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Brief Definitive Report

Triglyceride-rich Lipoproteins Prevent Septic Death in Rats

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Summary

Triglyceride-rich lipoproteins bind and inactivate bacterial endotoxin in vitro and prevent death when given before a lethal dose of endotoxin in animals. However, lipoproteins have not yet been demonstrated to improve survival in polymicrobial gram-negative sepsis. We therefore tested the ability of triglyceride-rich lipoproteins to prevent death after cecal ligation and puncture (CLP) in rats. Animals were given bolus infusions of either chylomicrons (1 g triglyceride/kg per 4 h) or an equal volume of saline for 28 h after CLP. Chylomicron infusions significantly improved survival (measured at 96 h) compared with saline controls (80 vs 27%, $P \leq 0.03$). Chylomicron infusions also reduced serum levels of endotoxin, measured 90 min (26 ± 3 vs 136 ± 51 pg/ml, mean ± SEM, $P \leq 0.03$) and 6 h (121 ± 54 vs 1,026 ± 459 pg/ml, $P \leq 0.05$) after CLP. The reduction in serum endotoxin correlated with a reduction in serum tumor necrosis factor, measured 6 h after CLP (0 ± 0 vs 58 ± 24 pg/ml, $P \leq 0.03$), suggesting that chylomicrons improve survival in this model by limiting macrophage exposure to endotoxin and thereby reducing secretion of inflammatory cytokines. Infusions of a synthetic triglyceride-rich lipid emulsion (Intralipid; KabiVitrum, Inc., Alameda, CA) (1 g triglyceride/kg) also significantly improved survival compared with saline controls (71 vs 27%, $P \leq 0.03$). These data demonstrate that triglyceride-rich lipoproteins can protect animals from lethal polymicrobial gram-negative sepsis.

One of the earliest and most consistent metabolic responses to endotoxin is an increase in plasma levels of triglyceride, due to an increase in triglyceride-rich very low density lipoprotein (VLDL) (1). This endotoxin-stimulated increase in circulating triglyceride-rich particles may have a protective function, as triglyceride-rich lipoproteins (VLDL and chylomicrons) have been shown to bind and inactivate endotoxin (2, 3). Previous work in our laboratory has demonstrated that both chylomicrons and VLDL can inhibit the detection of significant quantities of endotoxin ($\geq$1 ng of endotoxin/mg of particle triglyceride) by the limulus assay in vitro (4). Triglyceride-rich lipoproteins also reduce endotoxin toxicity in vivo. Chylomicrons and VLDL have been shown to protect animals from death when incubated with a lethal dose of endotoxin before administration (2, 3). Chylomicrons also prevent death in animals when given as a separate intravenous infusion just before endotoxin administration (3).

In addition to inhibiting endotoxin activity directly, triglyceride-rich lipoproteins also alter endotoxin metabolism, which may contribute to their protective effect in these animal models. When administered with chylomicrons, the clearance of endotoxin from plasma is accelerated (3), with increased endotoxin uptake by the liver (3), shunting of endotoxin to hepatocytes and away from hepatic macrophages (Kupffer cells) (3), and increased endotoxin excretion in bile (5). There is a concomitant decrease in serum TNF levels (3), suggesting that chylomicrons shield the organism from endotoxin-induced macrophage activation and cytokine secretion by accelerating endotoxin delivery to hepatocytes.

The purpose of the present study was to determine whether triglyceride-rich lipoproteins would lessen the toxicity of endotoxin generated endogenously during polymicrobial sepsis. We performed cecal ligation and puncture (CLP) in rats, a

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widely used model of intraabdominal sepsis that produces polymicrobial bacteremia (predominantly gram negative), endotoxemia, and a 60–80% mortality by 72 h (6, 7). Intermittent intravenous infusions of chylomicrons significantly improved survival after CLP while reducing serum levels of endotoxin and TNF. Infusions of a synthetic triglyceride-rich lipid emulsion also significantly improved survival after CLP.

Materials and Methods

Reagents and Solutions. Glacial acetic acid (Fisher Chemical Co., Fairlawn, NJ); NaOH (J. T. Baker, Inc., Pittsburgh, PA); apyrogenic, preservative-free 0.9% NaCl (Kendall McGraw Laboratories, Inc., Irvine, CA), and H_2O (Elkins-Sinn, Inc., Cherry Hill, NJ); 3% H_2O_2 (Cumberland Co., Smyrna, TN); heparin sodium salt (Sigma Chemical Co., St. Louis, MO); and Intralipid 20% intravenous lipid emulsion containing 20% soybean oil (triglyceride), 1.2% egg yolk phospholipid, and 2.25% glycerin were used as specified. The PBS used in all experiments was tested by a chromogenic modification of the limulus assay (8) as we have used previously (2) and was found to be free of detectable endotoxin (<10 pg endotoxin/ml).

