Chylomicrons Enhance Endotoxin Excretion in Bile

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Chylomicrons prevent endotoxin toxicity and increase endotoxin uptake by hepatocytes. As a consequence, less endotoxin is available to activate macrophages, thereby reducing tumor necrosis factor secretion. To determine whether the chylomicron-mediated increase in hepatocellular uptake of endotoxin results in increased endotoxin excretion into bile, we examined bile after endotoxin administration. A sublethal dose (7 μg/kg) of 125I-endotoxin was incubated with either rat mesenteric lymph containing nascent chylomicrons (500 mg of chylomicron triglyceride per kg of body weight) or an equal volume of normal saline (controls) for 3 h and then infused into male Sprague-Dawley rats. Bile samples were collected via a common bile duct catheter for 24 h. Infusion of endotoxin incubated with chylomicrons increased biliary excretion of endotoxin by 67% at 3 h (P<0.006) and by 20% at 24 h (P<0.01) compared with infusion of endotoxin incubated in saline. Endotoxin activity, as measured by the Limulus assay, was not detected in the bile of test animals. However, endotoxin activity was detected after hot phenol-water extraction of bile, demonstrating that endotoxin is inactive in the presence of bile but retains bioactivity after hepatic processing. Since the majority of an intravenous endotoxin load has been shown to be cleared by the liver, acceleration of hepatocyte clearance and biliary excretion of endotoxin may represent a component of the mechanism by which chylomicrons protect against endotoxin-induced lethality.

Endotoxemia stimulates changes in lipid metabolism, including an increase in plasma triglyceride (TG) (1, 13, 19, 26, 57). It has been proposed that the hypertriglyceridemia observed during gram-negative sepsis represents the mobilization of fat stores to fuel the body's metabolic response to the infectious challenge (3, 7, 30). However, there is data to suggest that TG-rich lipoproteins are components of the acute-phase response. One of the earliest and most consistent metabolic responses to endotoxin is an increase in plasma TG levels due to an increase in TG-rich very-low-density lipoprotein (VLDL) (13). This endotoxin-stimulated hypertriglyceridemia may have a protective function, since TG-rich lipoproteins (VLDL and chylomicrons) have been shown to bind endotoxin and inhibit its activity (11, 20, 21, 53). We have demonstrated that chylomicrons and VLDL, when incubated with lethal doses of endotoxin prior to intraperitoneal administration, inhibit detection of endotoxin by the Limulus assay in vitro (11, 20) and protect mice from death (21). Chylomicrons also prevent death in rats when given intravenously prior to a dose of endotoxin (22).

In addition to inhibiting endotoxin activity directly, TG-rich lipoproteins also alter endotoxin metabolism, which may contribute to their protective effect in these animal models. Specifically, the metabolic fate of the lipoprotein-endotoxin complex appears to be directed by the lipoprotein particle. When endotoxin is administered with chylomicrons, the clearance of endotoxin from plasma is enhanced, with shunting of endotoxin to hepatocytes and away from hepatic macrophages (Kupffer cells) (22). There is a concomitant decrease in plasma tumor necrosis factor levels, which correlates with improved animal survival (22).

Once taken up by hepatocytes, endotoxin appears to be secreted into bile (29, 31, 52). Maitra et al. found that the quantity of endotoxin excreted via the biliary system was small (<1% of the injected dose recovered at 3 h and <7% of the injected dose recovered at 48 h) (29). However, other investigators demonstrated that up to 60% of an intravenous load of radiolabeled endotoxin can be recovered in the stools of rats (15–17, 27, 28), suggesting that a significant portion of endotoxin processed in vivo is ultimately secreted into the gut, presumably via bile.

We hypothesized that the chylomicron-mediated uptake of endotoxin by hepatocytes would increase the biliary excretion of endotoxin. To test this hypothesis, we measured the amount of radiolabeled endotoxin present in the bile of rats injected with chylomicron-endotoxin complexes or with endotoxin in saline. We then examined the degree to which endotoxin is altered by hepatic processing and biliary excretion and determined its activity by the Limulus assay.

MATERIALS AND METHODS

Reagents and solutions. Chloroform, reagent-grade orthophosphoric acid, and glacial acetic acid were obtained from Fisher Chemical Co. (Fairlawn, N.J.); NaOH was from J. T. Baker Chemical Co. (Phillipsburg, N.J.); apyrogenic, preservative-free 0.9% NaCl was from Kendall McGraw Labs, Inc. (Irvine, Calif.); apyrogenic H2O was from Elkins-Sinn, Inc. (Cherry Hill, N.J.); 3% H2O2 was from Cumberland Co. (Smyrna, Tenn.); D-galactosamine hydrochloride was from Sigma Chemical Co. (St. Louis, Mo.). The phosphate-buffered saline used in all experiments was tested and found free of detectable endotoxin (<10 μg of endotoxin per ml).

