Endotoxin Susceptibility Following Hepatic Injury by Carbon Tetrachloride. (28980)

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Guinea pigs become abnormally susceptible to the lethal effects of bacterial endotoxin following a sublethal dose of carbon tetrachloride (CCl_4)(1). The most conspicuous morphologic feature of CCl_4 poisoning is damage to the liver, and the striking degree of this damage suggests that it may bear some relationship to the loss of normal resistance to endotoxin shown by these animals. This study was undertaken to define further this relationship.

Although it is known that injected endotoxin is rapidly cleared from the plasma, partly by association with platelets(2) and principally by sequestration in reticuloendothelial cells, notably of the liver and spleen (3,4), its ultimate fate is poorly understood. Evidently some host mechanism exists which renders it pharmacologically inert. The hepatic cell, because of its role in the detoxification of various foreign substances, would seem to be a likely site for this important function. Trapani *et al*(5) have recently shown that cell-free homogenates of rabbit liver can detoxify endotoxin, and Smith *et al* (6) have described a similar system in the dog, although in this animal splenic tissue was more active than liver. In the present study it was found that homogenates of normal guinea pig liver are capable of inactivating substantial amounts of endotoxin *in vitro*, whereas liver from CCl_4-treated animals is virtually inactive in this respect.

Materials and methods. Female Hartley strain guinea pigs weighing 300 to 400 g were used. CCl_4 treatment consisted of subcutaneous injection of 0.15 ml, a dose which caused significant liver damage but no mortality. Susceptibility to endotoxin was determined as the proportion of animals dead 24 hours after intravenous challenge. LD_{50} was calculated by the method of Reed and Muench(7).

Endotoxins of *Serratia marcescens* and *Shigella flexneri* were obtained from Difco Laboratories.

Complete gross and microscopic autopsies were done on animals sacrificed at various intervals from 6 to 168 hours after CCl_4 administration, using formalin fixation and hematoxylin-eosin staining.

Liver homogenates were prepared by a method similar to that used by Trapani *et al* (5) for rabbit liver. Animals were killed by a blow on the head and the livers quickly removed using aseptic precautions. The excised liver was cleared of blood by forcing 200-300 ml of cold saline into the hepatic vein, with drainage through the portal vein. It was then sliced, drained and weighed, and homogenized with 2.5 volumes of 0.01 M phosphate-buffered saline (pH 7.4) for 2 minutes (4 periods of 30 seconds each at 3-minute intervals) in a Waring blendor at 0-4°C. The resulting suspension was centrifuged at 2°C for 15 minutes at 1000 $\times$ g to remove nuclei and cell debris, and then for 20 minutes at 6000 $\times$ g to remove mitochondria. The term "homogenate" as used here refers to the supernatant obtained by this procedure. Only homogenates which were sterile after 48 hours' incubation in thioglycollate broth and on meat extract agar at 25° and 37° were used. Protein concentration of the 10% homogenate was 7.8-13 mg/ml for normal liver, 14.5-20 mg/ml for liver removed 48 hours after CCl_4 administration, and 13-13.5 mg/ml for liver removed 120 hours after CCl_4 treatment.

The endotoxin assay used in the experiments on endotoxin inactivation was based on the finding by Smith and Thomas(8) that the developing chick embryo is highly susceptible to the lethal effects of endotoxin. Endotoxin (20 μg) was incubated with various amounts of liver homogenate for 1 hour at 37°. The reaction was carried out at pH 6.5-7.0 (established by preliminary experi-