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 (≤5–10 pg/ml) by steam autoclaving followed by dry heating at 180°C for a minimum of 4 h, as previously reported (2).

Chylomicron Collection. Mesenteric lymph containing nascent chylomicrons was obtained by cannulation of the mesenteric lymph duct of male Sprague-Dawley rats (250–350 g, Bantin and Kingman, Inc., Fremont, CA) gavage fed a mixture of corn oil and milk, as previously described (3). Special precautions were taken to avoid the introduction of exogenous endotoxin during the collection process, as previously described (2). The triglyceride content of the mesenteric lymph was determined using a standard enzymatic assay (Sigma Chemical Co.) and varied between 75 and 100 mg triglyceride/ml.

Chylomicron Triglyceride Clearance. Male Sprague-Dawley rats (250–280 g) were anesthetized with pentobarbital (50 mg/kg i.p.) and catheterized via the ileofemoral vein. Animals received an intravenous bolus infusion of mesenteric lymph containing nascent chylomicrons (1 g triglyceride/kg) or Intralipid (1 g triglyceride/kg). 400-μl blood samples were drawn before and 5, 15, 60, 120, 180, and 240 min after infusion. After centrifugation (2,000 rpm for 10 min), the triglyceride content of plasma was determined using standard enzymatic assays. All animal procedures were performed in accordance with the guidelines of the Animal Studies Subcommittee (Department of Veterans Affairs Medical Center, San Francisco).

Sepsis Model (CLP). Male Sprague-Dawley rats (240–280 g) underwent placement of an internal jugular vein catheter (MRE-040 polyethylene tubing), which was tunneled to the back and connected to a subcutaneous port (Jelco intermittent injection cap; Critikon, Inc., Irvine, CA) while under pentobarbital anesthesia. The cannula and port were then flushed with 0.3 ml apyrogenic heparin (50 U/ml). The animals were allowed to fully recover overnight with free access to food and water.

24 h after insertion of the venous cannulae, the animals underwent CLP, as previously described (6, 7). Under light ether anesthesia, a 1-cm midline abdominal incision was made and the cecum was delivered into the wound. The avascular portion of the mesentery was sharply incised. The distal cecum was ligated with 4-0 silk suture (so as not to interrupt the flow of intestinal contents), and two punctures were made on the antimesenteric border of the distal cecum with an 18-gauge needle. The cecum was returned to the abdomen, and the abdominal wall was closed in layers. The rats were returned to their cages and allowed free access to food and water. Beginning at the time of CLP, animals received intravenous bolus infusions of either mesenteric lymph containing nascent chylomicrons (1 g triglyceride/kg, 75 mg triglyceride/ml), Intralipid (1 g triglyceride/kg, 200 mg triglyceride/ml), or normal saline (volume equivalent to that of the chylomicron dose). Bolus infusions were administered every 4 h for 28 h and were followed by heparin flush. The 4-h dosing interval allowed plasma triglyceride levels to return to baseline just before the subsequent dose of chylomicrons or Intralipid (Fig. 1). Animals were fed ad lib. throughout. The animals were observed continuously during the first 28 h after CLP. Mortality was also determined at 48, 72, and 96 h after CLP.

Serum Endotoxin and TNF. To determine endotoxin and TNF levels after CLP, the study was repeated as described above, with infusion of mesenteric lymph containing nascent chylomicrons or normal saline (controls). At various time intervals after CLP, the animals were killed and blood samples were taken from the aorta. After collection of serum, samples were assayed for endotoxin by a chromogenic modification of the limulus assay (8). Serum samples were diluted 1/10 with apyrogenic saline and heated at 75°C for 5 min before the limulus assay to remove the effect of serum inhibitors of the assay (8) and specifically to liberate lipoprotein-bound endotoxin (9). Serum TNF levels were measured via a cytotoxicity assay using WEHI 164 clone 13 cells with development using thiazolyl blue (10).

Statistical Analysis. Statistical significance for the mortality data was determined by chi square analysis, measured 96 h after CLP. Serum endotoxin and TNF levels were compared using Kruskal-Wallis analysis.