Endotoxin. Escherichia coli O55:B5 endotoxin (Difco Laboratories, Detroit, Mich.) was reconstituted with sterile, apyrogenic H2O to a concentration of 1 μg/ml and stored in 3-ml aliquots at −70°C. This preparation of endotoxin had a

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specific activity of 15 endotoxin units per ng (USP reference endotoxin).

**Depyrogenation.** To avoid contamination with exogenously derived endotoxin, all heat-stable materials used in the isolation, processing, and assay of solutions to be injected into the rats, including test tubes, flasks, stoppers, beakers, and pipettes, were rendered sterile and free of detectable endotoxin (≤5 to 10 pg/ml) by a combination of steam autoclaving and dry heating at 180°C for a minimum of 4 h as previously described (21).

**Radioiodination of endotoxin.** Radiolabeled endotoxin was prepared by the method of Ulevitch (45). Briefly, *E. coli* (OSS: BS) endotoxin was first derivatized by reacting with p-OH methylbenzimidate at alkaline pH and then radiolabeled with Na²¹¹I.

**Mesenteric lymph collection.** Mesenteric lymph was obtained by cannulation of the mesenteric lymph duct of gavage-fed male Sprague-Dawley rats (200 to 300 g; Bantin and Kingman, Fremont, Calif.) as previously described (22). Special precautions were taken to avoid the introduction of exogenous endotoxin during the collection process, as previously described (21). The TG content of the mesenteric lymph was determined by a standard enzymatic assay (Sigma). The lymph preparations contained no demonstrable apoprotein B-100 (VLDL) by Coomassie staining of gels after polyacrylamide gel electrophoresis (data not shown).

The amount of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in mesenteric lymph was measured in a pooled lymph sample from 10 animals. Chylomicrons were removed after centrifugation at 36,000 × g for 1 h at density (d) = 1.006. The LDL and HDL fractions were isolated by sequential centrifugation at 36,000 × g for 18 h at d = 1.063 and d = 1.21, respectively. Samples were dialyzed exhaustively against normal saline to remove KBr. The total cholesterol content of the samples was then measured by a standard enzymatic assay (Sigma).

**Biliary excretion of endotoxin.** Male Sprague-Dawley rats (314 ± 21 g) were anesthetized with pentobarbital (50 mg/kg of body weight intraperitoneally). A midline abdominal incision was made, and the common bile duct was cannulated (Clay Adams PE-50 catheter; Becton Dickinson and Co., Parsippany, N.J.). An infusion catheter was placed in the ileofemoral vein (Micro-Renathane type MRE-040 catheter; Braintree Scientific, Inc., Braintree, Mass.). The animals were given intravenous infusions of a sublethal dose of ¹²⁵I-labeled endotoxin (7 μg/kg). The ¹²⁵I-endotoxin was incubated with either rat mesenteric lymph containing nascent chylomicrons (chylomicron-treated group; 500 mg of chylomicron TG per kg) or an equal volume of normal saline (control group) at 37°C for 3 h in a shaking water bath prior to infusion. In the first group of experiments, D-galactosamine (375 mg/kg) was given intravenously just prior to endotoxin infusion.

Bile flow was observed for at least 15 min prior to the endotoxin infusion to assure unobstructed output. After the endotoxin infusion, bile samples were collected via the common bile duct cannula in 30-min aliquots for 180 min and then for an additional 21 h to complete a 24-h collection. The aliquot volumes were measured, and each sample was assayed for ¹²⁵I content by gamma counting. The experiments were repeated without D-galactosamine in a second group of animals.

Bile flow volume was measured in all four experimental groups in addition to a group of animals which received saline alone.

**Chylomicron TG clearance.** As the dose of chylomicron TG used in the above experiments was supraphysiologic, studies were performed to assess the clearance of this dose of chylomicron TG from the circulation. Male Sprague-Dawley rats (250 to 280 g) were anesthetized with pentobarbital (50 mg/kg), and a catheter was placed in the ileofemoral vein. Animals received an intravenous infusion of nascent chylomicrons (500 mg of chylomicron TG per kg). Four-hundred-microliter blood samples were drawn for plasma TG determination prior to chylomicron infusion and at 5, 15, 60, 120, and 180, and 240 min after chylomicron infusion. After centrifugation (2,000 rpm for 10 min) and collection of plasma, the TG content was determined by standard enzymatic assays (Sigma and Wako Chemicals USA, Inc., Dallas, Tex.).