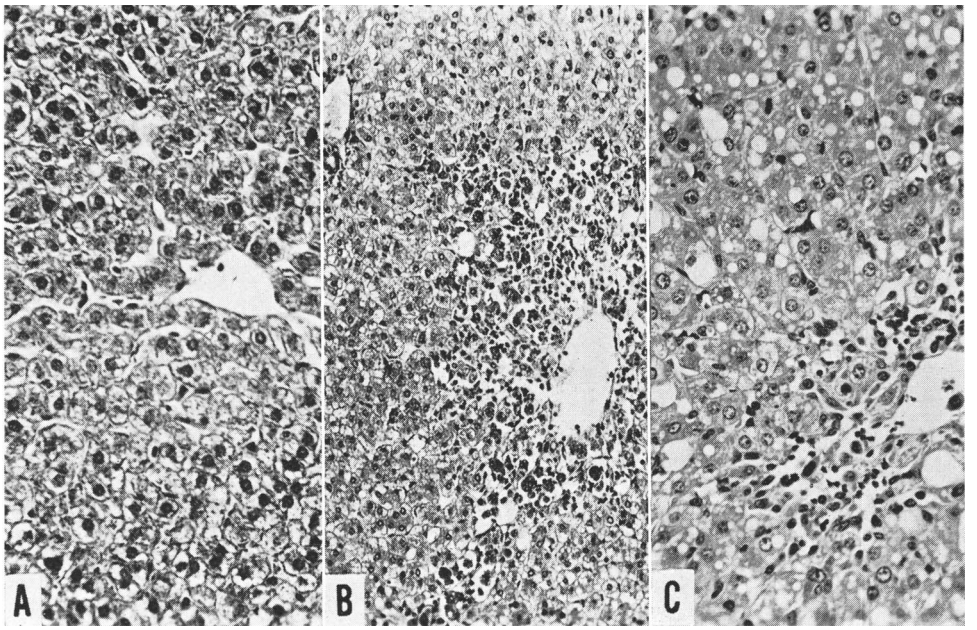

FIG. 1. A. Normal liver. B. Liver 48 hr after CCl_4, showing marked centrilobular necrosis and fatty metamorphosis. C. Liver 96 hr after CCl_4, showing slight residual centrilobular necrosis, midzonal mitotic activity and residual fatty metamorphosis.

ments as the optimum range). Total volume of reaction mixture was 2 ml. After incubation the reaction mixture was diluted with cold 0.01 M phosphate-buffered saline (pH 7.4) so that 0.1 ml contained approximately 2 chick embryo LD_{50}, assuming that no inactivation had taken place. This volume was then injected intravenously into 11-day old embryos using the technique described by Hook *et al*(9). Control groups of eggs received endotoxin which had been incubated in buffered saline and homogenates incubated without endotoxin, respectively. Eggs were incubated at 39.5°C for 20-24 hours and survival rates determined.

Carbon clearances were determined as described by Biozzi *et al*(10). Commercial India ink ("Pelikan," Gunther Wagner, Hanover) was centrifuged at 5000 rpm for 15 minutes to remove aggregated particles and the supernatant diluted with 2% gelatin solution in saline. Animals received 8 mg carbon per 100 g body weight and groups were bled at intervals up to 1 hour. Blood samples were lysed in 0.1% Na_2CO_3 and optical density was read on a Coleman spectrophotometer at 675 mμ.

Results. The morphologic effects of a single, sublethal dose of CCl_4 in guinea pigs have been reported by Phelps and Hu(11). Our findings were in essential agreement with these. The most pronounced changes were seen in the liver (Fig. 1) and adrenal. Parenchymatous degeneration of the cytoplasm of hepatic cells was seen as early as 12 hours after administration of 0.15 ml of CCl_4. Frank necrosis, manifested by nuclear pyknosis and lysis as well as cytoplasmic hyalinization, was evident by 24 hours, becoming maximal at 48 hours (Fig. 1B). These changes were strictly confined to the centrilobular zone. At 72 hours mitotic activity, indicative of hepatic cell regeneration, became apparent in midzonal areas and reached maximal intensity by 96 hours (Fig. 1C). Fatty metamorphosis appeared in midzonal and portal areas, becoming maximal at 72 hours and persisting as long as 120 hours. By 120 hours fatty metamorphosis was the only abnormality seen, and at 168 hours the livers were indistinguishable from those of normal controls.

In the adrenal, marked necrosis of the zona reticularis was present at 24 hours. This

TABLE I. Lethality of Endotoxin Following CCl_4 Administration.

Endotoxin (μg)	Normal animals	Interval between CCl_4 administration* and intravenous endotoxin challenge (hr) 0	6	12	24	48	72	96	168
2500	17/17†								
500	19/27								
100	0/22	7/23	8/23	13/15	15/15	15/15	15/15	3/15	2/13
20				0/8	10/16	20/21	8/16		
4				0/8	7/16	15/22	0/16		
.8				0/8	2/8	4/16	1/8		
LD_{50} (μg)	334			51	6.7	2.2	18.2	>100	

* .15 ml subcutaneously. † Deaths/total.

was followed by a burst of mitotic activity in the zona fasciculata which reached a peak at 96 hours. By 168 hours the glands were normal in appearance.

