes, in every lobule appeared mildly swollen (approximately 33% larger than normal by morphometry) with a partly clear, irregularly vacuolated cytoplasm and nuclei seemingly suspended in the cleared central area (Fig. 2). Narrow uneven rims of amphophilic cytoplasm appeared compressed against the cell membranes and nuclei or extended as delicate bridges between the two (Fig. 3). The vacuolation appeared as clear or very pale staining spaces of irregular contour,

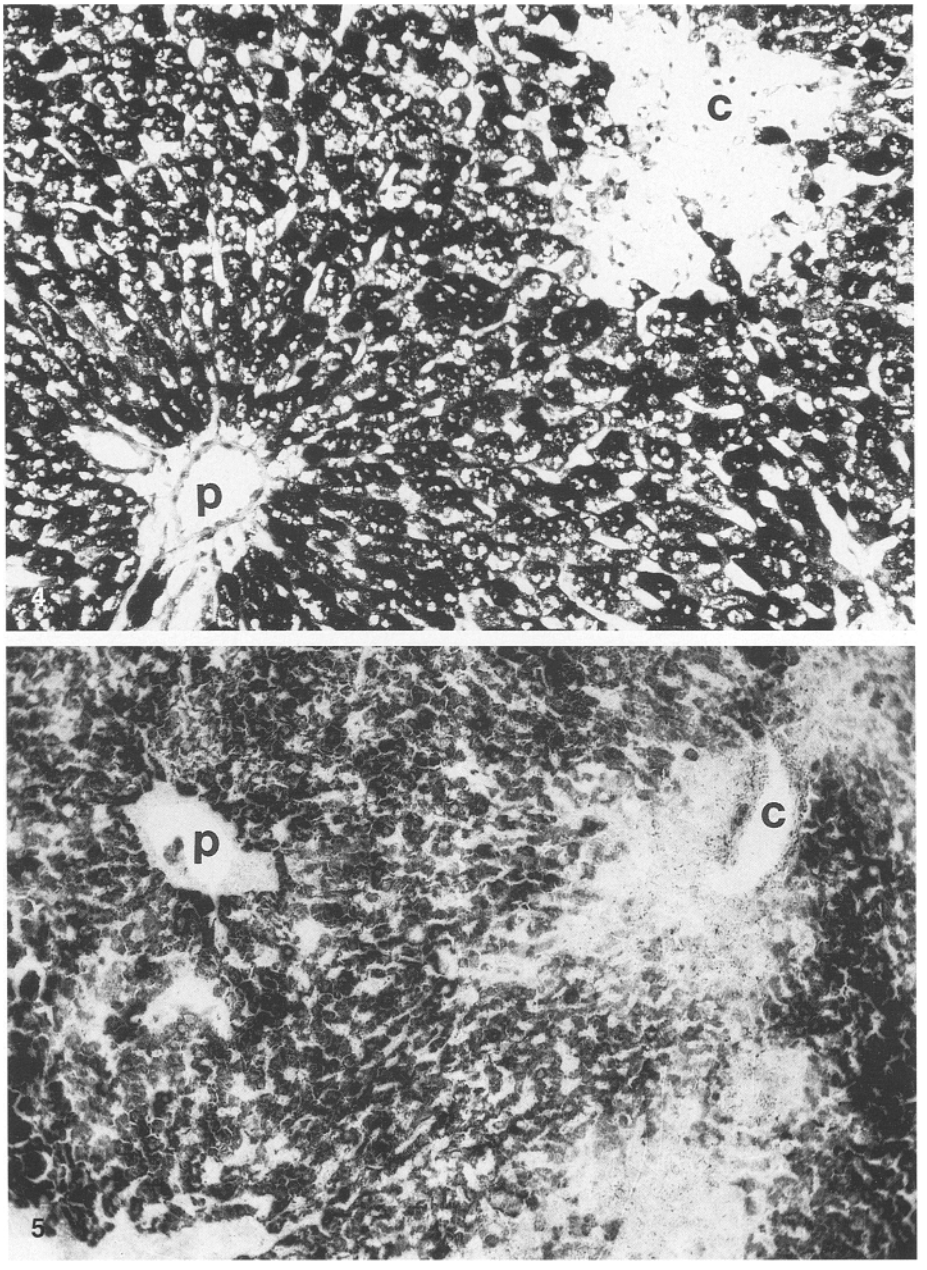

Fig. 4 Vacuolated cells around the portal tract (*p*) are loaded with glycogen. Necrotic and degenerated hepatocytes in the centrilobular area (*c*) show complete lack or significant loss of glycogen. PAS, formol-alcohol-fixed tissue, ×620

Fig. 5 Glucose-6-phosphatase is absent in the necrosed and degenerated centrizonal (*c*) liver cells but is present in normal amounts in vacuolated cells around the portal tract (*p*). Cryostat section, snap-frozen fresh tissue, ×250

