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Mitochondrial Changes in Hepatocytes of Rats Chronically Exposed to Vinyl Chloride and Ethanol

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Chronic exposure of male rats to 600 ppm vinyl chloride (VC) or VC and 5% ethanol (EtOH) in drinking water induced ultrastructural changes in the mitochondria of hepatocytes. After 6 months of VC/EtOH treatment, hepatocyte mitochondria often contained rigid tubular parallel cristae and dilated cristae with dense inclusions; ethanol ingestion alone did not induce these morphological changes. In animals receiving VC alone, large flocculer densities in the mitochondrial matrix were seen occasionally after 6 months of VC inhalation. The numbers and severity of changes in mitochondria increased with duration of exposure and age. The greatest responses observed in mitochondria occurred with the combined treatment of VC/EtOH.

INTRODUCTION

It has been well documented that inhalation of vinyl chloride (VC) by man (Creech and Johnson, 1974) and laboratory animals (Maltoni and Lefemine, 1975) induces angiosarcoma of the liver. The toxicity of VC is attributed to reactive metabolites, chloroethylene oxide and 2-chloroacetaldehyde, which also elicit mutagenic and toxic responses in selected bacteria (Malaveille *et al.*, 1975; McCann *et al.*, 1975) and mammalian cells (Huberman *et al.*, 1979). At equal concentrations in bacterial systems chloroethylene oxide was more mutagenic, although less toxic than 2-chloroacetaldehyde.

The hepatocyte, which is predominantly responsible for the metabolism of VC, has been reported to undergo morphologic changes and significant cellular destruction following acute exposures of rats to VC (Du *et al.*, 1979; Feron *et al.*, 1979; Feron and Kroes, 1979). In an attempt to elucidate some of the intracellular mechanisms involved in VC toxicity and carcinogenicity before destruction of parenchymal elements, a model of chronic VC inhalation exposure and ethanol ingestion was devised (Radike *et al.*, 1977). This report describes the light and electron microscopic alterations in the mitochondria of the liver parenchymal cells resulting from chronic exposure of rats to VC or VC/EtOH.

MATERIALS AND METHODS

Male Sprague–Dawley rats were used in two separate long-term studies on the effects of ingested ethanol (EtOH) on VC-induced carcinogenesis. Animals (480) were divided into four groups: group one received filtered air and tap water (Air/H_2O); group two received filtered air and 5% EtOH (v/v) in the drinking water (Air/EtOH); group three inhaled 600 ppm VC, 4 hr/day, 5 days/week (VC/H_2O);

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and group four inhaled VC and ingested EtOH (VC/EtOH). All animals were fed Ralston Purina rat chow *ad libitum* except during exposure to VC, when food and water were withheld. Animals in groups three and four were held in individually compartmented stainless-steel cages for VC exposure by inhalation. Ingestion of EtOH began 4 weeks prior to the initiation of VC inhalation and was continued until death or killing of the animal.

In the first experiment, animals were treated with VC for 1 year (80/group). Unless the animals appeared to be moribund, they were killed 1½ years postexposure. In the second experiment (40/group), sacrifices were scheduled at 3, 6, 9, and 12 months after the initiation of VC inhalation. The method of killing, procedures for obtaining paraffin-embedded tissues, special staining procedures for light microscopy, and the evaluation of the types of tumors which developed have been reported (Radike *et al.*, 1981).

Preparation of tissues for electron microscopy was as follows: as quickly as possible after pentobarbital anesthesia, pieces of the liver were removed and diced into 1-mm cubes. Tissues were fixed in Chick fix (Russell, 1972) then post-fixed in phosphate-buffered 1% osmium tetroxide for 2 hr. In addition, duplicate tissues were fixed directly in phosphate-buffered osmium tetroxide. All tissues were dehydrated stepwise through graded ethanols and embedded in Spurr (Spurr, 1969). Toluidine blue-stained sections (0.5 μm) were examined with light microscopy and thin sections on naked grids were contrasted with uranyl acetate and lead citrate for transmission electron microscopy. A total of 20 animals each from the Air/H_2O, Air, EtOH, and VC/EtOH groups and 19 from the VC group (79 of 480 animals) were prepared for electron microscopy.