**Gel electrophoresis of endotoxin in bile.** One percent agarose gel electrophoresis was performed on bile samples from chylomicron-treated animals and saline-treated animals (controls). After electrophoresis, each lane of the gel was cut into 1-cm² sections, and these were measured for ¹²⁵I activity by gamma counting. Bile samples were taken from four animals in each group, and each sample was run in triplicate. Bile samples were tested from each 30-min time period from 0 to 180 min.

The electrophoretic patterns were compared with that of native radioiodinated endotoxin and with that of native endotoxin incubated in bile in vitro for 3 h at 37°C. Bile for the in vitro incubation was obtained from animals which did not receive endotoxin or D-galactosamine. The concentration of endotoxin added to bile in vitro was based on the concentration of endotoxin recovered in bile during the first 3 h of the in vivo experiments described above.

**Biologic activity of endotoxin in bile.** As described previously (20), endotoxin activity was assayed by a chromogenic modification of the *Limulus* lysate assay (23). Bile samples from the chylomicron-treated and control groups were tested both before and after hot phenol-water extraction. To assess the amount of endogenous endotoxin activity in bile, samples collected prior to endotoxin injection were tested before and after extraction.

**Bile extraction.** As described by Westphal et al. (56), collected bile and a 90% phenol solution were heated together (1:1, vol/vol) at 65°C for 30 min. The mixture was chilled with ice and ice water, separating the solution into two phases, an aqueous supernatant (lipopolysaccharide and nucleic acid fractions) and a phenol infranatant (protein fraction). The mixture was centrifuged at 2,000 rpm for 15 min; the supernatant was exhaustively dialyzed against sterile saline and tested for endotoxin activity by the *Limulus* assay.

**Statistical analysis.** Statistical significance between treatment groups was determined by using the Student's *t* test. All studies in animals were approved by the Animal Studies Subcommittee of the Veterans Administration Medical Center, San Francisco, Calif. (protocol 91-087-01) prior to experimentation.

**RESULTS**

**Biliary excretion of endotoxin.** After 3 h, rats injected with ¹²⁵I-endotoxin–chylomicron complexes had a 67% increase in total biliary excretion of endotoxin compared with rats given ¹²⁵I-endotoxin in saline (12.7% ± 1.2% versus 7.6% ± 0.3% of the total injected endotoxin dose, *P* ≤ 0.006; Fig. 1A). Twenty-four hours after injection, the increase in total biliary excretion of endotoxin by the chylomicron-treated group was still significant (20% more than controls; Fig. 1A).
The difference between the groups was significant as early as 30 min after endotoxin injection, but the difference decreased with time (Fig. 1B).

We measured the biliary excretion of endotoxin initially by using D-galactosamine, as our previous lethality experiments had used this well-accepted method to sensitize animals to the toxic effects of endotoxin (21, 22). However, because D-galactosamine has potential effects on hepatocyte function (9, 33, 54) and lipid metabolism (2, 32, 42), we repeated our studies in a second group of animals without D-galactosamine.

Rats that did not receive D-galactosamine also showed enhanced biliary excretion of endotoxin, although the absolute amounts excreted were somewhat less than those of animals receiving D-galactosamine. Rats injected with 125I-endotoxin–chylomicron complexes had a 62% increase in biliary excretion of endotoxin compared with rats given 125I-endotoxin in saline (6.8% ± 0.7% versus 4.2% ± 0.9% of the total injected load into the bile by 3 h postinjection, P = 0.04; Fig. 2A). Again, after 24 h, the increase in biliary excretion of endotoxin by the chylomicron-treated group was still significant (26% more than controls; Fig. 2A). In contrast to the animals that were given D-galactosamine, the increase in biliary excretion of endotoxin in the chylomicron-treated group did not become significant until 150 min after endotoxin infusion (Fig. 2B).

Chylomicron TG clearance (Fig. 3). To determine whether the rate of clearance of chylomicrons from the circulation was rapid enough to account for the appearance of endotoxin in the bile, we measured plasma TG after an infusion of chylomicrons. Infusion of 500 mg of chylomicron TG per kg produced a plasma TG concentration of 883 ± 74 mg/dl at 5 min. The TG was rapidly cleared, falling to 148 ± 13 mg/dl at 1 h after infusion and returning to baseline by 4 h after infusion.
Effect of endotoxin, chylomicrons, and D-galactosamine on bile flow (Fig. 4). Bile flow did not differ significantly between chylomicron-treated and control animals or between animals given D-galactosamine and those not given D-galactosamine. There was also no significant difference in bile flow between the experimental groups and the group of animals that received saline alone.