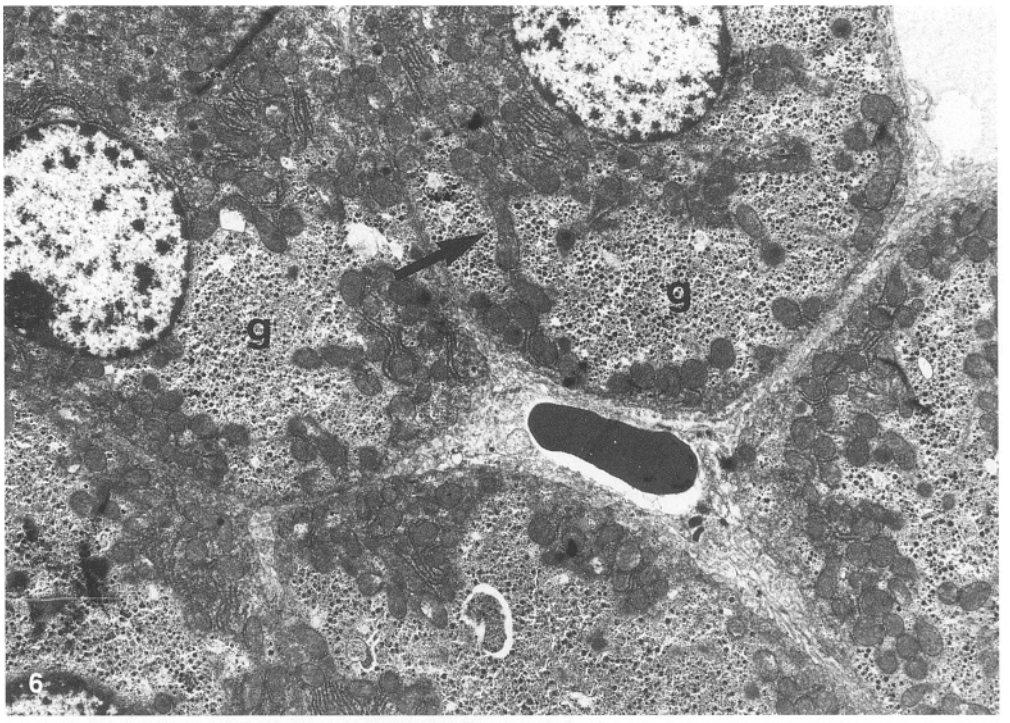

Fig. 6 Electron micrograph shows glycogen aggregates (*g*) in the hepatocyte of a rat 48 h after a small dose of CCl_4. Both monoparticulate (alpha) and rosette (beta) forms are seen. The cell organelles are decreased in these areas and are clustered along the cell or nuclear membrane, sometimes bridging between the two (*arrow*). Compare with light microscopic appearance in Fig. 1. ×5200

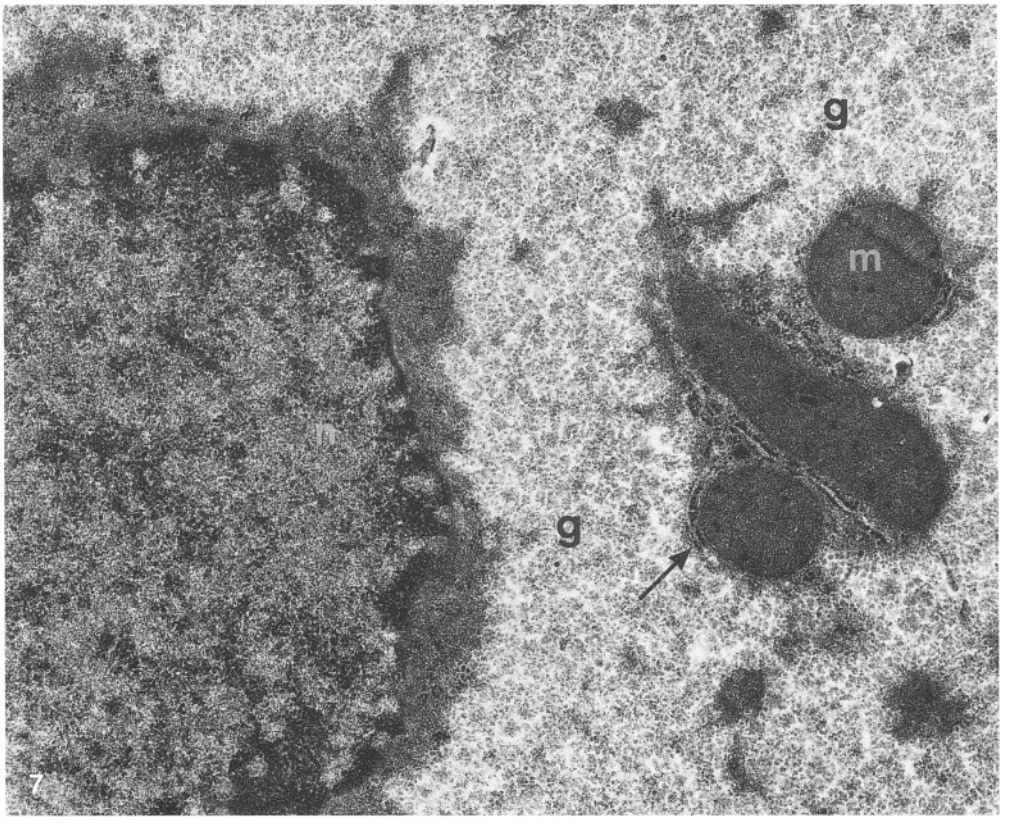

Fig. 7 Glycogen (*g*) in a vacuolated liver cell reveals marked decrease in electron density and predominant monoparticulate forms (compare with ribosome particles, *arrow*). Note absence of smooth ER (*n* nucleus, *m* mitochondrion). ×30500

distinct from the uniformly rounded lipid vacuoles. This appearance of the cytoplasm contrasted with the seemingly solid amphophilic appearance of cells in the more central lobular zone immediately outside the damaged parenchyma (Fig. 3). A transition zone of intermediate cell types with lesser vacuolations was present between the apparently solid and the prominently vacuolated cells (Figs. 2, 3). Vacuolation was most obvious in formalin-fixed tissue and less so in formol-alcohol-fixed and frozen tissues.

