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Influence of Carbon Tetrachloride on Circulating Endotoxin after Exogenous Administration of Endotoxin in Rats (41003)

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Abstract. To ascertain the influence of carbon tetrachloride on the ability of the liver to remove circulating endotoxin, groups of rats were injected ip with endotoxin obtained from *Escherichia coli* 026 and also received carbon tetrachloride orally. The serum concentrations of endotoxin at different time intervals were measured by an immunoradiometric assay specific for the endotoxin used. It was shown that carbon tetrachloride delayed the removal of endotoxin by the liver. The effect was dependent on the dose of carbon tetrachloride given and was seen as early as 1 hr after the endotoxin injection.

Introduction. Evidence exists that enterically derived endotoxins are continuously presented to the liver for detoxification. Studies using the *Limulus* amebocyte lysate assay (LAL) demonstrated endotoxin in the portal circulation only, in over 50% of patients without liver disease while patients with hepatic injury had detectable lipopolysaccharide (LPS)[1] in the systemic circulation as well (1). Thus, it appears that normal function of the hepatic reticuloendothelial system (RES) is necessary for detoxification of endotoxin from the gut. Many factors such as alcohol and fatty infiltration depress the ability of the RES to remove colloids (2,3). It was also shown that animals given small amounts of carbon tetrachloride (CCl_4) became very sensitive to otherwise innocuous amounts of exogenous endotoxin (4), and liver homogenates from rabbits given CCl_4 lost their ordinarily potent endotoxin-detoxifying capacity (5).

It has been postulated that enteric endotoxin which cannot be detoxified by a damaged liver reaches the systemic circulation and produces various clinical manifestations as well as additional hepatic injury leading to further impairment of its clearance (6). Until recently, the endotoxin-detoxifying capacity of liver could not be accurately measured because of the lack of suitable quantitative assay. Recently, our laboratory developed an immunoradiometric assay (IRMA) for endotoxin from *Escherichia coli* 026 which allows quantitation in the circulation of an exogenously administered, marker endotoxin. The effect of liver injury on endotoxin removal from the circulation can thus be definitely measured, and we report here such a study.

Materials and methods. Animals. Female inbred albino rats (Holtzman Co., Madison, Wisc.) weighing 140–160 g were used in all the experiments. The animals were kept in individual cages for 7–14 days before the experiments and were given Purina Lab Chow (Ralston Purina Co., St. Louis, Mo.) and water *ad libitum* until 24 hr prior to CCl_4 administration.

Endotoxin. The LPS was prepared from *E. coli* 026: B6, (Boivin type, Difco Laboratories, Detroit, Mich., lot No. 649593) and was freshly reconstituted in pyrogen-free isotonic saline (Abbott Laboratories, North Chicago, Ill.) for ip administration. Carbon tetrachloride (J. T. Baker, Chemical Co., Phillipsburgh, N.J.) diluted in an equal volume of olive oil, was given by gavage.

Experimental design. In one experiment, three groups of 10 rats of similar weight received different amounts of CCl_4 (0.25, 0.05, and 0.0125 ml/100 g wt, respectively) and a constant dose (0.1 mg/100 g wt) of endotoxin. Control animals received the same amount of endotoxin and olive oil. In another experiment, two groups of 30 rats received two different doses of LPS (0.001

[1] In this paper the terms endotoxin and LPS are used interchangeably.

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and 0.025 mg/100 g wt, respectively) while the amount of CCl_4 was the same in each group (0.05 ml/100 g wt.)

In these experiments, the oral administration of CCl_4 or oil was followed immediately by endotoxin injection ip. Groups of 10 rats were bled out under sodium pentobarbital anesthesia, by aspiration from the aorta at 1, 6, 24, and 48 hr after the endotoxin injection.

Finally, in another experiment, two groups of 15 rats were given 0.05 ml/100 g wt of CCl_4 or oil and 2 hr later received 0.05 mg/100 g wt endotoxin ip. The rats were bled aseptically from the retroorbital sinus at 1, 6, and 24 hr after endotoxin administration.

The sera from all the experiments were separated under sterile conditions and stored at −20° until assayed.

Chemical assay. Alanine aminotransferase (SGPT) was determined as previously reported (8) and expressed in Sigma–Frankel units (SFU) (Sigma Technical Bulletin No. 410); 1 SFU/ml = 0.48 IU per liter. Endotoxin was quantitated by the IRMA as previously described (7). In brief, serum samples were incubated overnight in wells of microtiter plates precoated with the IgG fraction of rabbit antiserum to endotoxin from *E. coli* 026. The wells were then washed and incubated with the same antibody labeled with ^{125}I. Each individual well was counted in a gamma scintillation counter, and the endotoxin concentrations were calculated from a standard curve plotted from known amounts of endotoxin.

