Fine Structural Changes in Hepatocytes after Simultaneous Treatment with Single Doses of 3'-Methyl-4-dimethylaminoazobenzene and Cycloheximide

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SUMMARY

Male rats were given single doses p.o. of 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB), either alone or at the same time as single s.c. injections of cycloheximide. They were killed either 24 or 48 hr after treatment or at intervals up to 21 months, and their hepatic tissues were examined by electron microscopy. Whereas 3'-MeDAB alone induced an early, marked peripheral displacement of organelles in the cytoplasm of the hepatocytes, together with a reduction in hyaloplasmic electron density, combined treatment with both chemicals failed to produce this acute toxic effect. However, the fine structure of the hepatic cells of rats given this combined treatment with cycloheximide and 3'-MeDAB closely resembled that obtained by chronic exposure to carcinogenic azo dyes. Of the changes thus produced, the granular endoplasmic reticulum in particular became permanently altered, both quantitatively and morphologically. Other persistent changes included mitochondrial abnormalities and glycogen depletion. Cycloheximide appears to protect the liver cell against the nonspecific acute toxic action of 3'-MeDAB, while facilitating the expression of effects that may possibly be associated with the carcinogenic action of this azo dye. Although this experimental model does not result in the appearance of tumors, it demonstrates that a single exposure to a carcinogen may induce permanent changes that are similar to those observed during carcinogenesis.

INTRODUCTION

The hepatocarcinogenic azo dyes (22-24, 27, 28) are known to induce a number of changes in the fine structure of the rat hepatocyte during their chronic administration (1, 15, 30, 35, 37, 38), many of which have also been observed in the cells of azo dye-induced hepatomata (21, 26, 27). However, the early effects of acute exposure to azo dyes such as 3'-MeDAB on the fine structure of the liver have received comparatively little attention hitherto, although there is some evidence (2, 14, 15, 16) that they may foreshadow the changes that arise during prolonged treatment. In view of the widely held belief that tumor initiation may be a relatively short process, the earliest changes induced by carcinogens are of interest. Unfortunately, the administration of acute doses of some agents, including 3'-MeDAB, results in toxic liver changes (11, 29, 36) which may include considerable disorganization of the fine structure of the hepatocytes (14). This may mask any more subtle effects that might be due to the carcinogenic action of the agent.

In previous studies (11, 12), it had been demonstrated that the acute histopathological damage resulting from acute intoxication with 3'-MeDAB and some other carcinogens could be prevented by simultaneous treatment with CHM. This raised the possibility that toxic damage at the level of fine structure may also be prevented by CHM, without necessarily inhibiting specific effects of 3'-MeDAB. The present study was carried out to investigate this possibility by electron microscopic examination of the livers of rats treated simultaneously with 3'-MeDAB and protective doses of CHM. In addition to acute studies, long-term experiments were also performed to ascertain the effects of this treatment on tumor induction or other liver changes. No such long-term studies appear to have been made hitherto at the ultrastructural level.

MATERIALS AND METHODS

Animals

Adult male Leeds rats, bred in this laboratory, were maintained on Oxoid 41B diet and water ad libitum.

Chemicals

3'-MeDAB (Koch-Light, Colnbrook, England) was administered p.o. as a suspension in olive oil, at concentration of 0.5 g/ml. The sublethal doses used, 150 and 300 mg/kg body weight, were those given in a previous study of CHM protection (11). The dose of 3'-MeDAB that killed 50% of rats has been reported to be 500 mg/kg body weight (41). CHM (Actidione; Upjohn Company, Kalamazoo, Mich.) was given s.c. into the flank, as single doses of 1.5 mg/kg. It was prepared as a sterile 0.2% aqueous solution. This dose induces a 90% inhibition of protein synthesis (44), without either inhibiting RNA synthesis (18) or producing liver cell...
damage (39, 40), and is known to protect a variety of tissues from toxic damage (4, 11, 12, 18, 19).

Experimental Treatment

**Acute Experiments.** Five groups of rats, each consisting of 6 animals, were used. Each rat of Group 1 received a single p.o. dose of 3'-MeDAB, 150 mg/kg body weight. Group 2 rats were similarly treated with 300 mg/kg. Group 3 rats received 3'-MeDAB, 150 mg/kg, immediately followed by a single s.c. dose of CHM, 1.5 mg/kg. Rats of Group 4 were given 3'-MeDAB, 300 mg/kg, and CHM, 1.5 mg/kg. Group 5 rats received CHM alone.

Three rats from each group were sacrificed, by stunning, 24 hr after treatment and the remainder were sacrificed at 48 hr. Samples of hepatic tissue were immediately removed for processing for electron microscopy and histology.