The vacuolated areas of cytoplasm of hepatocytes in the outer lobular zone regularly stained positive for gly-

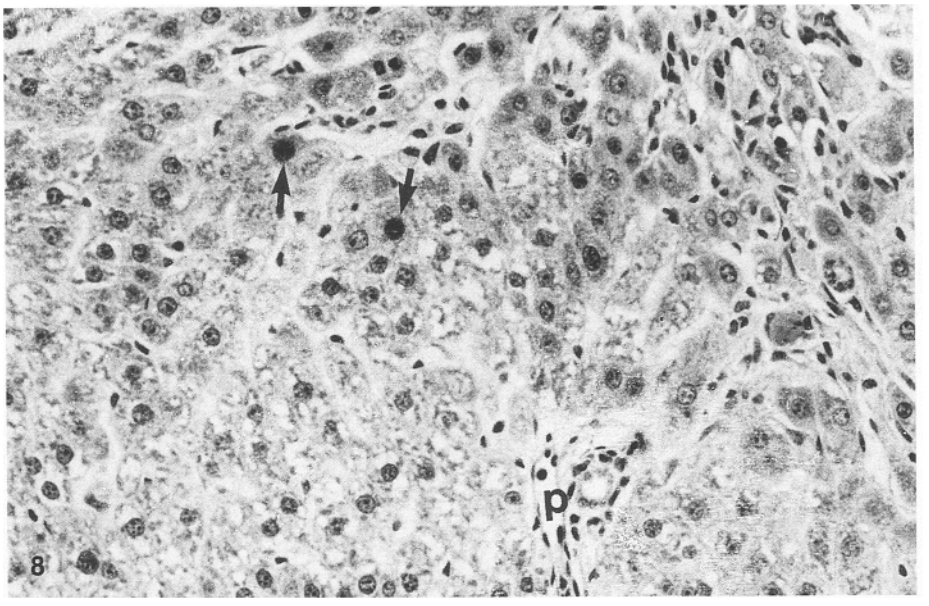

Fig. 8 Some hepatocytes just outside the pericentral zone show PCNA labelling in the nuclei (*arrows*). Vacuolated cells around the portal tract (*p*) are free of labelling. ×700

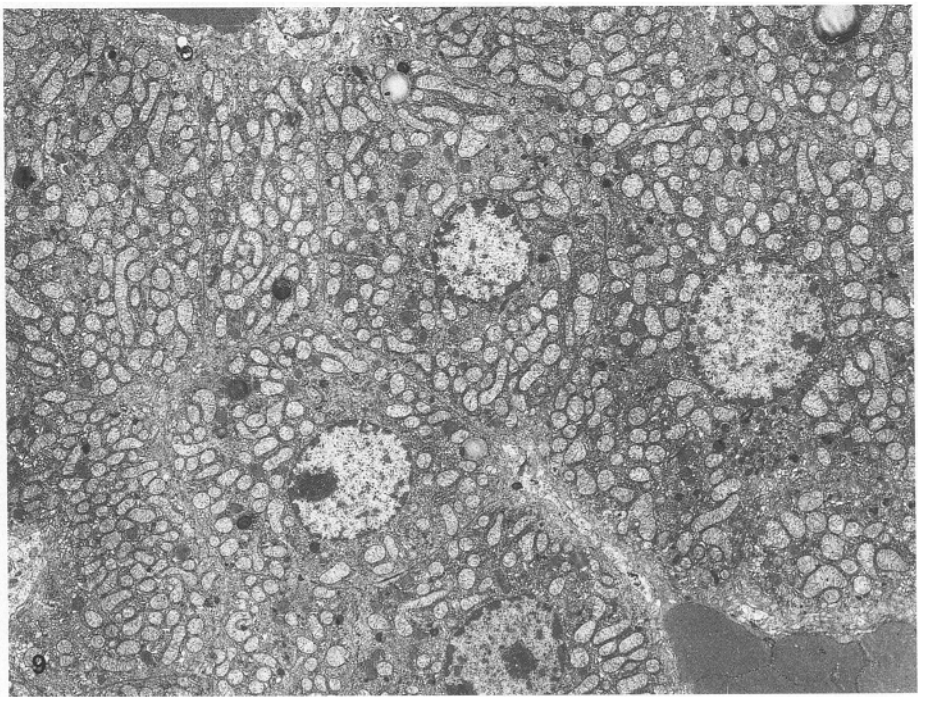

Fig. 9 Ultrastructure of hepatocytes of a rat primed with small dose CCl_4 and starved for 24 h before sacrifice. Note almost complete absence of glycogen, crowding of mitochondria and some increase of smooth ER. Compare with Fig. 7. ×6200

cogen, in contrast to the cytoplasm of injured cells in the centrizonal areas, which were always PAS negative (Fig. 4). The staining was best appreciated in alcohol-fixed and frozen tissue sections. The larger vacuoles appeared bright red and globular, while the smaller ones were granular and had a magenta colour. Oil red O stain did not show any lipid in the vacuolated cells. A few small lipid globules were seen in several of the central necrotic areas. In sections stained for G-6-Pase the centrizonal areas of necrosis were uniformly devoid of the enzyme, while it was present in normal amounts in the vacuolated cells (Fig. 5).

