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Experimental acute poisoning in mice induced by emestrin, a new mycotoxin isolated from *Emericella* species

Kiyoshi Terao,[1] Emiko Ito,[1] Ken-ichi Kawai,[2] Kohei Nozawa[2] & Shun-ichi Udagawa[3]
[1] *Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Inohana 1-8-1, Chiba, 280, Japan*; [2] *Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo, 142, Japan*; [3] *National Institute of Hygienic Sciences, Kamiyoga, 1-18-1, Setagaya-ku, Tokyo, Japan*

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Abstract

The effects of emestrin (EMS), a secondary metabolite of the *Emericella* species, on male ICR mice were examined. The intraperitoneal LD_{50} values of EMS were 17.7 and 13.0 mg/kg at 24 and 48 hr, respectively. The target organs of EMS were the heart, liver and thymus. In doses over 30 mg/kg the experimental animals died from cardiac failure shortly after the injections. Several survivors that were given EMS in doses under 20 mg/kg showed severe centrilobular necrosis in the liver at 24 hr. Marked degeneration of mitochondria was seen in electron micrographs of both cardiac muscle cells and hepatocytes. In the degenerated hepatocytes, prominent proliferation of RER, membrane-limited inclusions containing both ribosome-like granules and RER, and fenestrated lamella-like structures were observed. Massive necrosis of lymphocytes was always observed in the cortical layer of the thymus of the survivors within 24 hr, while bilateral adrenalectomized mice showed no discernible pathomorphological changes in the lymphoid tissues. Pretreatment of mice with diethyl maleate increased the incidence and severity of hepatic necrosis, whereas that with either cysteine or $CoCl_2$ reduced the severity of centrilobular necrosis of the liver. Pretreatment with phenobarbital had no significant effect on EMS-induced hepatic lesions.

Introduction

Emestrin (EMS) was isolated by Seya *et al.* in 1985 from mycelial acetone extracts of the mold *Emericella striata*. Its structure and absolute configuration have been determined as a macrocyclic epidithiodioxopiperizine derivative [10 11], (Fig. 1). At the same time, EQ-1 named by Maebayashi *et al.* [5] for a toxic metabolite of *E. quadrilineata*, *E. acristata*, and *E. paravathecia* was identified with EMS by Seya *et al.* [10, 11].

Species of the genus *Emericella* are distributed widely throughout tropical and subtropical countries. EMS-producing species have been isolated from soil of a date-plantation in Iraq [5], from cumin (the seeds of *Cuminum cymium* L) collected in Nepal [10, 11], and from various spices imported into Japan from India, South East Asia and China [12]. EMS was also obtained from the culture of *E. foveolata*, a new species from an Indian herbal drug (*Tribulus terrestrias* L).

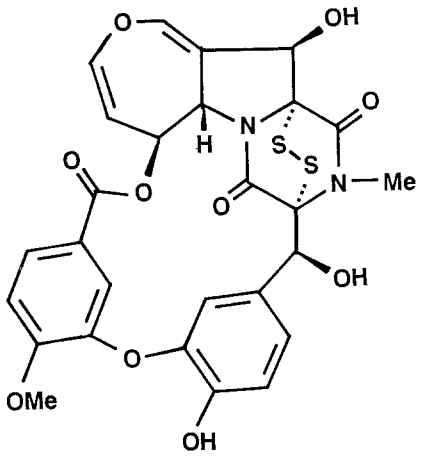

Fig. 1. Chemical structure of emestrin.

Only limited information is available regarding the biological activities of EMS. In an *in vivo* system, Maebayashi *et al.* [5] reported a high lethality in mice after i.p. injection of EMS in propylene glycol at a dose of 500 mg/kg. In an *in vitro* system of isolated murine hepatocyte mitochondria, K. Kawai of Nagoya Women's University observed a potent suppressive effects of EMS on respiratory systems (personal communication, 1987). However, no precise toxicological studies have been reported on the target organs of experimental animals after administration of EMS. The present studies were undertaken to investigate the morphological changes in heart, liver, and lymphoid tissues in male ICR mice after the administration of EMS, and to demonstrate the effects of various modifying factors on EMS-induced lesions.