**Long-term Experiments.** A group of 27 rats was given a single p.o. dose of 3'-MeDAB, 300 mg/kg body weight. A 2nd group received the same dose of 3'-MeDAB together with CHM, 1.5 mg/kg. A number of animals from each group were killed at the following times after treatment: 4 at 5 months, 3 at 9 months, 6 at 12 months, 12 at 17 months, and 4 at 21 months.

At each of these times 3 rats that had received a single dose of CHM alone were also sacrificed. The livers of all animals were examined by light and electron microscopy.

Treatment of Tissues

The hepatic tissue was placed in ice-cold 4% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.2 (32), and minced into 1-cm mm blocks. Fixation was continued for 4 hr at 0–4°C, and the tissue was then washed for at least 16 hr at 0–4°C in 0.1 M sodium cacodylate in 0.25 M sucrose. Postfixation was carried out for 2 hr in phosphate-buffered 1% osmium tetroxide at 0–4°C (25). The tissue was dehydrated in a graded ethanol series at ambient temperature and embedded in Epon 812, essentially by the method of Luft (20).

For electron microscopy thin sections were cut with diamond knives on a Sorvall MT-1 Porter-Blum ultramicrotome, mounted on naked copper grids, and double-stained with saturated uranyl acetate in 50% ethanol and Millonig's lead tartrate (26). They were examined in a Philips EM 300 electron microscope at an accelerating voltage of 80 kV. In order to facilitate orientation of the tissue used for electron microscopy and for high-resolution light microscopy, thicker (0.25–0.5μm) parallel sections were cut in the same way, mounted on glass slides, and stained with 1% methylene blue in 1% borax.

For routine histopathology paraffin-embedded samples of liver were used, stained with hematoxylin and eosin or periodic acid-Schiff.

RESULTS

Acute Effects

The early light microscopic changes that are seen in the rat liver during CHM protection of 3'-MeDAB-intoxicated rats have been fully described in a previous paper (11). In brief, 3'-MeDAB, in the doses used here, causes severe glycogen depletion and a pronounced "vacuolation" in the hepatocytes, which is seen in high-resolution light microscopy (Fig. 1) to be due to the presence of large perinuclear areas of cytoplasm that are virtually free from organelles. The effects are more severe at 48 hr than at 24 hr and after 300 mg/kg than after 150 mg/kg. Cells showing the most pronounced cytoplasmic changes also frequently have dark, shrunken, irregular nuclei. A small amount of periporal necrosis can be observed in some animals treated with the higher dose of 3'-MeDAB. CHM, which alone appears only to produce loss of hepatic cell glycogen, prevents the peripheral displacement of organelles by 3'-MeDAB (Fig. 2), as well as any nuclear changes. There is no evidence of necrosis or any liver damage up to 48 hr after combined treatment with CHM and either dose level of 3'-MeDAB.

**3'-MeDAB Controls.** The pathogenesis of the acute fine structural lesion induced in rat hepatocytes by a single dose of 3'-MeDAB has been the subject of earlier reports (2, 14). The present study confirmed that the characteristic changes in all rats (14) include severe glycogen depletion and the progressive development of large clear cytoplasmic areas of reduced hyaloplasmic density (Fig. 3), which may ultimately encircle the nuclei and displace the organelles to the cell periphery. During the period investigated, there was no invasion of these clear areas by the agranular endoplasmic reticulum. The granular endoplasmic reticulum was dispersed and reduced in quantity (Fig. 3), while the Golgi apparatus became atrophic. Nuclear shrinkage occurred in the most severely affected hepatocytes. The changes were more pronounced after 48 hr and after the higher dose of 3'-MeDAB, thus being both time and dose dependent. However, the differences were quantitative rather than qualitative. Similar, but less marked, differences in the degree of damage were observed between individual rats. However, up to 48 hr no frankly necrotic cells were observed in the samples studied.

**CHM Controls.** The hepatocytes of all rats treated with CHM, 24 or 48 hr previously, exhibited severe glycogen depletion, the glycogen areas being infiltrated and replaced by elements of the proliferating agranular endoplasmic reticulum (Fig. 4). The size of individual parallel arrays of granular endoplasmic reticulum cisternae was somewhat reduced, but this organelle showed little change otherwise.

The Golgi zones usually possessed normal granular contents, but they were sometimes displaced from their periportal sites. The morphology and disposition of the remaining organelles appeared to be normal and no nuclear changes were observed.

**Combined 3'-MeDAB and CHM Treatment.** The simultaneous administration of 3'-MeDAB and CHM resulted in changes that were clearly different from those induced by either agent alone. In particular (Fig. 5), there was no sign of the clear cytoplasmic areas or peripheral displacement of organelles which follow 3'-MeDAB treatment, although glycogen depletion was similarly severe. The fine structure of the hepatocytes was essentially independent of the dose of 3'-MeDAB given and did not appear to change between 24
and 48 hr. All rats showed the same responses in their hepatic cells.