MGP stain for ribonucleoprotein particles was positive in the cytoplasmic strands and perinuclear mantles of vacuolated cells but negative in the vacuoles themselves. Necrotic cells in the centre of lobule did not stain. In semithin sections of glutaraldehyde-fixed Epon-embedded tissue, the vacuolated areas in the cytoplasm of periportal hepatocytes stained lightly with toluidine blue, brightly positive with PAS and negative with MGP.

Ultrastructurally, the vacuolated areas of light microscopy showed moderate to marked excess of glycogen in the forms of both alpha (rosetted) and beta (nonrosetted) particles with paucity of other organelles (Fig. 6). As in light microscopy, groups of mitochondria and endoplasmic reticulum profiles were packed against the cell membranes or appeared clustered in focal areas of cytoplasm. In some areas the electron density of glycogen

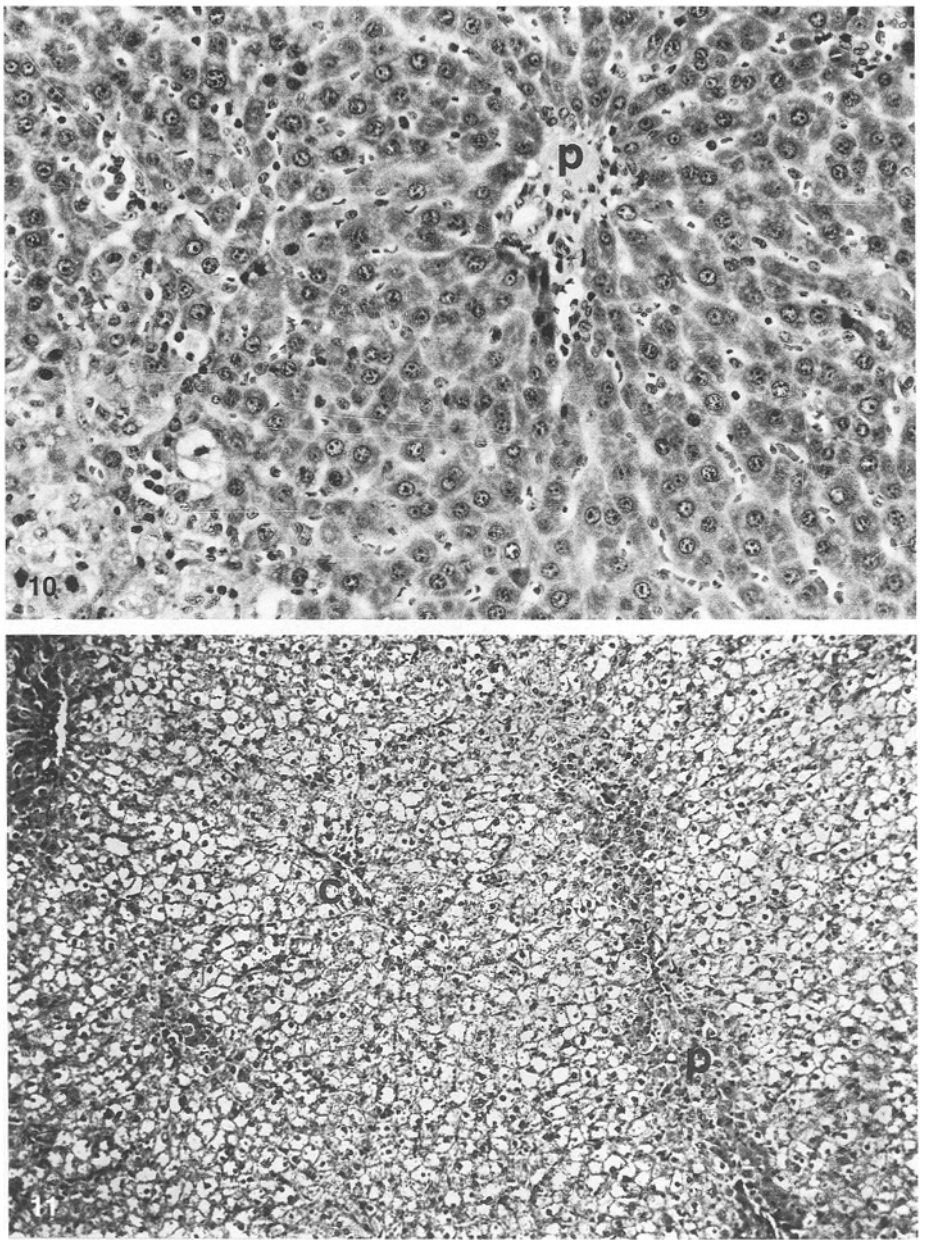

Fig. 10 Liver of an animal given a small dose of CCl_4 72 h before sacrifice and starved for the last 24 h. Hepatocytes around portal tract (*p*) show no cytoplasmic vacuolation. The necrotic cells in the centrizonal area are seen in the *lower left corner*. HE, formalin-fixed tissue section, ×620

Fig. 11 Liver of a rat 72 h after intoxication with aflatoxin B_1. Narrow zones of necrosis are seen around portal areas (*p*), while hepatocytes in a wide mantle around the central vein (*c*) reveal the characteristic vacuolation seen in periportal hepatocytes after CCl_4. Compare with Fig. 1. HE, formalin-fixed tissue section, ×290

particles was significantly less than normal. Beta particles constituted the predominant component in some cells (Fig. 7). Glycogen areas did not reveal smooth endoplasmic reticulum (SER) but sometimes had polysomal aggregates and rough ER in the neighbourhood (Figs. 6, 7). Mitochondria and other organelles of these cells appeared qualitatively normal. The nuclei often had prominent nucleoli. Centrilobular hepatocytes presented varying grades of degenerative changes together with conspicuous glycogen loss.