Material and methods

Experimental animals. Thirty male ICR mice weighing 20–25 g were used for the determination of lethality and 70 mice of similar weights were used for morphological studies.

EMS. EMS was prepared from mycelium of *E. striata* according to the method of Seya *et al.* [10].

Determination of lethality. The lethality of the toxin was assayed in mice by i.p. injection. The LD_{50} value was calculated according to the method of Lichfield and Wilcoxon at 24 and 48 hr after EMS injection.

Sequential studies of EMS-intoxication. EMS was dissolved in 50% aqueous N,N-dimethylformamide (DMFA) (Kanto Chemical Co, Inc, Tokyo, Japan) solution. For sequential studies, a total of 47 mice were divided into 5 groups. Group 1: Five mice given 0.1 ml of 50% DMFA solution intraperitoneally served as control. Group 2: Four mice given 50 mg/kg of EMS i.p. were sacrificed at 10 min. Group 3: Eight mice were given 30 mg/kg of EMS intraperitoneally. At 1 and 2 hr after the injections four mice were killed. Group 4: Fifteen mice were given 20 mg/kg of EMS i.p., and then at 2, 6, and 24 hr after the injections 5 mice were sacrificed. Group 5: Ten mice were given 10 mg/kg of EMS intraperitoneally. Five mice were killed at 24 and 48 hr after the treatment.

A separate group of 5 mice were given EMS at a dose of 60 mg/kg orally by intubation, and were sacrificed 24 hr later.

Adrenalectomized animals. Six male ICR mice (23–25 g in body weight) were bilaterally adrenalectomized under ether anaesthesia. The animals were supplied with drinking water containing 0.9% sodium chloride *ad libitum* for two weeks, after which they were used for the experiment.

Effects of various treatments on EMS-poisoning. A total of 35 male ICR mice were used for this experiment. The severity of injuries in the liver and thymus induced by EMS only was compared with that of animals given $CoCl_2$, phenobarbital (PB), diethyl maleate (DEM) or cysteine before administration of EMS. Each group consisted of 5 mice. Groups 1 and 2: Mice were given $CoCl_2$ (10 mg/l, Nakarai, Kyoto, Japan) in distilled water for 3 consecutive days *ad libitum*, or were given PB (75 mg/kg, Sigma Chem Co. St Louis) for 3 consecutive days intraperitoneally. Sixteen hours after the last administration of these two

agents, the animals were given with 18.0 mg/kg of EMS i.p. Group 3: DEM (0.6 ml/kg, Nakarai, Kyoto, Japan) was given i.p. 2 hr before the administration of EMS (18 mg/kg). Group 4: A solution of cysteine-HCL (Nakarai, Kyoto, Japan) in distilled water (200 mg/kg) was likewise given i.p. to another group 15 min prior to EMS (18 mg/kg). Groups 5–7: Three groups given EMS alone, or DEM alone, or cysteine only, and they served as control. All the animals in this experimental series were sacrificed 24 hr after being given EMS.

Light microscopic observations. All animals examined for morphological observations were killed at various time intervals after the administration of EMS. The animals were killed under light ether anesthesia and all visceral organs were removed and fixed in 10% neutral formalin for light microscopy. Paraffin sections of the various organs were prepared and stained with hematoxylin and eosin, and periodic acid Schiff (PAS).

Electron microscopic observations. For transmission electron microscopy, a 2% paraformaldehyde-glutaraldehyde solution was perfused from the left ventricle of the heart, and then pieces of the heart muscle, liver, thymus and spleen were fixed in this cold solution for 12 hr. Postfixation was done with 1% OsO_4 at room temperature for 2 hr. The specimens were dehydrated in a series of graded concentrations of ethanol and embedded in Epon 812, and then cut with a diamond knife on a Porter II ultratome. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H 700H transmission electron microscope. Semi-thin sections of the Epon-embedded specimens were prepared for light microscopy.