In contrast to the minimal changes induced by CHM in the granular endoplasmic reticulum (Fig. 6), combined drug treatment gave rise to dispersal and fragmentation of this organelle (Figs. 5, 7, and 8) and an apparent reduction in its quantity. It was almost entirely present as single cisternae, either lying free or, more commonly, encircling the mitochondria. These changes were more pronounced than those that followed simple 3'-MeDAB intoxication and also included focal loss of attached ribosomes (Figs. 6 and 7) and some localized cisternal dilation (Fig. 7).

The Golgi zones were typically small and devoid of granular contents (Figs. 7 and 9) and were often situated in the perinuclear cytoplasm. However, many hepatocytes contained increased numbers of lysosomes or residual bodies (Figs. 5 and 7).

A moderate hypertrophy of the agranular endoplasmic reticulum was observed (Fig. 10). This did not appear to be more pronounced at 48 hr than at 24 hr. The hypertrophy was too marked to be accounted for by an "unmasking" effect of glycogen depletion. Many cells contained large lipid droplets.

The bile canaliculi were sometimes distorted in form, with dilated lumina, and exhibited a localized loss of microvilli (Figs. 5 and 9). Intercellular pegs were frequently observed (Fig. 8).

The nuclei were normal in size and shape, but many of the nucleoli showed minor degrees of microsegregation (Fig. 1).

**Long-Term Effects**

The histological appearance of the livers of rats killed from 5 to 21 months after a single dose of 3'-MeDAB, with or without CHM, was unremarkable, except for weak periodic acid-Schiff staining which was most apparent after combined treatment and indicated a low glycogen content. Treatment of rats with CHM alone did not induce any long-term changes. No liver tumors were found and there was no overt hyperplasia or bile duct cell proliferation. A single rat had developed a small hemangiosarcoma, attached to the cecum, by 21 months after combined treatment.

**Combined 3'-MeDAB and CHM Treatment.** The fine structure of the hepatocytes remained consistently abnormal up to 21 months after treatment, there being little significant difference between animals killed earlier or later in the experiment, except for mitochondrial changes which became rather more pronounced with time. Glycogen depletion was severe, there being an almost total absence of normal glycogen areas (Fig. 12). Some hepatocytes contained abnormally large numbers of lysosomes, and the Golgi zones were frequently displaced from their pericanalicular sites.

The granular endoplasmic reticulum was clearly reduced in quantity and was present as single, short cisternae, either lying free or closely encircling the mitochondria (Figs. 12 and 13). Parallel arrays of rough-surfaced cisternae were rarely observed. Small areas of agranular endoplasmic reticulum were present, but it was not readily apparent that the reticulum was hypertrophied. It seemed more probable that its conspicuousness was due to an unmasking effect resulting from loss of glycogen. The cell surface and its modifications showed no consistent alterations. The nuclei and nucleoli appeared to be normal in morphology. The alterations described were observed in the overwhelming majority of the hepatocytes of all rats. However, there was, in contrast, considerable variation in the mitochondrial population. Mitochondria were frequently abnormally abundant. They were usually either apparently normal in appearance or grossly enlarged (Fig. 13). In some cells they were closely packed, irregular in outline, and with abnormal cristae (Fig. 14).

**3'-MeDAB Controls.** The long-term fine structural changes observed after treatment with 3'-MeDAB alone were qualitatively similar to those induced by combined treatment. However, they were observed only in a minority of hepatic cells. Most cells contained relatively normal glycogen areas and prominent parallel arrays of granular endoplasmic reticulum cisternae. Mitochondrial abnormalities were also less common than after treatment with both 3'-MeDAB and CHM.

**CHM Controls.** Rats treated with CHM alone had livers that were histologically and ultrastructurally normal at 5 months and thereafter for the duration of the experiment.

**DISCUSSION**

The present investigation has confirmed that the most conspicuous and immediate hepatic cell change induced by acute 3'-MeDAB intoxication is a peripheral displacement of organelles, with a reduction in electron density of the hyaloplasm (12). It is clear that CHM prevents both this change and the nuclear irregularity and shrinkage that can result from acute exposure to 3'-MeDAB (11, 14). This result is in close accord with previous light microscopic observations on CHM protection of the rat liver against 3'-MeDAB-induced damage (11). This protection was previously attributed specifically to the inhibition of protein synthesis by CHM (11). This view is strongly supported by evidence that, in the doses used in the present study, CHM selectively inhibits peptide chain termination or release (31) without affecting RNA synthesis (3) and protects other tissues as well as the liver against various cytotoxic agents (4, 11, 12, 18, 19). In addition, a different compound, tenuazonic acid, which inhibits protein synthesis in the same way as CHM (34), also exerts a similar protective effect (4, 18). It is thus possible that CHM protection is due to the prevention of a type of "lethal synthesis" in the target cells, as suggested by Lieberman (17).