At 72 h after CCl_4 dosing, the vacuolar changes in periportal hepatocytes were almost always more prominent than at 48 h. The centrizonal necrotic area, however, was reduced in extent with repair beginning. Balloon cells were exceptional. The light microscopic, histochemical and ultrastructural features of vacuolated cells were identical to those described earlier. Glycogen globules were generally larger and more prominent.

In animals in group 1a (small dose of CCl_4) PCNA positivity was almost exclusively seen in the nuclei of cells with solid cytoplasm, just outside the small centrizonal area of necrosis. Higher numbers of labelled nuclei were seen at 48 than at 72 h after intoxication. Nuclei of vacuolated cells in the outer part of the lobule were rarely, if at all, labelled (Fig. 8). Cells with prominent cytoplasmic vacuolation were consistently PCNA negative.

Small-dose CCL_4 intoxication with periodic fasting

In animals starved for 24 h before sacrifice (group 1b) cytoplasmic glycogen disappeared almost completely, as

Table 3 Necrogenic effect of large dose CCl_4 on livers with and without vacuolated hepatocytes

Experimental group	Priming toxin/vehicle	Vacuolation in biopsy[a]	Necrosis following large dose of CCl_4[b]
5a	CCl_4	–	41.4% of lobule±12.2 Range 26–64%
5b (fasting 24 h before large dose of CCl_4)	CCl_4	–	89.4% of lobule±7.1 Range 73–100%
5c	Olive oil	–	85.7% of lobule Range 24–100%
6	AFB_1	Present (5) Absent (5)	(0/5) (5/5)
7	AF	Present (6) Absent (9)	(0/6) (8/9)

[a] Figures in parenthesis indicate number of animals
[b] 5a vs 5b and 5c: P=0.001; 5b vs 5c: NS

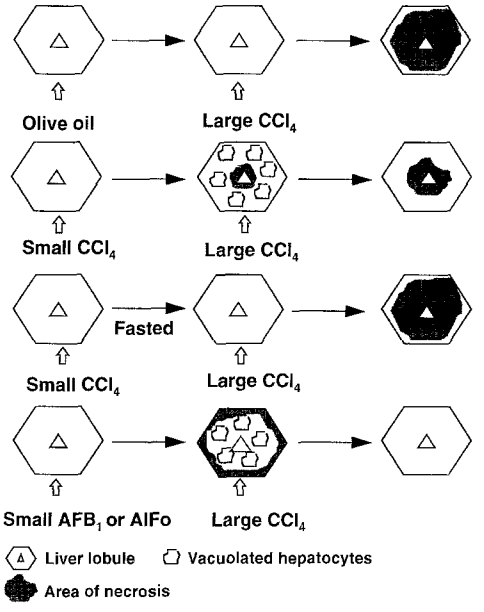

Fig. 12 Occurrence of vacuolation in hepatocytes after small doses of toxins correlates with resistance to necrogenic effect of large dose of CCl_4

seen by light microscopic PAS stain and under the electron microscope (Fig. 9), and correspondingly, vacuolation of periportal hepatocytes was significantly less and in a good proportion was almost totally absent (Fig. 10). Of the 12 rats sacrificed at 48 h after CCl_4 administration, 6 showed only mild vacuolation of cytoplasm, while in the other 6 in this group and in all 12 sacrificed at 72 h after CCl_4, no vacuolation was seen. Qualitative histochemical and ultrastructural features of glycogen in the starved animals were similar to those in the non-starved animals, however. The centrizonal necrosis, was nevertheless somewhat more prominent, and in some animals peripheral lobular hepatocytes contained small lipid droplets.

In animals fasted for the first 24 h after CCl_4 administration (group 1c) there was only a mild reduction in the vacuolation of peripheral hepatocytes compared with group 1a animals in the corresponding time to sacrifice (48 h). The central necrotic zone was wider and mitosis more frequent.

AFB_1 and AlFo intoxication

A narrow zone of necrosis involving two to four rows of periportal hepatocytes was evident at sacrifice in 70% and 50% of animals that received AFB_1 and AlFo, respectively (groups 2, 3a, 3b). In these animals centrizonal liver cells showed prominent vacuolations (Fig. 11), whereas in those without necrosis vacuolation was mild or absent. All vacuolated and necrotic cells showed morphological and histochemical features identical to those in the CCl_4-treated animals described earlier. All control animals (groups 4a, 4b) showed normal morphology and histochemistry of liver cells at 48 and 72 h.

Hepatocellular vacuolation and toxic effect of large dose of CCl_4

The effects of a large necrogenic dose of CCl_4 were tested on livers showing vacuolation following priming by small doses of toxins as well as on livers in which the vacuolation was either halted or did not become manifest (Table 3, Fig. 12). In animals in group 5a given an unrestricted diet, necrosis affected the inner 41.4±12.2% (range 26–64%) of each lobule. In fasted animals in group 5b, however, necrosis was much more extensive, affecting about 89.4±7.1% (range 73–100%) of the lobular parenchyma in a manner identical to that seen in group 5c control animals primed with the vehicle (olive oil) alone (85.7±6.5%, range 24–100%). Only a small rim of periportal cells was spared. One or two rows of balloon cells delineated the necrotic or necrobiotic parenchyma from the surviving parenchyma. Differences in the extent of necrotic areas between the animals primed with a small dose of CCl_4 and controls (primed with olive oil) were highly significant (P<0.001), while necrosis in the latter and in CCL_4-primed but starved animals were virtually identical.