Quantitative analysis of the liver injuries. Areas of hepatic necrosis were measured by an Automatic Image Analysing System (Nikon Luzex 2, Nikon, Japan).

Results

LD_{50}. The LD_{50} value of EMS was 17.7 mg per kilogram of body weight at 24 hr after i.p. injection and 13.0 mg/kg at 48 hr. Most of the dead animals showed a marked retention of ascites and effusion of fluid into the thorax and pericardial cavity. Severe congestion of whole organs was also noted.

Sequential pathomorphological changes. Mice given EMS either intraperitoneally or orally showed essentially the same morphological changes in the target organs, although the injuries induced when the former method of administration was used were 2 to 3 times more severe. For this reason, the present study describes the results in mice following i.p. injections.

Animals given 30 mg/kg or more died within 2 hr after the beginning of the experiment. Shortly after the injection all animals showed dyspnea and cyanosis. The most severely affected organ of these animals was the heart. At the ultrastructural level, condensed necrotic cells accompaning swollen mitochondria with irregular arrangement of the crista were occasionally seen to be located between less affected ones in the myocardium. In contrast, fine fibrils such as myosin and actin bundles, as well as the other microorganelles did not show any abnormalities. Marked edema was usually observed between bundles of myofibrils in the affected neighbouring cells (Fig. 2). On the other hand, mice given 20 mg/kg or less of EMS did not show such characteristic clinical signs and morphological findings in the cardiac tissue.

One of the characteristic features of EMS-intoxication at the dose of 20 mg/kg was severe centrilobular necrosis of the liver (Fig. 3a). Desquamation of the endothelium of the central veins from the hepatic cord was often seen, and in such cases there were numerous red corpuscles in the subendothelial tissue (Figs. 3b). Marked mitochondrial swelling was usually seen in electronmicrographs of the hepatocytes in the centrilobular regions (Fig. 4a). Unusual proliferation of granular endoplasmic reticulum (RER) in the hepatocytes around the necrotic regions was