Gel electrophoresis of endotoxin in bile (Fig. 5). Gel electrophoresis of bile samples from chylomicron-treated and control animals displayed almost identical patterns, suggesting that hepatic processing of endotoxin is not altered by chylomicron binding. However, gel electrophoresis of bile samples from both chylomicron-treated and controls differed from that of native endotoxin. The electrophoretic mobility of the molecule increased after hepatic processing and biliary excretion. Bile samples from various collection time periods all showed similar electrophoretic migration patterns (data not shown).

We found that native endotoxin incubated in bile in vitro displayed an electrophoretic pattern similar to that of endotoxin excreted into the bile of experimental animals, suggesting that the change in electrophoretic mobility of endotoxin in bile is due to association with compounds in bile rather than an effect of hepatic processing.

Biologic activity of endotoxin in bile (Table 1). Bile collected from animals prior to the injection of endotoxin showed no evidence of endotoxin activity, as determined by the chromogenic Limulus assay. Bile collected after the injection of endotoxin showed endotoxin activity only after phenol extraction. It would appear that endotoxin is inactive in the presence of bile but that endotoxin is excreted into bile with its lipid A moiety intact.

Characterization of rat mesenteric lymph. Chylomicrons were the predominant lipoprotein in the lymph. Pooled mesenteric lymph samples contained 75 mg of chylomicron TG per ml, 0.032 mg of LDL total cholesterol per ml, and 0.016 mg of HDL total cholesterol per ml.

**DISCUSSION**

The ability of lipoproteins to inhibit endotoxin activity has been demonstrated both in vitro and in vivo (8, 12, 21, 22, 34–37, 46–48, 53, 55). While early investigations focused on

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**TABLE 1. Chromogenic Limulus assay of bile samples**

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<thead>
<tr>
<th>Time of bile sample collection</th>
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<tr>
<td></td>
<td>Chylomicron treated</td>
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<tr>
<td>Before injection of 125I-endotoxin</td>
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* a+, >100 pg of endotoxin per ml.

*b Extraction of endotoxin by the hot phenol-water method of Westphal et al. (56).
the interaction of endotoxin with cholesterol-rich lipoproteins (34-36, 46-48, 55), we have demonstrated that TG-rich lipoproteins also inhibit endotoxin activity in vitro (11, 20) and improve survival of animals during endotoxemia (21, 22).

The protective effect of TG-rich lipoproteins results, at least in part, from their ability to redirect endotoxin metabolism. Chylomicron binding accelerates endotoxin clearance from plasma and increases endotoxin uptake by the liver (22). Autoradiographic data suggest that chylomicron binding also alters the cellular distribution of endotoxin uptake within the liver (22). Previous studies have demonstrated that the liver clears the bulk of an intravenous endotoxin load and that hepatic macrophages (Kupffer cells) appear to be the predominant cell type involved in the clearance process (6, 15, 17, 18, 22, 24, 31, 38, 40, 51). Hepatocyte uptake of endotoxin does occur but is normally less than that of Kupffer cells (6, 15, 17, 18, 22, 24, 31, 38, 40, 51). When bound to chylomicrons, however, endotoxin is taken up preferentially by hepatocytes (22). We hypothesized that the increase in hepatocyte uptake of chylomicron-bound endotoxin would result in increased endotoxin excretion in bile.

In this study, we found that biliary excretion of endotoxin was significantly increased when endotoxin was administered with chylomicron-endotoxin complexes compared with endotoxin in saline. This finding further supports our autoradiographic data demonstrating that chylomicrons increase endotoxin uptake by hepatocytes (22). The difference in biliary excretion of endotoxin between the chylomicron-treated and control groups was most striking early, within the first 3 h after administration of endotoxin. Similarly, the majority of injected chylomicron TG was cleared from the circulation within 3 h. Since hepatocytes are the major site of chylo-
micron remnant uptake (25, 43), our findings would be consistent with the hypothesis that the chylomicron directs the clearance of chylomicron-bound endotoxin. Because chylo-
microns are rapidly cleared by hepatocytes, chylomicron-bound endotoxin is delivered more rapidly into the bile.