In none of the 5 animals primed with AFB_1 that had vacuolation of centrizonal hepatocytes in the biopsy at 60 h was necrosis found at 24 h after the large dose of CCl_4. In contrast, in each of the animals treated according to the identical regimen that did not have vacuolation in the biopsy, centrizonal necrosis involved approximately 60% of the lobule (Table 3). Similar findings were ob-

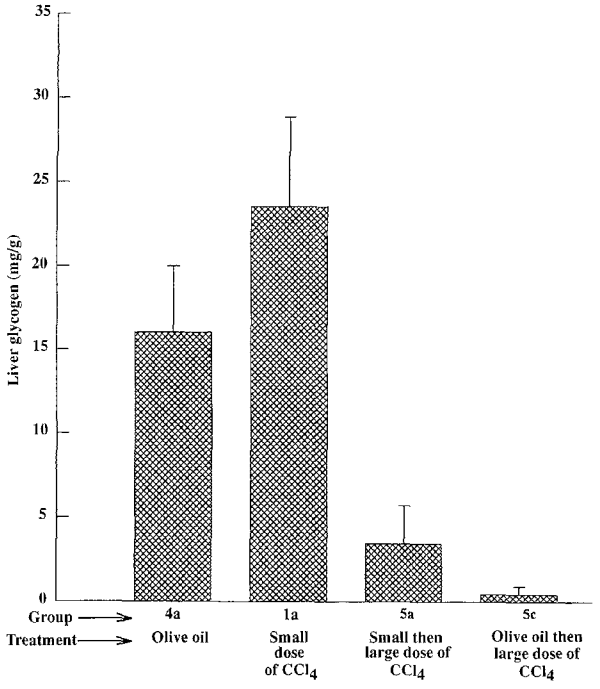

Fig. 13 Liver glycogen in control and various experimental animals

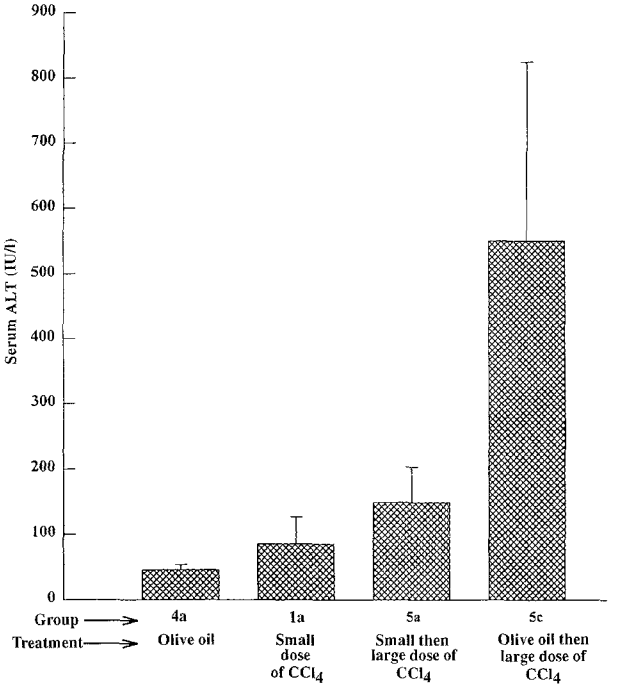

Fig. 15 Serum ALT levels in control and various experimental animals

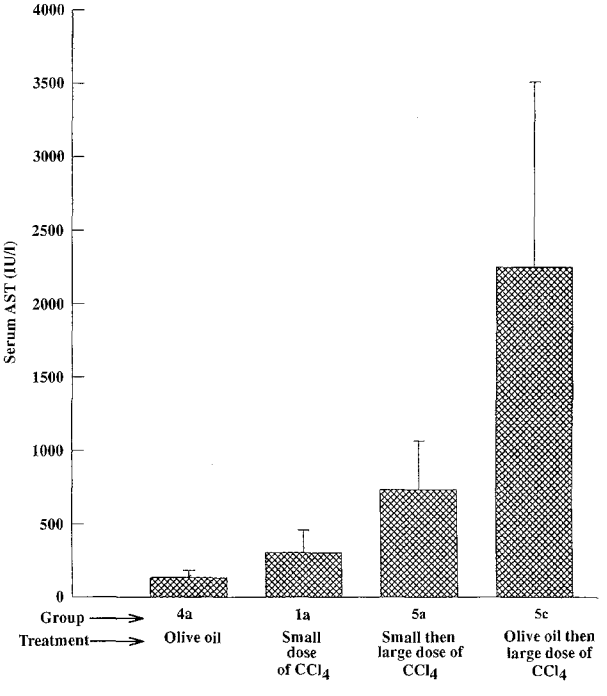

Fig. 14 Serum AST levels in control and various experimental animals

served in AlFo-primed animals with or without vacuolation of centrilobular hepatocytes in the biopsies at 48 h. Whereas 8 of the 9 without vacuolation showed necrosis involving the inner third of each liver lobule, none of the 6 with vacuolation showed necrosis.