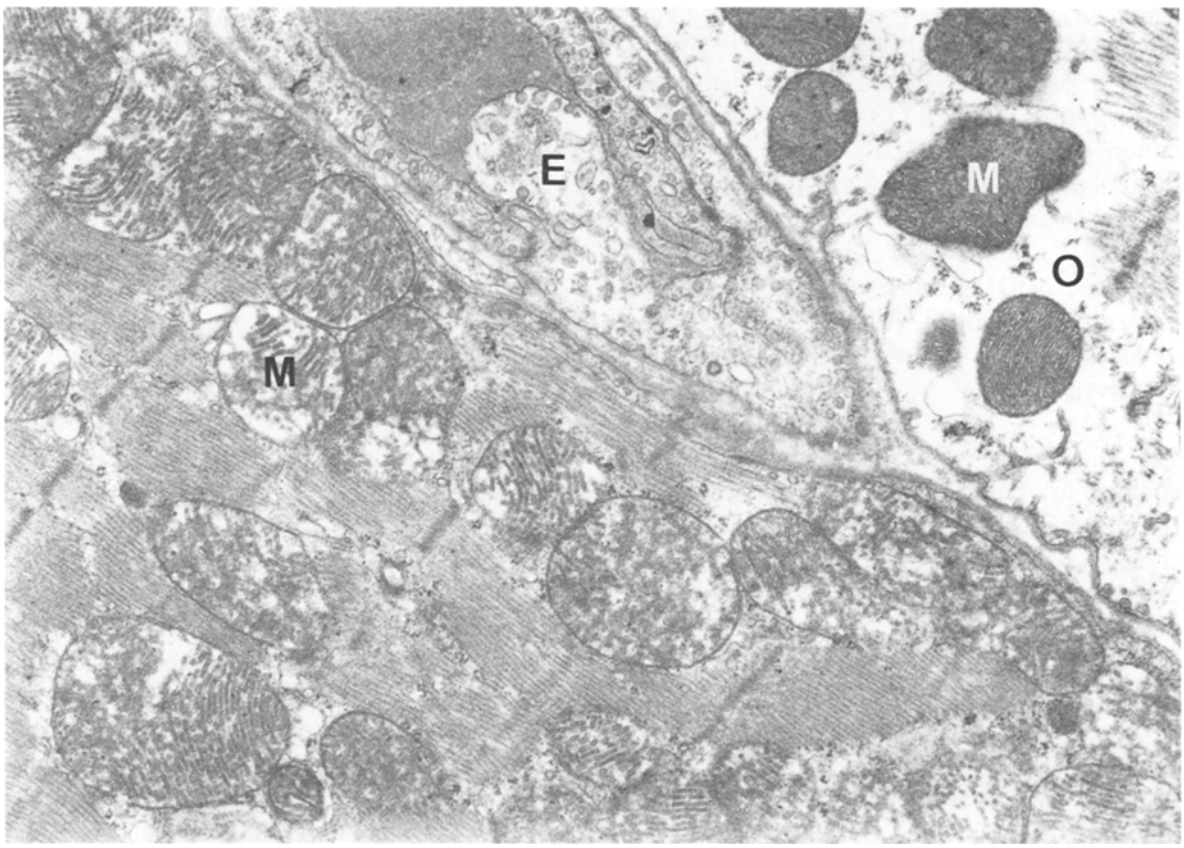

Fig. 2. An electron micrograph of cardiac muscle form a mouse given 30 mg/kg of EMS 1 hr prior to sacrifice. All mitochondria (M) in a muscle on the left side are degenerated. There is marked edema (o) in the cytoplasm of a neighbouring muscle cell. E: endothelium of the capillary. × 20000.

always noted (Fig. 4a). Inclusion bodies with membrane-limited fine fibrils containing clusters of free ribosomes and RER were often seen in the cytoplasm of the hepatocytes (Fig. 4b). Membrane whorl-formation of RER up to 7 μm in diameter occasionally were seen in the hepatocytes in the central regions of the hepatic lobules (Fig. 4c). In the cytoplasm of almost all of the hepatocytes surrounding degenerated hepatocytes there were numerous ribosome-like granules between the proliferated RER (Figs. 4a & d). In our experimental conditions, there was no discernible proliferation of agranular endo-plasmic reticulum (SER) in the hepatocytes.

Degeneration of lymphoid tissues was another prominent result of injection of EMS in mice. Twenty-four hours after the injection of EMS, there was massive necrosis of lymphocytes in the cortical layer of the thymus (Fig. 5a). In contrast to the lymphocytes, epithelial reticular cells in the cortical layers survived. The large lymphocytes in the medulla were relatively resistant to EMS treatment. Numerous macrophages with residual bodies were seen in the cortical layer of the thymus. Fourty-eight hours after a single injection of 20 mg/kg of EMS, almost all lymphocytes had disappeared from the cortical layer of the thymus. At that time the weight of the spleen had become markedly reduced (about 50% of control). Histologically, a reduction in the number of lymphocytes in the periarterial lymphoid sheath as well as in the red pulp was noted (Figs. 5b & c). To clarify the effects of EMS on lymphoid tissue, we compared the effect of the toxin on the lymphoid