The rate of endotoxin excretion into the bile was greater than that found by Maitra et al. (29), who used the β-hydroxy-
mystic acid of lipid A as a marker. The increased biliary excretion of endotoxin in our study may be explained by several observations. First, the dose of endotoxin we used (7 μg/kg) was significantly less than that used by Maitra et al. (170 to 540 μg/kg) (29). Other investigators have shown that the fraction of an intravenous endotoxin load initially cleared by the liver decreases as the dose of endotoxin increases (5, 38). Thus, when lower doses of endotoxin are used, the liver may process a greater proportion of the circulating endotoxin and excrete it into the bile. Second, variation in the labeling method may account for some of the differences.

Animals receiving D-galactosamine excreted slightly more endotoxin into the bile than animals who did not receive D-galactosamine. This was an unexpected finding, as the percentage of chylomicron-bound endotoxin taken up by hepatocytes is not affected by D-galactosamine (22). The toxic effects of D-galactosamine include depletion of hepatic UTP and a decrease in hepatic biosynthesis of macromolecules (9). However, when D-galactosamine is given at a dose similar to the one used in this study, it does not grossly decrease concentrations of ATP, GTP, and CTP (10) and thus may not affect the energy requirement necessary for hepatic processing of endotoxin.

The increase in biliary excretion of endotoxin was not merely a product of bile flow volume. Previous studies have demonstrated that intravenous endotoxin infusion in vivo and in the perfused rat liver can cause cholestasis (41, 49, 50). We did not observe the cholestatic effects of endotoxin in this study; however, we used a lower dose of endotoxin than in previous studies showing endotoxin-induced cholestasis (41, 49, 50). Bile volume was also not affected by D-galactosamine.

Our gel electrophoresis and Limulus assay data suggest that endotoxin is modified by some compound(s) in bile, so that its electrophoretic mobility on agarose gel is altered and its activity is reduced. Our findings are consistent with those of previous investigators, who have demonstrated that bile salts disaggregate endotoxin and decrease its pyrogenicity in a reversible fashion (39). The electrophoretic patterns of endotoxin in bile collected in vivo and endotoxin incubated in bile in vitro were similar, suggesting that the increase in electrophoretic mobility of endotoxin in bile is attributable to modification of endotoxin by substances in bile rather than extensive degradation of the endotoxin molecule by hepatocytes. Freudenberg and Galanos have shown that partial deacylation of endotoxin does occur in the liver but that the toxic properties of the molecule (pyrogenicity, lethality, local Schwartzman reaction, and Limulus activity) are retained after hepatic processing (14). Our Limulus assay data show that endotoxin is inactive in the presence of bile, a finding that is consistent with that of other investigators (29, 39, 52). After extraction, however, the excreted endotoxin retained its activity. This is further evidence against extensive hepatic degradation of endotoxin prior to biliary excretion. Although limitations in the Limulus assay prevent quantitation of small reductions in endotoxin activity in bile, our data, and those of others, suggest that endotoxin is excreted into the gut with at least a portion of its intrinsic activity intact but that its toxic properties are mitigated by bile and the intestinal mucosal barrier.

While the redirection of endotoxin metabolism by chylomicrons may contribute to the protective effect of the chylomicrons in animal models, the extrapolation of these results to the clinical situation of gram-negative sepsis has potential limitations. To date, studies demonstrating the ability of lipoproteins to reduce endotoxin-induced toxicity in vivo (21, 22, 46, 47) have employed purified endotoxin, usually isolated by phenol extraction (56). However, during gram-negative sepsis, only a fraction of endotoxin may be released from bacterial wall membranes (4, 44). Depending on their size, bacterial membrane fragments may not bind as readily to lipoproteins (4, 36), which would render lipoproteins less effective in modifying the host response to gram-negative sepsis. In addition, extrinsic labeling of endotoxin may cause minor alterations in its metabolism, although many investigators have shown endotoxin uptake by the liver and excretion in bile by using a variety of labels (29, 31, 52). It remains to be determined whether TG-rich lipoproteins would lessen the toxicity of endotoxin generated endogenously during polymicrobial gram-negative sepsis.

In summary, this study shows that chylomicron binding increases the rate of biliary excretion of radioiodinated endotoxin. This finding supports our previous data demonstrating that chylomicrons redirect endotoxin to hepatocytes and away from Kupffer cells and provides further insight into the mechanism by which chylomicrons alter endotoxin metabolism. By rapidly delivering endotoxin to hepatocytes, chylomicrons may shield the organism from excessive macrophage activation and cytokine release. A portion of the endotoxin load is then excreted in bile, where it is inactive, and ultimately returned to the gut. The hepatic processing of
CHYLOMICRONS ENHANCE LPS EXCRETION IN BILE