Hydropic degeneration of the epithelium of the proximal and distal tubules of the kidney appeared transiently, but this change never proceeded to necrosis and the epithelium usually regained normal appearance within 48 hours. No consistent changes were seen in any other organs examined.

CCl_4 treatment markedly enhanced the susceptibility of guinea pigs to *S. flexneri* endotoxin (Table I). Some enhancement was evident when the CCl_4 and endotoxin were given simultaneously, but this effect was much more pronounced when CCl_4 administration preceded endotoxin challenge, being maximal when the 2 injections were separated by 48 hours. At this time the LD_{50} was 2.2 μg, approximately 1/150 of the LD_{50} for normal animals. By 72 hours the animals were somewhat more resistant, and by 96 hours they were quite resistant, the LD_{50} being in excess of 100 μg.

In Table II the LD_{50} at various times after CCl_4 is compared with the time course of the histologic changes produced by CCl_4. It is evident that animals were most susceptible when hepatic necrosis was maximal. The appearance of intense regenerative activity coincided with the return of relatively normal resistance to endotoxin.

Other experiments demonstrated that CCl_4-treated animals were at least 100 times more susceptible than normals to the lethal effect of *S. marcescens* endotoxin.

Experiments were performed to compare the ability of normal and CCl_4-damaged livers to inactivate endotoxin *in vitro*. *S. marcescens* endotoxin was incubated with cell-free homogenates prepared from normal livers and from livers removed at various intervals after CCl_4 treatment. Residual toxicity of the reaction mixtures was assayed by injection into chick embryos.

Liver homogenates prepared from normal animals possessed considerable endotoxin-detoxifying activity (Fig. 2). The activity was proportional to the amount of homogenate in the reaction mixture. In contrast, homogenates made from animals treated with CCl_4 48 hours before sacrifice had almost no activity. Livers removed 120 hours after CCl_4 treatment showed activity approaching that of normal liver. The amount of liver required to protect 50% of the chick embryos used in the assay (ED_{50}) was 9.1 mg for normal animals, 2300 mg (by extrapolation) for animals given CCl_4 48 hours before sac-

TABLE II. Comparison Between Endotoxin Susceptibility and Morphologic Changes in the Liver at Various Intervals After CCl_4 Administration.

Morphologic change	Time (hr) 24	48	72	96	120
Necrosis	++	++++	++	+	0
Mitosis	0	+	+++	++++	0
Fatty metamorphosis	++	++	++++	+++	++
Endotoxin LD_{50} (μg)	6.7	2.2	18.2	>100	