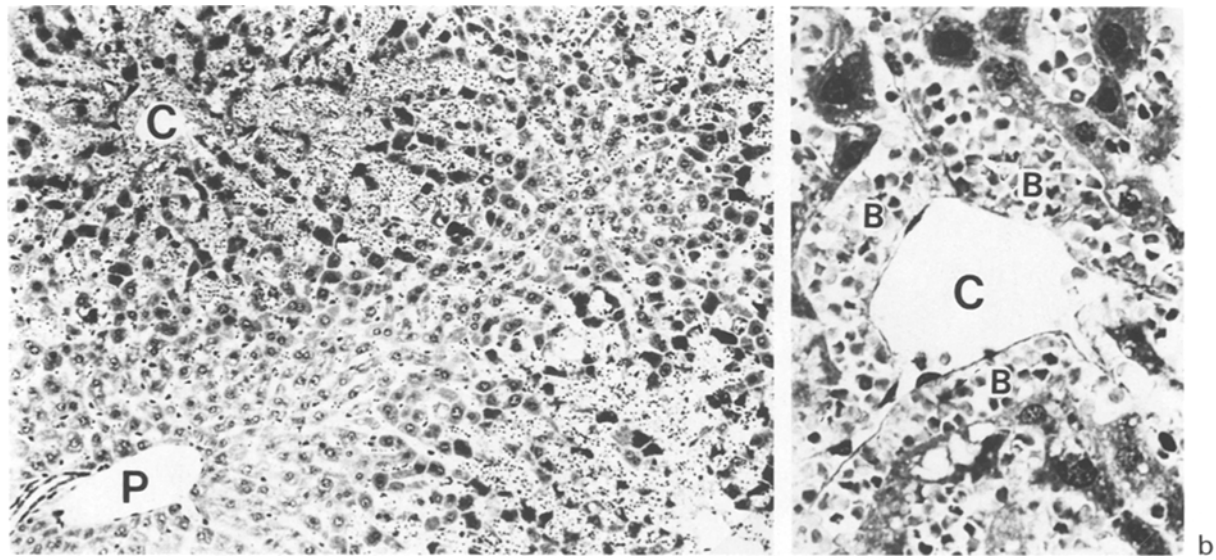

Fig. 3a. A photomicrograph of the liver of a mouse given 20 mg/kg of EMS intraperitoneally (24 hr after injection). Note, the severe central necrosis and severe congestion. C: Central vein, P: Portal vein. HE × 300.
Fig. 3b. A part of centrilobular region in Fig. 3a. Marked hemorrhage (small B) can be seen beneath the endothelium of central vein (C). HE × 1200.

tissue of the EMS-treated control mice with those of adrenalectomized animals. In spite of EMS injection, no discernible pathomorphological changes were seen in the thymus and spleen of the adrenalectomized mice (Table 1).

Effects of various pretreatments on EMS-induced lesions. The effects of various pretreatments on EMS-induced lesions in the liver are shown in Table 2. Pretreatment of mice with DEM increased the incidence and severity of hepatic necrosis. In contrast, pretreatment with cysteine or $CoCl_2$ reduced the severity of central necrosis of the liver. A dose of 18 mg/kg EMS caused only degenerated hepatocytes that were sporadic in the central region of the liver in mice pretreated with cysteine or $CoCl_2$, while mice injected with a similar dose of EMS alone demonstrated severe central hepatic necrosis. The effect of PB on the hepatic lesions induced by EMS was quantitatively not significant. Proliferation of SER was demonstrable in mice pretreated with PB, but no such phenomenon was seen in the other treated with EMS.

In contrast to the hepatic necrosis, thymic

Table 1. EMS-Induced injuries of the thymus of mice

Treated with EMS*	Incidence of mice with thymic lesions
Adrenalectomized mice	0/6
Non-operated mice	6/6

*18 mg/kg of EMS was injected i.p. 24 hr before sacrifice.

Table 2. The effects of various pretreatments on hepatic necrosis induced by emestrin

Treatment	Area of hepatic lesion (mm^2)[a]
EMS* only	60.9 ± 7.9
PB** + EMS	66.0 ± 30.0
$CoCl_2$ + EMS	3.6 ± 1.0
DEM*** + EMS	87.6 ± 7.0
Cysyeine**** + EMS	2.7 ± 1.0

*EMS: 18 mg/kg of emestrin, **PB: 75 mg/kg of phenobarbital for 3 days, ***DEM: 0.6 ml/kg of diethyl maleate, ****200 mg/kg of cysteine. [a] Effect of treatment is significant ($p < 0.05$) with Student's t test.