At 72 h after intoxication with the small dose of CCl_4, liver glycogen increased by approximately 50% (23.53±5.53 mg/g) over values (16.02±3.97 mg/g) in control animals in group 4a given olive oil only (P=0.009; Fig. 13). In animals in group 5c primed with olive oil before intoxication by a large dose of CCl_4, there was a marked drop of glycogen to about 3% of normal (0.44±0.48 mg/g). In rats primed with the small dose of CCl_4 72 h earlier, the same large dose caused a drop in glycogen to about 22% of normal (3.44±2.26 mg/g). This difference is significant (P=0.016).

Levels of serum transaminases 72 h after the small dose of CCl_4 (group 1a) and 24 h after the large dose CCl_4 in animals that received olive oil 72 h before the toxin (group 5c) were respectively twice and more than 12 times those in control animals (Figs. 14, 15) receiving olive oil only (group 4a). Rats that received the same large dose of CCl_4 72 h after priming with the small dose of CCl_4 (group 5a) showed serum AST and ALT levels 4–5 times those in nonintoxicated controls, i.e. significantly different from those in group 5c animals (P=0.027 and 0.028, respectively).

Discussion

Within 48–72 h after administration of a small dose of hepatotoxic agent conventional histological preparations of the liver revealed a characteristic cytoplasmic clearing or vacuolation in the swollen hepatocytes away from the area of necrosis in the lobule. This change was consistent in the case of CCl_4 and involved the major part of parenchyma outside the centrilobular zone of injury. In animals with periportal necrosis after AFB_1 or AlFo, vacuo-

lation became manifest in hepatocytes located more centrally in the lobule. Histochemical and ultrastructural observations demonstrated convincingly that cytoplasmic vacuolation is represented almost exclusively by conspicuous aggregates of glycogen particles. The amount of glycogen in these cells contrasted sharply with the significantly reduced content in the degenerated and necrotic cells (Fig. 4) and was in excess of glycogen in normal hepatocytes. This was confirmed by our quantitative assays for liver glycogen. Considering the significant decrease in hepatocellular glycogen in the degenerated and necrotic centrilobular zone in CCl_4-dosed animals, the excess of glycogen in the individual vacuolated cells over normal ones would certainly be more than 50%.

The glycogen in vacuolated hepatocytes was also qualitatively different in some ways from that in normal cells. Not only did it appear more globular and red, rather than magenta colour, with PAS stain on light microscopy, but also ultrastructural examination showed that a significant part of it was in the nonrosetted beta particle form and often distinctly less electron dense. This relatively atypical particulate material was, however, confirmed to be glycogen by its strongly positive staining with PAS in corresponding semithin sections. It is possible that its morphological differences from normal glycogen are due to delayed breakdown or rapid synthesis. That the vacuolated appearance of the cells is due to accumulation of glycogen was further substantiated by our findings in rats starved for 24 h before sacrifice. Cytoplasmic vacuolation and demonstrable glycogen were both absent or significantly reduced. It is known that during starvation glycogen is rapidly removed from liver cells [2, 3, 6, 35]. An interesting aspect of the excessive glycogen accumulation in our animals is the almost complete absence of associated SER profiles. With the present experimental set-up, excessive accumulation of glycogen is not accompanied by SER proliferation. Current information indicates that while an increase in SER generally accompanies glycogen increase [3], the role of this organelle in synthesis of glycogen is debated [6]. In fact, in several other situations glycogenosis is not accompanied by SER proliferation [31]. In a recently reported study [17], livers of rats fed on a liquid diet containing ethanol showed vacuolated hepatocytes in the periportal areas, and these were histologically indistinguishable from those seen in our animals. The authors, however, interpreted the vacuolation as being due to microvesicular fatty change even though no biochemical, histochemical or morphological support was provided for this conclusion.

The vacuolated hepatocytes in our animals are not damaged liver cells showing degenerative changes, nor do they represent cells regenerating in response to hepatic injury. CCl_4-induced injury to hepatocytes is known to result in rapid loss of G-6-Pase enzyme, dilatation of ER profiles, accumulation of lipid, loss of cytoplasmic glycogen and disruption of cell organelles [14, 30]. All these features were consistently seen in the damaged cells located in the central lobular area in CCl_4-treated animals and in the periportal area in the AFB_1 and AlFo-primed animals. In contrast, all vacuolated hepatocytes had a full complement of G-6-Pase, excess of glycogen, no increase of lipid, and morphologically normal ER and mitochondria. Liver cells preparing to divide after hepatic necrosis induced by standard necrogenic doses of CCl_4 or after two-thirds partial hepatectomy and their progeny do not exhibit prominent cytoplasmic vacuolation, as observed in our present study [5]. Also, after CCl_4 injury cell replication involves hepatocytes in the immediate vicinity of the central necrotic area [5, 10, 22]. This was confirmed in our own material by positivity for PCNA in the nuclei of such hepatocytes (Fig. 8). Vacuolation affected more peripherally located liver cells, sparing the survivors adjoining the centrizonal area of necrosis. Mitosis and PCNA labelling were seen in these latter cells and not in the vacuolated ones (Fig. 8). A recent study on CCl_4-intoxicated rats showed that expression of the *c-Ha-ras* gene, which precedes cellular DNA duplication and cell division, increases markedly in the hepatocytes of the immediate perinecrotic parenchyma [34].