RESULTS

The ultrastructure of the liver, particularly the parenchymal cells of the Air/H_2O group, was similar to that amply described in the literature (Fig. 1).

Mitochondria in all experimental groups demonstrated greater heterogeneity in size and shape than did controls. Numbers of mitochondria with changes and the severity of changes increased with the duration of exposure and age. The ratio of surface/volume appeared to increase in the experimental groups with time.

Mitochondria of ethanol-treated animals were rounded with an increase in the number of marginated cristae and an increase in heterogeneity in size and shape. Some areas of parallel cristae were seen in animals which received ethanol for 12 months and longer. Not seen were the intracristal inclusions which were so common in the animals treated with ethanol and vinyl chloride together. In addition to minimal changes in the mitochondria, the cytoplasm of hepatocytes demonstrated a modest increase in the amount of smooth endoplasmic reticulum.

Vinyl chloride inhalation alone did not induce mitochondrial changes which were observed following VC/EtOH treatment. Intracristal inclusions in their group were never demonstrated unequivocally, although dilated cristae with some indication of dense inclusions were encountered. Mitochondria as the main component in autophagocytic bodies in the cytoplasm of VC-treated animals were common.

Mitochondria in the VC/EtOH groups were predominantly indented and cup-shaped (Fig. 2), whereas those resulting from VC exposure alone were more often

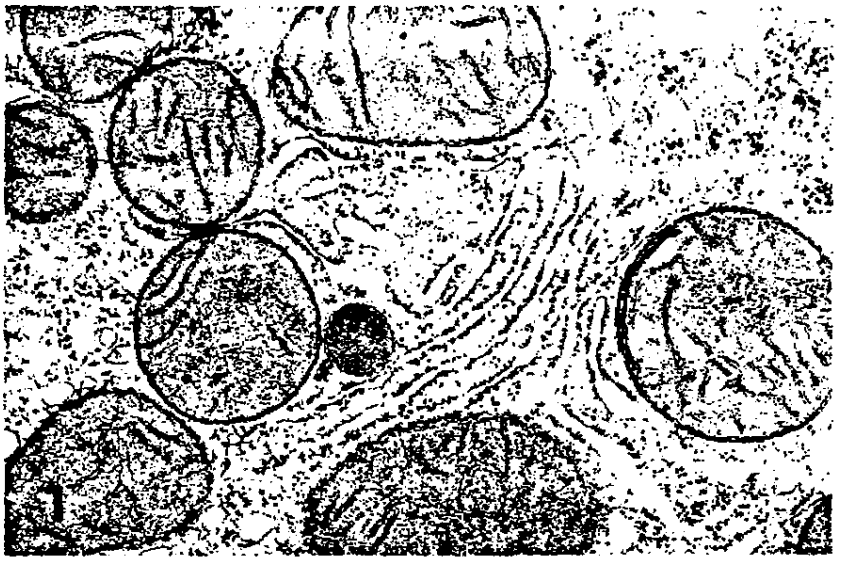

FIG. 1. Liver of a control rat which was age-matched to animals treated with vinyl chloride and ethanol for a year. Normal mitochondria, rough endoplasmic reticulum, microbodies, and smooth endoplasmic reticulum prevail. Glycogen is present. Cristae within mitochondria are lamellar and the complement of intramitochondrial granules is usual. 9000×.