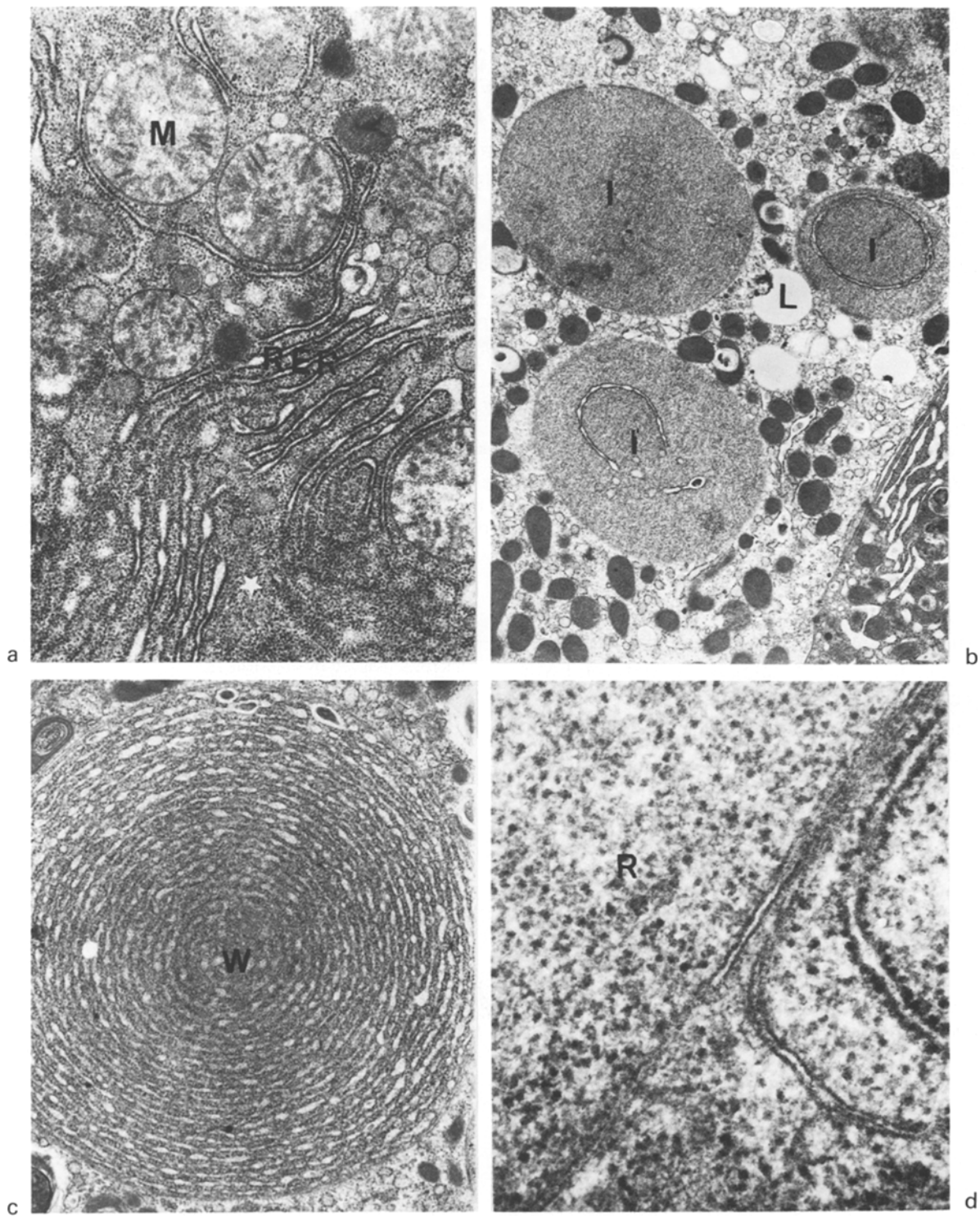

Fig. 4. a–d. Electron micrographs of hepatocytes of a mouse injected i p with 20 mg/kg of EMS and then sacrificed 24 hr later. *4a:* Swelling of mitochondria (M) is prominent. Proliferation of rough surfaced endoplasmic reticulum (RER) and free ribosomes (white star) is noted. × 20000. *4b:* Three membrane-limited inclusions (I) are seen in a hepatocyte. Two of them contain RER. L: Lipid droplet. × 8500. *4c:* Membrane whorl (W) in a hepatocyte. × 13000. *4d:* Free ribosomes (R) in a hepatocyte. × 75000.