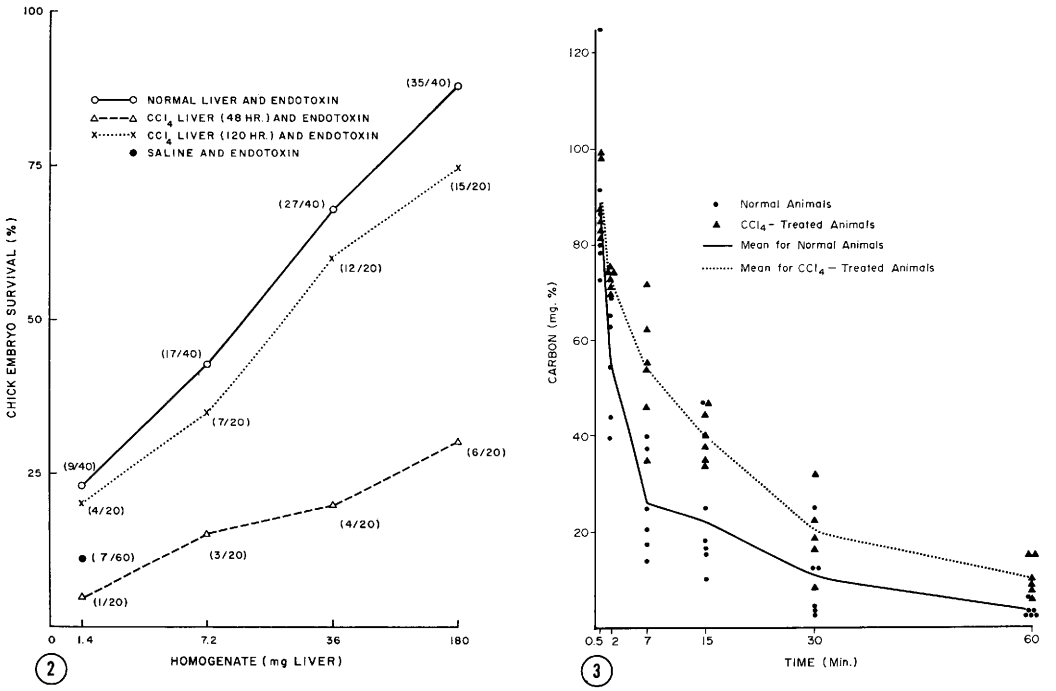

FIG. 2. Effect of incubation with liver homogenates on toxicity of *S. marcescens* endotoxin for 11-day chick embryo. Pooled data from 4 experiments. The values in parentheses represent surviving embryos/total tested. Each embryo received 0.0125 μg endotoxin intravenously (2 LD_{50}), assuming no inactivation had taken place.

FIG. 3. Carbon clearance by normal and CCl_4-treated animals. Pooled data from 3 experiments. Each animal received 8 mg carbon intravenously per 100 g body wt.

rifice, and 21.2 mg for animals given CCl_4 120 hours before sacrifice. Comparison of the slopes of the lines by the method of least squares revealed a highly significant difference between the normal animals and each of the CCl_4-treated groups ($p<0.001$ for each comparison).

Because blockade of the reticuloendothelial system is known to increase susceptibility to endotoxin, animals were tested for their ability to remove injected carbon particles from the bloodstream 48 hours after CCl_4 administration. Removal was rapid in both normal and CCl_4-treated animals, somewhat more so in the former (Fig. 3). Analysis of variance applied to the data revealed that the lines described by the means of the two groups were essentially quadratic in form, and the slopes were similar, but the difference between the two groups was highly significant ($p<0.001$).

The distribution of carbon in the organs of CCl_4-treated animals differed somewhat from that seen in normal animals. In normal animals large amounts of carbon were seen in phagocytic cells of the liver and spleen, but in no other organs. CCl_4-treated animals showed less carbon in the liver and more in the spleen, and appreciable amounts were seen in the sinusoidal lining cells of the adrenal.

Because of the lesions seen in the zona reticularis of the adrenal cortex after CCl_4 treatment, and the known susceptibility of adrenalectomized animals to endotoxin, experiments were carried out to determine if adrenal damage by CCl_4 could be responsible for the increased susceptibility to endotoxin. Testosterone propionate in oil, 5 mg subcutaneously each day for 3 days, failed to protect CCl_4-treated guinea pigs against challenge 48 hours later with small doses of endotoxin (0.8-20 μg). Cortisone acetate, 3 mg given subcutaneously in divided doses 2 hours before and 4 hours and 10 hours after endotoxin challenge, actually enhanced the lethal-

ity of endotoxin for CCl_4-treated guinea pigs. Plasma 17-hydroxycorticoid levels (kindly determined by Mr. Joseph Bruton, Dept. of Metabolism) of CCl_4-treated animals before and after a lethal dose of endotoxin did not differ from those of normal controls.

Discussion. Guinea pigs poisoned with CCl_4 are extremely susceptible to the lethal effects of bacterial endotoxin, and this susceptibility is greatest during the period of maximal hepatic necrosis. After regeneration of the liver has taken place the animals are again resistant to endotoxin.

A striking parallel is noted between the response of the intact animal and the ability of cell-free liver homogenates to detoxify endotoxin. Homogenates of normal liver possess considerable endotoxin-detoxifying activity, whereas homogenates of livers removed 48 hours after CCl_4 administration show very little activity. If the liver is removed 120 hours after CCl_4 treatment, when hepatic regeneration is virtually complete, the endotoxin-detoxifying activity of the homogenate approaches that of normal liver.

These temporal relationships suggest that CCl_4 may induce endotoxin susceptibility by damaging the liver, possibly by rendering it incapable of detoxifying endotoxin. Inactivation of endotoxin by liver appears to be an enzymatic process(5), and CCl_4 is known to disrupt a number of enzymatic functions of the liver(12), possibly by damaging hepatic ribosomes and interfering with protein synthesis(13,14). Healing of the hepatic lesion is associated with renewed endotoxin-detoxifying activity *in vitro,* and with a return of normal resistance by the animal to endotoxin.