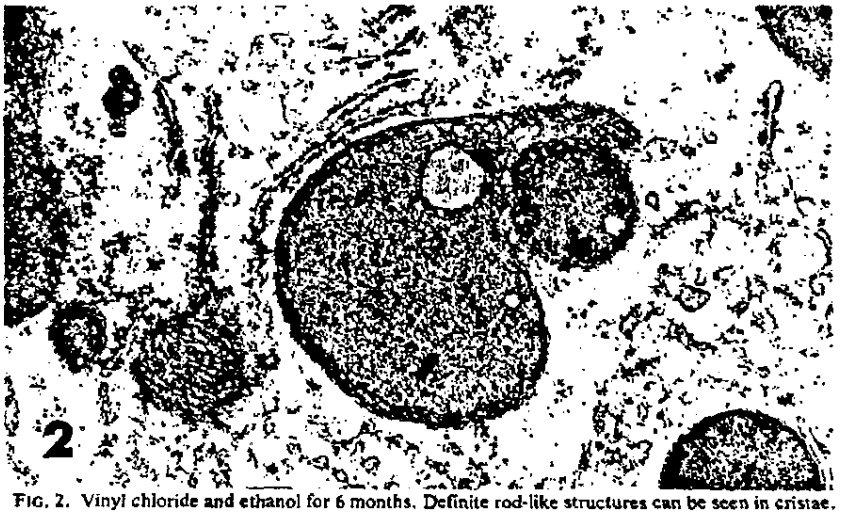

FIG. 2. Vinyl chloride and ethanol for 6 months. Definite rod-like structures can be seen in cristae. Note lack of normal lamellar cristae and parallel organization of tubular cristae on the thin indented portion of the mitochondrion. 35,000×.

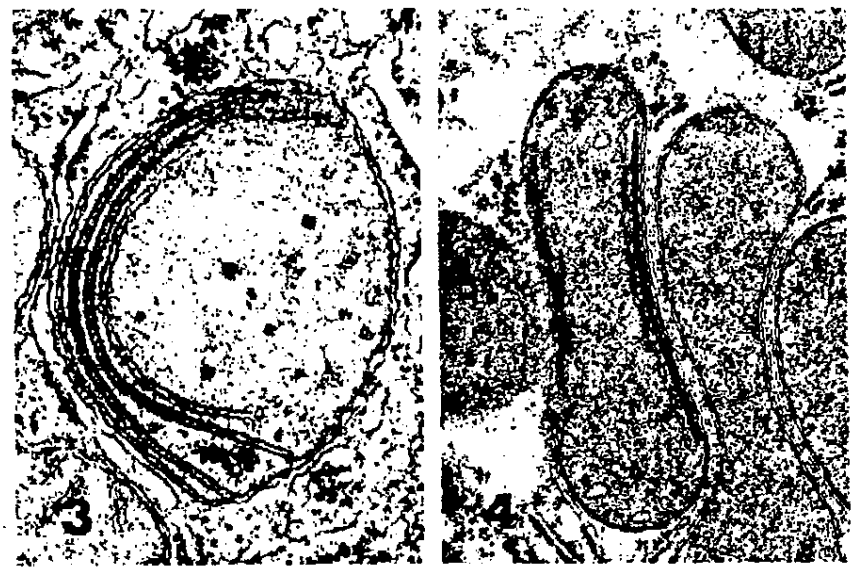

FIG. 3. Vinyl chloride and ethanol for 6 months. Tubules within the matrix have deformed the mitochondrion. Note the coiled nature. At opportune areas, a dense central line can be observed. Cross sections of the tubules are visible in the lower corner of the mitochondrion. A central core in these particular tubules is not resolved. 59,000×.

FIG. 4. Vinyl chloride and ethanol for 6 months. Longitudinal tubular structures in the mitochondria of the hepatocytes. Cristae not yet transformed are usually tubular, instead of lamellar. 36,000×.

elongated. Parallel cristae in the indented areas were common. Pseudoinclusions of cytoplasm or portions of other mitochondria resulted from section artifact. Animals exposed to VC or VC/EtOH had mitochondria which often demonstrated rigid parallel cristae (Figs. 3, 4). Cross sections of these structures confirmed their tubular nature. In extreme cases, rigid cristae showed a periodicity and a faint central line. Increases in membrane density of cristae, periodicity, and the central line were not seen in animals receiving EtOH alone or in Air/H_2O controls. These changes first appeared at 6 months after initiation of treatment in the VC/EtOH group in a small number of animals and by 12 months they were found routinely.

The mitochondria of VC/EtOH-treated animals showed peculiar intracristal densities which, on very opportune sections, had a regular substructure. Some of these were stacked (Fig. 2), and others were coiled (Fig. 5). Dilated cristae with inclusions were seen in VC/EtOH animals after 6 months of exposure and were not seen in animals after VC or ethanol alone or filtered air.

In animals which received VC alone, another type of density, not associated with the cristae, was found in the matrix of the mitochondria. These floccular densities were observed at 6 months after initiation of VC and after 1½ years of recovery (Fig. 6).