Vacuolated liver cells generated after small priming doses of hepatotoxins seem to be unaffected or less affected by a large necrogenic dose of CCl_4. Normally, centrizonal hepatocytes are preferred targets of CCl_4 injury [14, 15, 29, 30]. A large dose of CCl_4, however, failed to induce necrosis in those of our AFB_1- and AlFo-primed animals in which vacuolated cells were generated in the centrizonal area. In CCl_4-primed animals presence of vacuolation limited the subsequent necrosis to 41.4% of the lobular parenchyma, whereas in similarly primed but starved rats excretion of accumulated glycogen and vacuolation made the cells susceptible to necrosis, which thus affected 89.4% of the lobule, as in control animals (Table 3, Figs. 12). Serum transaminase elevations in rats challenged with a large dose of CCl_4 after priming with the small dose of the toxin were also significantly less pronounced than those in control animals (Figs. 14, 15). These findings substantiate our earlier preliminary data [10] showing that development of vacuolated liver cells in rats given a small dose of CCl_4 was associated with tolerance to a subsequent large dose of the chemical. Tolerance of animals and their livers to the hepatotoxic effect of chemicals following prior exposure to the same or another agent has been reported earlier, but the subcellular basis of this was not investigated [7, 8, 12, 13, 16, 18, 23, 24].

The pattern of cellular events extending centrifugally from the centre of peak hepatocellular damage appears to show a gradient from necrosis, through replication for cell replacement to an apparently adaptive change aimed at protection against toxic injury immediately afterwards. Vacuolated cells of identical constitution were generated after priming with all three hepatotoxic agents, CCl_4, AFB_1 and AlFo, and in all instances the change was equally associated with protection against subsequent assault with CCl_4. The induction of this cellular change therefore appears to be nonspecific in terms of

not only the initiating agent but also possibly the agent against which protection is acquired. However, it is known that CCl_4 is activated to its toxic product by cytochrome P-450 housed in the ER [9, 15, 30] and that induction of this enzyme by agents such as phenobarbitone is accompanied by SER proliferation and heightened susceptibility to CCl_4 hepatotoxicity [9, 15]. The reduction of ER profiles including SER in the vacuolated cells of some of our animals may be one way of explaining the tolerance to CCl_4. However, presence of excess glycogen appears to be consistently related to reduced susceptibility to CCl_4 injury, irrespective of the SER load. Changes in the levels of such substances as glutathione and prostacyclines, which help to reduce the lipid peroxidation that is crucial in the cell injury induced by hepatotoxins, may also be playing important parts. The molecular mechanism of tolerance to injury acquired by the glycogen-loaded, vacuolated cells remains to be elucidated.

Cells morphologically and histochemically identical to the vacuolated hepatocytes observed in the present study have been regularly encountered in animals subjected to various experimental modalities for studies on hepatocarcinogenesis [4, 11, 19, 25, 32, 33, 36]. These cells have been variously named "clear cells" [3, 19], vacuolated cells [19] and type-I cells [25], and in most instances large glycogen deposits have been observed in them [4]. In fact, in our own earlier study [25], the atypical ultrastructural features of glycogen, which were similar to those seen in the present investigation, misled us and we assumed that this material was possibly not glycogen. Several studies have now shown that chemical carcinogen-induced premalignant cell populations in in vivo hyperplastic nodules or in vitro cell cultures are resistant to the cytotoxic action of a variety of chemicals, including CCl_4 [7, 8, 12, 13, 18]. It has been proposed that during chemical carcinogenesis initially emerging altered hepatocytes represent an adaptive rather than a true preneoplastic change [13] in response to the hepatotoxic oncogenic agents. In our studies on hepatocarcinogenesis after one or more doses of oncogens in rats and monkeys the vacuolated type-I altered hepatocytes grouped in small foci or localised areas were the first to emerge [25, 32, 33]. We have encountered identical cells evolving before the emergence of preneoplastic nodules in material examined in a recent study reported from our laboratory on thioacetamide-induced hepatic fibrinogenesis in rats [1]. With time cells of this type are replaced by other altered cell types occurring in nodules or larger tumours [25, 32, 33]. Thus, it is reasonable to assume that as in the present study on acute toxic injury, vacuolated glycogen-rich liver cells emerge early to resist the damaging action of the toxic carcinogen in experimental hepatocarcinogenesis.

Our earlier observations on subacute hepatic failure in humans [27, 28] indicate that in this situation vacuolation of a proportion of hepatocytes may be an adaptive change to provide protection against the liver injury. A direct correlation between presence of vacuolated cells and recovery from hepatic coma was striking. We have recently been able to observe glycogen overloading and lack of PCNA labelling in vacuolated hepatocytes in this material as well as in archival biopsies of nonfatal acute viral hepatitis (unpublished data). Ultrastructural studies on acute viral hepatitis generally highlight ER dilatation and damage in swollen liver cells, but glycogen excess in several cells has been reported [37]. Bannasch earlier pointed out that under the light microscope glycogen accumulation is often mistaken for hydropic degeneration [3]. These findings, supplemented by those of the present study, strongly suggest that the swollen hepatocytes with nonlipid cytoplasmic vacuolation seen in acute and subacute liver injury are cells that have been adapted for the toleration of further injury rather than being damaged cells with hydropic change. This may also be true of some cells in chronic injury. Unlike other pathologic alterations, such as atrophy and hypertrophy, which are cellular adaptations to chronic stress or injury, the vacuolatory swelling seems to be a relatively short-lived, acute-phase adaptive response to milder forms of injury.

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