The cytoplasmic "clearing" which results from acute 3'-MeDAB intoxication is probably caused by changes in cellular hydration as well as glycogenolysis. Certainly, it is not simply due to loss of glycogen, as is shown by its lack of similarity to the severe, typical glycogen depletion produced by CHM alone or combined with 3'-MeDAB. DL-Ethionine and 2-acetylaminofluorene also induce a similar cytoplasmic clearing which can be prevented by CHM (Ref. 13; B. Flaks and W. F. Basley, unpublished observations), indicating that this represents a nonspecific toxic lesion.
As in previous studies (39, 40), CHM itself caused only minimal fine structural changes, although proliferation of the agranular endoplasmic reticulum was considerably more pronounced in the present experiment. However, none of the effects of this agent persisted in the long-term experiments. 3'-MeDAB alone did not induce hypertrophy of the agranular endoplasmic reticulum, probably because of the toxicity of the doses used. This view is reinforced by evidence from 1 study of acute 3'-MeDAB intoxication (2), in which less severe liver cell damage was accompanied by proliferation of this organelle, the induction of which by chronic exposure to low levels of azo dyes is well known (15, 30, 33, 35-38).

The early morphological alterations of the granular endoplasmic reticulum following simultaneous treatment with 3'-MeDAB and CHM were considerably greater than those induced by 3'-MeDAB alone. This may have been due to the action of CHM in preventing the peripheral dispersal of cytoplasmic organelles and thus the masking of such changes. However, there is no evidence that this nonspecific cytoplasmic clearing requires the metabolic activation of the azo dye. Thus, the enhanced changes of the granular cytomembranes may be the result of an increased rate of metabolism, and activation, of 3'-MeDAB by the CHM-induced hypertrophic agranular endoplasmic reticulum.

The early granular endoplasmic reticulum changes persisted for at least 21 months following a single exposure to 3'-MeDAB. Thus, they do not appear to be spontaneously reversible. Furthermore, it is apparent that the early enhancement of these alterations by CHM protection is reflected in their greater prominence and frequency, in long-term studies, compared with their relatively less common occurrence in rats that had originally received 3'-MeDAB alone. There is a striking similarity between these granular endoplasmic reticulum changes and those observed during the chronic administration of carcinogenic azo dyes to the rat (1, 15, 30, 36-38), as well as in azo dye-induced hepatic cell carcinomata (21, 36, 37). Similar changes in this organ-elle are also induced during hepatocarcinogenesis by other types of compounds, including 2-acylaminofluorene (6-8, 37), and may become irreversible (5). The failure of a noncarcinogenic isomer of 2-acylaminofluorene, 4-acylamino- fluorene, to effect such alterations indicates that they may be significant to the neoplastic process in the liver (9, 10). Mitochondrial pleiomorphism, of the type seen in the present long-term experiments, is also a common feature of azo dye- and aminofluorene-induced hepatocarcinogenesis. The fact that such a change was not observed during the acute stages of intoxication suggests that it is secondary to the granular endoplasmic reticulum lesion.

Other fine structural similarities between the effects of acute combined CHM and 3'-MeDAB treatment and chronic exposure to azo dyes include increased numbers of lysosomes (15, 30, 36, 37), lipid accumulation (36-38), glycogen depletion (15, 30, 37), nucleolar segregation (37), and of the Golgi apparatus (33, 36, 37). However, there is evidence from other studies (6-10) that few of these are necessary features of hepatocarcinogenesis; in the present study, with the exception of increased lysosomal abundance, all were transient effects. Nevertheless, it would appear that the acute toxic effects of 3'-MeDAB may indeed interfere with the expression of fine structural alterations which are potentially related to its carcinogenic action and that CHM can overcome this interference, with significant long-term consequences. Although no hepatic cell tumors were induced by the treatments used here, the induction of persistent cellular changes may have some relevance to problems of human exposure to chemical carcinogens. Similar persistent fine structural alterations may also arise in human tissues as a result of a single subcarcinogenic exposure, without producing histologically detectable damage, and may alter their susceptibility to subsequent carcinogenic stimuli.

The use of CHM as a protective agent appears to provide a means of facilitating the study of early changes produced by single doses of azo dyes and other carcinogens.

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REFERENCES


Hepatocyte Changes after Simultaneous 3'-MeDAB and CHM

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