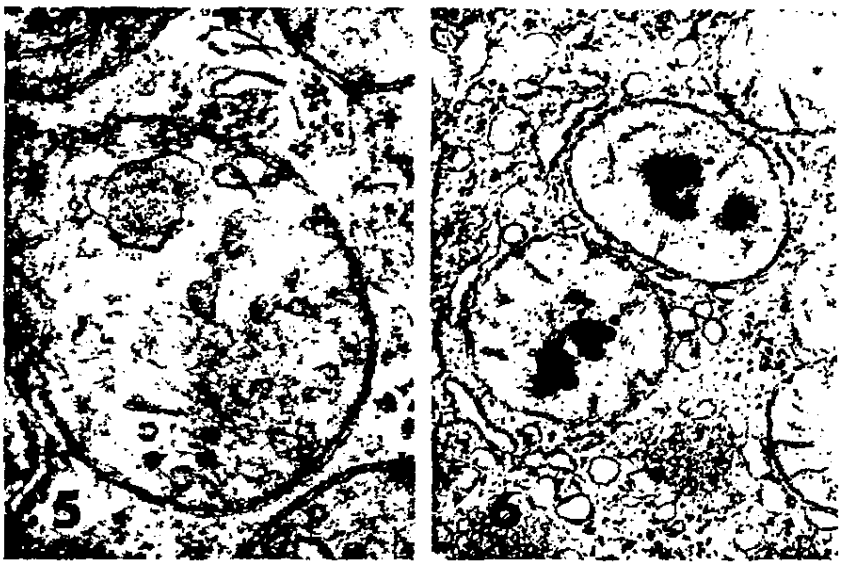

FIG. 5. Vinyl chloride and ethanol for 6 months. Intracristal inclusions in mitochondria occasionally appeared helical; or coiled. 62,000×.

FIG. 6. Vinyl chloride alone for 1 year and recovery for 1½ years. A less common alteration in mitochondria was floccular densities within the matrix. 3000×.

Cytoplasmic changes which were observed at the early sacrifices (3 and 6 months) in the VC and VC/EtOH groups included a mild increase in the density of filaments at the plasmalemma of the hepatocytes and a conspicuously close association of the mitochondria with the filaments adjacent to tight junctions (desmosomes) (Fig. 7). These anchor mitochondria were encountered less frequently in the 3-month Air/H_2O controls. Mitochondria associated with the plasmalemma in VC and VC/EtOH groups were noted by light microscopy of semithin sections by an increase in their numbers at the periphery of the hepatocytes. In lesions of parenchymal cells which developed in these rats, hepatocytes also demonstrated long, peripherally located mitochondria of which a large number were associated with desmosomes, sometimes at more than one point of anchoring. Mitochondria also showed an increase in touching (two or more mitochondria in close contact along their outer membranes). These morphologic changes appeared in the early stages of exposure and in lesions; other mitochondrial changes (parallel cristae, tubular transformations, intracristal inclusions, and matrical densities) did not.

DISCUSSION

Mitochondria with modified cristae have been seen in animals exposed to toxic agents other than VC or VC/EtOH. The administration of polychlorinated biphenyls in the rat resulted in cristae which were dense, noticeably rigid, and

FIG. 7. Vinyl chloride and ethanol for 12 months. Anchor mitochondria were a regular feature of the animals which received VC/EtOH. Here two mitochondria from adjacent hepatocytes show a peculiar arrangement next to the filaments of a desmosome. Such configurations were frequently seen in hepatocellular lesions; however, this was visualized in the "normal" portion of the liver. 37,000×.

parallel to the long axis of the mitochondrion (Burse *et al.*, 1974), but dilated cristae with inclusions were not mentioned. Confer and Stenger (1966) reported a central sheaf of cristae in mitochondria of hepatocytes after carbon tetrachloride exposure; they did not observe, however, an increase in density, or tubular or coiled cristae with a central core. Weinstein *et al.* (1972) observed bizarre-shaped mitochondria resulting from dichloromethane inhalation. Some of the changes bear at least a superficial resemblance to the mitochondrial alterations seen in VC/EtOH-exposed animals.