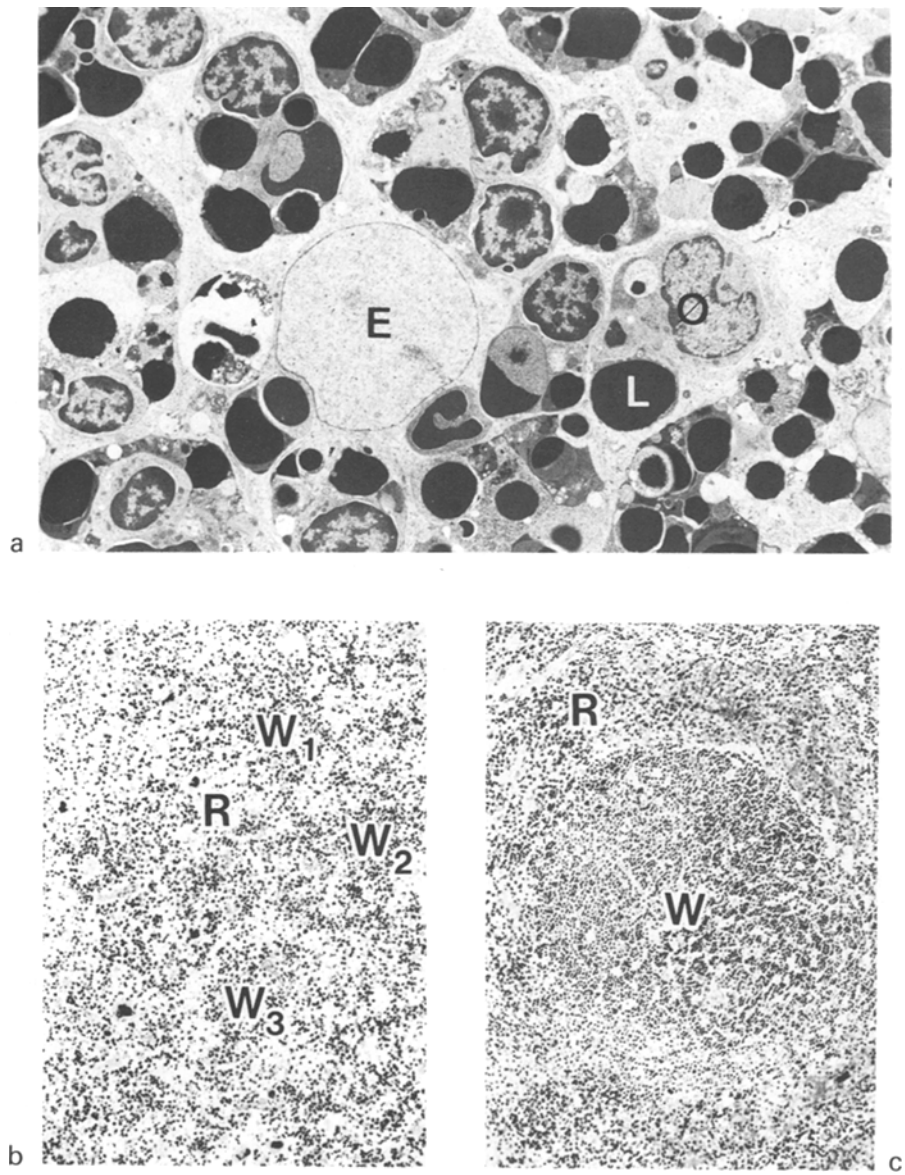

Fig. 5a. An electron micrograph of the cortical layer of the thymus from a mouse 24 hr after the injection of 20 mg/kg of EMS. Almost all lymphocytes are necrotic, while epithelial reticular cells (E) are intact. L: debris of lymphocyte, Φ: macrophage with phagocytic remnant. × 3000.
Fig. 5b. A photomicrograph of the spleen 48 hr after the administration of 20 mg/kg of EMS. Marked atrophy of white (W_1–W_3) as well as red pulp (R) is noted. HE × 300.
Fig. 5c. A photomicrograph of the spleen from a control mouse W: white pulp. R: red pulp. HE × 300.

lesions were constantly present in all experimental animals to a similar degree, and they were quite independent of the various pretreatments.