Histology. Liver specimens were fixed in 10% buffered Formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, and the amounts of fatty infiltration and necrosis were separately and blindly graded on a scale from zero to four. In grade 1, up to 25% of the hapatic lobule (low-power field) showed necrosis; in grade 2, between 25 and 50%; in grade 3, between 50 and 75%; and in grade 4, more than 75% of a hepatic lobule showed necrosis or fatty infiltration, respectively.

Statistics. The results were analyzed statistically by a two-tail Student's *t* test.

Results. When a constant dose of endotoxin (0.1 mg/100 g wt) was administered ip, the simultaneous oral administration of increasing amounts of CCl_4 raised the amounts of endotoxin detected in the circulation 1, 6, 24, and 48 hr later. The differences in the amounts of circulating endotoxin between animals which received oil alone and those which received CCl_4 were greater at 6 hr after endotoxin injection, even when a low dose of CCl_4 (0.0125 ml/100 g wt) was given. After a larger dose of CCl_4, a clear difference between the endotoxin concentrations was seen as early as 1 hr after endotoxin administration in rats which received CCl_4 at the same time, compared to animals which received oil alone (Fig. 1).

Figure 2 depicts the influence of CCl_4 on the amounts of circulating endotoxin when the CCl_4 dose was kept constant while decreasing amounts of endotoxin were given. Endotoxin doses of 0.025 mg and 0.001 mg/100 g wt raised the concentrations of circulating endotoxin at 1, 6, and 24 hr after the simultaneous administration of CCl_4, compared with control animals which received oil alone.

In another experiment, the time between

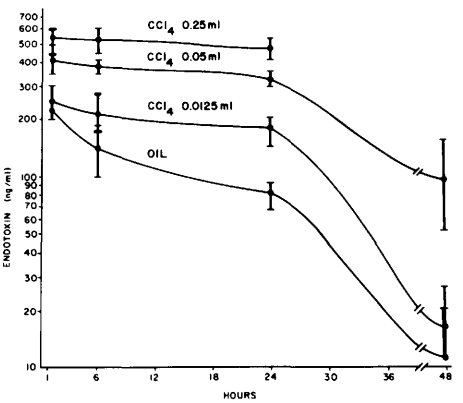

FIG. 1. Endotoxin concentrations in the circulation of rats after CCl_4 or oil administration. Each point represents the mean ± SEM of 10 rats. Endotoxin (0.1 mg/100 g wt) was given at the same time as CCl_4. At 48 hr no animals survived in the group given 0.25 ml CCl_4/100 g wt. Statistical significance between each group which received CCl_4 and the group which received oil: for CCl_4 0.25 ml/100 g wt, $P < 0.001$ at 1, 6, and 24 hr; for CCl_4 0.05 ml/100 g wt, $P < 0.001$ at 6 and 24 hr; for CCl_4 0.0125 ml/100 g wt, $P < 0.005$ at 24 hr.

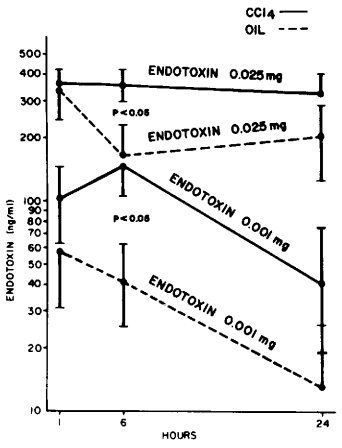

FIG. 2. Endotoxin concentrations in the circulation of rats after CCl_4 or oil administration. Each point represents the mean ± SEM of 10 rats. CCl_4 (0.05 ml/100 g wt) was given at the same time as endotoxin.

the CCl_4 gavage and the LPS injection was lengthened to 2 hr. Rats were bled at 1, 6, and 24 hr after injection of 0.1 mg/100 g wt of endotoxin. Significantly higher levels were found in the CCl_4 groups at all three time intervals (Fig. 3). Control groups of rats which received CCl_4 and no endotoxin had no detectable endotoxin in their blood at 3, 8, and 26 hr after CCl_4 administration.

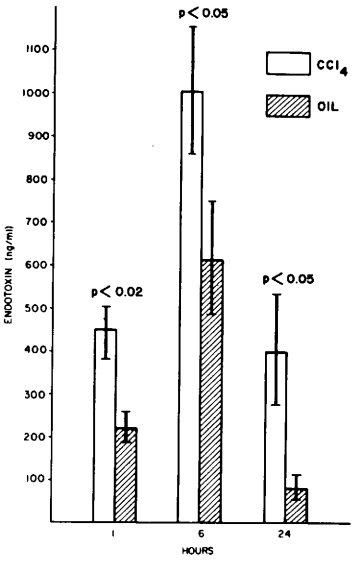

FIG. 3. Endotoxin concentrations in the circulation of rats after CCl_4 or oil administration. Columns represent the average ± SEM of 15 rats bled at 1, 6, and 24 hr after endotoxin injection (0.05 mg/100 g wt) ip. CCl_4 (0.05 ml/100 g wt) was given 2 hr before the endotoxin.