Occasionally, similar mitochondrial structures have been observed in normal tissues. Tubule-like structures with a dense central core seen in spermatids (Fukumoto, 1979) are somewhat similar to inclusions in VC/EtOH-treated mitochondria. Elongation of the organelle accompanied the presence of regularly arranged parallel cristae. It has also been reported that in a small number of cases the normal pineal gland and brown adipose tissue (interscapular) of pre- and neonatal rats have unusual mitochondrial inclusions (Barnard and Lindberg, 1969). It is evident that alignment of mitochondrial cristae represents a common response to toxic substances, however, intracristal inclusions appear in sufficiently few experimental and normal conditions to make their occurrence after VC/EtOH exposure remarkable.

Feron *et al.* (1979) examined mitochondria after high doses of VC (5000 ppm) and they stated that the swelling and irregularity in mitochondrial shape were not specific for vinyl chloride. Their exposures were very high in comparison to the 600 ppm used here. After high doses of VC, greatly enlarged pale mitochondria and spotty degeneration of the hepatocytes were reported (Du *et al.*, 1979). We found that at low doses necrosis was not a prominent feature of hepatocyte morphology, though hepatocellular carcinomas, angiosarcomas, and other tumors developed in rats after prolonged exposure to low doses of VC and VC/EtOH (Radike *et al.*, 1981).

In hepatocytes of animals treated with ethanol alone, the low level (5%) EtOH used in this study did not produce the megamitochondria commonly observed in animals receiving high concentrations of ethanol nor the filaments of Mallory bodies characteristic of the response of human hepatocytes. These effects of ethanol on mitochondria, *in vivo* and *in vitro*, have long been recognized (Rubin, 1973).

At this time, no correlation can be drawn between the structural changes in mitochondria reported here in rats concomitantly exposed to VC and ethanol and the increased incidence of hepatic angiosarcomas and carcinomas which were observed (Radike *et al.*, 1981).

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Effect of Subacute Exposure to NO_2 on Lymphocytes Required for Antibody Responses

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BALB/c mice were continuously exposed to 0.4 and 1.6 ppm NO_2 for 4 weeks and the effects on lymphocytes which are required for primary and secondary antibody responses to sheep red blood cells were examined *in vitro*. The primary antibody response was significantly suppressed by both concentrations of NO_2, whereas the secondary antibody response was slightly stimulated by 1.6 ppm NO_2 exposure. In reconstitution experiments no significant differences were observed in the activities of T and B lymphocytes from mice exposed to 1.6 ppm NO_2.

INTRODUCTION

Many investigators have reported the effect of short- and long-term exposure to low levels of NO_2 on immune defense mechanisms (Fenters *et al.*, 1971, 1973; Ehrlich *et al.*, 1975). Maigetter *et al.* (1978) have already shown the depression of mitogenic responses in both T and B cells by a long-term exposure to 0.5 ppm NO_2. Recently, the effect on the immunological function of exposure to 10 ppm NO or NO_2 for 2 hr daily on weekdays, for varying periods up to 30 weeks, was examined by Holt *et al.* (1979). They indicated that the immune functions, e.g., serum antibody responses, T cell mitogen, and graft-versus-host responses, were suppressed by chronic exposure but were enhanced by shorter exposure. *In vitro* analysis of effects of acute exposure to NO_2 on antibody responses was performed in our laboratory and indicated that the acute exposure to 20 ppm NO_2 for 12 hr markedly suppressed the primary antibody response, which was mainly caused by the inactivation of B cells (Fujimaki *et al.*, 1981).

In this paper, to extend these studies the effect of subacute exposure to relatively low levels of NO_2 on lymphocytes required for antibody responses to SRBC was investigated *in vitro*. The primary antibody response was significantly suppressed by exposure to 0.4 and 1.6 ppm NO_2 for 4 weeks, but the secondary antibody response was stimulated by exposure to 1.6 ppm NO_2.

MATERIALS AND METHODS

Mice. BALB/c male mice, purchased from Charles River Japan, Inc., were used and they were 7 weeks old at the commencement of the exposure.

NO_2 exposure. Mice were continuously exposed to 0.4 and 1.6 ppm NO_2 for 4 weeks in a stainless-steel and glass chamber (1800 liters) made by Koito Kogyo Co., Ltd., which was provided with 60 air changes per hour, and the concentra-

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