Discussion

Apparently the target organs of EMS in male ICR mice were the heart, liver and lymphoid tissues. Moreover, the direct target organelles of the mycotoxin were the mitochondria in hepatocytes

and cardiac muscle cells. Using isolated rat liver mitochondria, Ishizaki *et al.* [3] demonstrated that EMS strongly inhibited ATP synthesis and caused an uncoupling of oxidative phospholiration and a depression of respiration just as usual uncoupling agents do. Furthermore, they showed that EMS elicited a conspicuous swelling of mitochondria in an *in vitro* system. These results regarding *in vitro* toxicity reflect the mechanism of the *in vivo* EMS-intoxication of the present studies. Several investigators [1, 2, 7, 8, 9, 13] have reported the acute toxicity of mycotoxins belonging to the epipolythiodioxopiperadine (ETP) class in rodents. Chaetocin, a secondary metabolite of *Chaetomium* spp, contains two disulfide structures in its molecule. The mycotoxin produced peritoneal adhesions and focal necrosis in the liver after i.p. injection of 0.6 to 10 mg/kg in male ICR mice [9]. Gliotoxin is one of the secondary metabolites of the mold *Gliocladium* sp. and several other fungi and contains ETP in its structure. Frame & Carlton [2] examined the acute toxicity of gliotoxin in hamsters, and found that the principal histopathological alterations with oral doses of over 20 mg/kg at 12 hr were hepatic necrotizing and proliferative cholangitis with marked lymphoplasmacytic pericholangitis. Mullbacher *et al.* [7] demonstrated that gliotoxin possesses potent immunomodulating properties. Waring *et al.* [13] found that the DNA of cells treated with gliotoxin showed extensive double-stranded cleavage and they suggested that this is characteristic of apoptosis, a programmed form of cell death. Sporidesmin, a mycotoxin in the fungus *Pithomyces chartarum*, also contains an ETP in its molecule. This mycotoxin produced hepatic lesions similar to those produced by gliotoxin [1]. In contrast, EMS intoxication of the mouse in the present study showed neither cholangitis nor pericholangitis.

Pretreatment with $CoCl_2$, an inhibitor of NADPH-cytochrome P-450, markedly reduced the hepatic necrosis by EMS. However, there were quantitatively no significant differences between the hepatic injuries of mice treated with EMS alone and lesions induced by EMS in mice pretreated with PB, a well known inducer of cytochrome P-450. Therefore, it is not clear whether the hepatotoxicity of EMS is activated by the cytochrome P-450 system.

Interestingly, the presence of cysteine, an SH-containing amino acid, prevented EMS induced hepatic injuries, and the pretreatment with DEM, a potent inhibitor of SH containing amino acid, aggravated the necrosis of the centrilobular lesion of the liver induced by EMS. It has been well documented that a number of activated chemical substances in a body bind covalently with nucleophilic substances such as SH-containing amino acids [4, 6].

Although the mode of action of EMS is not known, the mycotoxin must have some effect on RNA metabolism of hepatocytes. Morphological evidence such as development of abundant free ribosomes, membrane whorls with ribosome-like granules, and marked proliferation of RER in the cytoplasm of the hepatocytes may be closely related with a modified RNA metabolism.

Interestingly, the thymic injuries were independent of the pretreatment with a variety of drug metabolism mediated agents. Furthermore, bilaterally adrenalectomized mice showed no discernible pathomorphological changes after the administration of EMS. Nagarajan [8] has confirmed that the bridged disulfite structure of the molecule of gliotoxin may play an important role in the biological activities of the mycotoxin. The bridged disulfite structure in the EMS molecule may also play an important role in the mode of action of hepatic lesions induced by EMS. However, the mechanism involved in thymic injuries is different from that of hepatic necrosis, although the precise process of the intoxication at the molecular level remains obscure.

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