The degree of liver damage, as assessed by the concentrations of SGPT in the serum and by liver histology indices at 24 hr following endotoxin injection, was not much different in rats which received CCl_4 and endotoxin [1326 ± 184 (SEM)] SFU from rats which received only CCl_4 (1233 ± 258 SFU). The necrosis index was 2.5 ± 0.1 in the former group and 2.7 ± 0.1 in the latter group, while the index of fatty infiltration was 2.3 ± 0.2 in the rats which received CCl_4 and endotoxin, and 1.7 ± 0.2 in the animals given only CCl_4.

Discussion. Endotoxin or the LPS cell wall is constantly produced in the gut from death of enteric gram-negative organisms. This toxic material is regularly absorbed and low-grade portal vein endotoxemia is a normal state of humans (8). The possibility that liver injury from a variety of agents might impair the liver's ability to detoxify ordinarily innocuous amounts of LPS, leading to further liver injury and spill-over into the systemic circulation, has been a prime interest of this laboratory (6). However, the work has been hampered in the past by the lack of a suitable quantitative technique to sequentially measure circulating endotoxin. The LAL is, at best, semiquantitative, while a strain-specific IRMA developed in our laboratory now allows us to study the clearing of an exogenously administered LPS in states of liver injury.

Historically, the presence of LPS in the gut, rather than the intact bacteria, has been shown to be crucial in the development of choline-deficient cirrhosis in rats, as the addition of purified *E. coli* 026 LPS to the drinking water of these animals abolished the protective effect of orally administered neomycin (9). Since a marked synergism exists between CCl_4 and endotoxin injury, and because liver from CCl_4-treated rabbits almost completely lost the ability to detoxify endotoxin *in vitro* (4, 5), prior studies in our laboratory were undertaken to demonstrate that modification of endotoxin toxicity significantly reduced liver damage from CCl_4. By the administration of increasing doses of a single endo-

toxin, rats became tolerant or resistant to large doses of a variety of heterologous LPS. These endotoxin-resistant rats, in turn, were significantly protected against both the biochemical and histologic liver injury of CCl_4, when compared to nontolerant controls (10). Polymyxin B is a specific antiendotoxin that physically disrupts LPS and renders it nontoxic (11). Pretreatment with Polymyxin B was compared to gentamicin, an antibiotic without antiendotoxin properties but with a similar antibacterial spectrum in modifying the liver injury induced by CCl_4. In the group given Polymyxin B prior to CCl_4 challenge, virtually no liver injury resulted at a dose which caused significant biochemical and histologic damage in gentamicin pretreated and control rats (12).

In the experiments described here we studied, by use of IRMA, the clearance of graded doses of *E. coli* 026 endotoxin administered ip in groups of rats given various amounts of CCl_4. It should be mentioned that we have not seen a linear relationship between the amount of endotoxin administered and the concentration of circulating endotoxin, i.e., a 10- or 25-fold increase in the amount of endotoxin given was reflected in only a severalfold increase in the concentration of circulating endotoxin.

The reason for this nonlinear relationship is not clear but this is likely due to the detoxifying ability of the liver which is not fully saturated at the doses used in the experiments just described. Since endotoxin is cleared by the RES, and the Kupffer cells of the liver represent the major concentration of these fixed macrophages (13), early injury to the Kupffer cells might be expected to delay endotoxin removal, and possibly account for the known synergistic effect of CCl_4 and LPS. We demonstrated a significant prolongation of endotoxin clearance occurring soon after CCl_4 administration. The greater the dose of CCl_4 administered with the endotoxin, the more profound the inability to take up the LPS. At the doses of the two substances which were lethal for the animals, virtually no endotoxin was cleared in 24 hr (Fig. 1) but with even a small sublethal dose of CCl_4, impairment of clearance was still obvious. Furthermore, when the CCl_4 dose was kept constant, even very small, usually innocuous doses of LPS were not removed from the circulation.

It is noteworthy that the ability to clear LPS was significantly diminished as early as 1 hr after CCl_4 administration, at a time when evidence of biochemical or histologic damage to the hepatocytes was not observed. This finding supports the hypothesis that the earliest lesion in CCl_4 injury may be Kupffer cell damage, and that this early injury may lead to hepatocyte damage because of the inability to remove enterically absorbed endotoxin.

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