Since CCl_4 also damages other organs, it is impossible to be certain that the endotoxin susceptibility is due to hepatic injury. However, the only other organs in which lesions were consistently seen in this study were the adrenal and kidney. Adrenal insufficiency does not appear to be involved because neither cortisone nor testosterone offered any protection against endotoxin, and plasma 17-hydroxycorticoid levels were normal.

CCl_4-treated animals show a slight but significant impairment in the removal of carbon particles from the circulation. This may reflect a specific inability of the liver to remove particulate matter in a normal manner, or simply a shunting of blood away from the liver, since at autopsy these animals were found to have less carbon in the liver and more in the spleen and adrenal when compared with normal controls. In view of the important role of the liver in removing endotoxin from the blood, shunting away from this organ might be highly significant, especially if hepatic detoxification is a major physiologic mechanism in the handling of endotoxin by the animal body.

Summary. Guinea pigs are abnormally susceptible to endotoxin during the stage of hepatic necrosis following a sublethal dose of CCl_4. CCl_4-damaged liver possesses little endotoxin-detoxifying activity *in vitro* as compared with normal liver. After hepatic regeneration has taken place liver tissue is again able to detoxify endotoxin, and the animals are again resistant to endotoxin. CCl_4-treated animals show slight impairment in clearance of injected carbon particles from the blood, probably because of diminished removal by the liver, but no evidence of adrenal insufficiency.

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Studies on Peripheral Blood Catecholamine Levels During Hemorrhagic Shock in Dogs.* (28981)

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Watts(1) showed that bleeding dogs to a mean arterial pressure of 38 mm Hg increased the peripheral arterial blood epinephrine (as the base) from 0.9 to 20.5 μg/l. Several investigators(2-5) have substantiated this observation and have also shown that there is a significant increase in arterial norepinephrine in dogs during shock. These results from different laboratories obtained by several methods for catecholamine determination show there is a 5-10-fold increase in norepinephrine and a 50-100-fold increase in epinephrine during hemorrhagic shock in dogs. In the present experiments peripheral blood catecholamines in dogs have been determined under a variety of conditions, to establish more precisely the origin and physiological role of these vasopressor agents during hemorrhagic shock. These studies include:

(1) Comparison of values obtained by simultaneous determinations of arterial epinephrine levels by bioassay and fluorimetry. (2) Arterial epinephrine—norepinephrine levels at decreasing increments of arterial pressure. (3) Effect of pentobarbital, chloralose and local anesthesia on arterial blood epinephrine levels during shock. (4) Simultaneous estimation of epinephrine in blood from the inferior vena cava above the adrenals, the femoral artery and the femoral vein, to obtain information on the secretion and disappearance of epinephrine during hemorrhagic shock.

Methods. Dogs of both sexes weighing from 8.2 to 15.9 kg were anesthetized with pentobarbital (30 mg/kg), chloralose (80 mg/kg), by infiltration of 1% procaine in the area to be cannulated. The dogs were bled from the femoral artery into a pressure compensator to a mean blood pressure of 40 mm Hg either by 30 mm Hg increments or by 50 ml increments at 10-minute intervals. Carotid blood pressure was monitored by means of a Sanborn 150 M recorder with a Statham transducer. Control arterial blood samples for bioassay were collected at the beginning of each experiment and after each bleeding. The method of Gaddum and Lembeck(6) as modified by Franko *et al*(7) was used for bioassay of epinephrine in 0.5 ml plasma or whole blood samples. To compare the bioassay method with the fluorimetric method four to eight 50 ml blood samples corresponding to the samples for bioassay were collected in chilled polyethylene centrifuge tubes containing 50 mg ethylenediaminetetraacetic acid disodium salt (Eastman) and immediately centrifuged at 4°C and 2500 rpm for 25 minutes. The plasma was decanted, the volume measured and stored at 4°C until assayed. The fluorimetric method of Vendsalu(8) was used until it was found that catecholamine adsorption on aluminum oxide according to the procedure of Goldfien *et al*(9) followed by the trihydroxyindole procedure of von Euler and Lishajko (10) was just as adequate and less time consuming. In several experiments the epinephrine levels in blood from the superior vena cava, femoral artery and femoral vein were compared. The samples were drawn

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