Results

Chylomicron Triglyceride Clearance (Fig. 1). Infusion of 1 g triglyceride/kg of chylomicrons or Intralipid produced an early

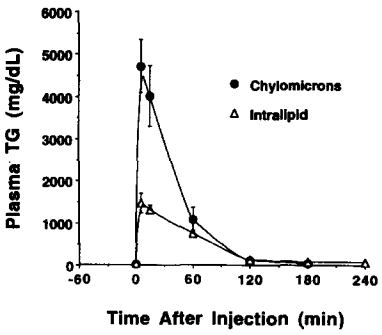

Figure 1. Chylomicron/Intralipid triglyceride clearance. Rats received an intravenous infusion of chylomicrons (1 g triglyceride/kg) or the synthetic triglyceride-rich lipid emulsion Intralipid (1 g triglyceride/kg). Serial blood samples were obtained and plasma was assayed for triglyceride. Data are mean ± SEM of four animals per group. Values were estimated from an enzymatic assay for glycerol. Therefore, these values may be 20% or more higher than actual circulating plasma values during chylomicron or Intralipid infusions.

rise in plasma triglyceride, but the triglyceride was rapidly cleared from the circulation. The maximal elevation of plasma triglyceride, as measured 5 min after infusion, was greater with infusion of chylomicrons than with Intralipid, but the clearance patterns were otherwise similar. Plasma triglyceride levels returned to baseline 4 h after infusion. Plasma triglyceride levels were measured by determining glycerol levels. Because the infusions of triglyceride with chylomicrons or Intralipid can raise plasma glycerol levels, the plasma triglyceride levels may be overestimated by 20% or more.

Survival after CLP (Fig. 2). Survival, measured 96 h after CLP, was significantly improved in those animals receiving chylomicrons (80%) and Intralipid (71%) compared with controls (27%) ($P \leq 0.03$ vs controls for both chylomicron- and Intralipid-treated groups). All deaths occurred within the first 72 h, with the majority of deaths in all groups occurring between 24 and 48 h after CLP. All animals appeared ill initially, with ruffled fur, huddling behavior, and diarrhea. By 96 h after CLP, survivors had returned to normal behavior patterns, while piloerection and diarrhea had ceased.

Serum Endotoxin after CLP (Fig. 3). Serum endotoxin levels were significantly less in chylomicron-treated animals than in controls at both 90 min and 6 h after CLP. Serum samples were diluted and heated before the limulus assay to remove the effect of serum inhibitors of the assay (8) and to liberate lipoprotein-bound endotoxin (9). Thus, the observed reduction in endotoxin activity represents a decrease in total circulating endotoxin rather than inhibition of the activity of lipoprotein-bound endotoxin.

Serum TNF after CLP (Fig. 4). Serum TNF was also significantly reduced by chylomicron treatment at 6 h after CLP (undetectable in chylomicron-treated animals.) At 90 min after CLP there was also a trend towards a reduction in TNF levels, but the difference did not reach statistical significance ($P = 0.19$).

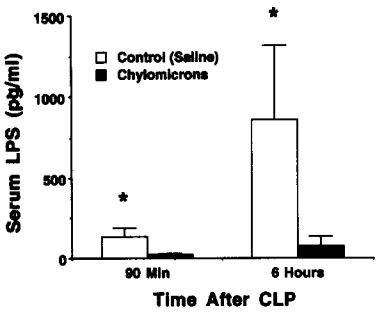

Figure 3. Serum endotoxin (LPS) levels after CLP. Rats underwent internal jugular vein catheter placement and CLP as described above. Intravenous infusions of mesenteric lymph containing nascent chylomicrons (1 g triglyceride/kg) or an equivalent volume of normal saline (controls) were administered at the time of CLP and every 4 h thereafter. Animals were killed either 90 min ($n = 16$ in each group) or 6 h ($n = 6$ in each group) after CLP for serum collection. Serum endotoxin levels were determined by a chromogenic modification of the limulus lysate assay (8). Data represent mean ± SEM for each group. * $P < 0.05$ versus saline controls.

Discussion

These data demonstrate that repeated doses of triglyceride-rich lipoproteins prolong survival while reducing serum endotoxin and TNF levels during lethal gram-negative sepsis. We chose to use CLP as a septic model because it creates a polymicrobial infection (7) similar to that seen in humans with intraabdominal sepsis. In addition, serum levels of endotoxin and inflammatory cytokines are lower and are sustained longer after CLP (7) than after single injections of bacteria or endotoxin. The prolonged release of inflammatory mediators during CLP more closely resembles the pattern seen in septic patients (11).

Lethality of the CLP model may vary as a function of the volume of stool in the cecum at the time of ligation and the amount of stool mechanically expressed before returning

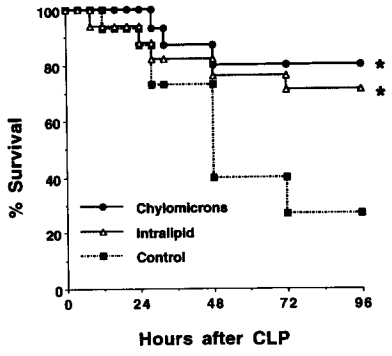

Figure 2. Survival of rats after CLP. Animals underwent placement of indwelling venous catheters, and 24 h later they underwent CLP. Beginning at the time of CLP, animals received intravenous infusions of either mesenteric lymph containing nascent chylomicrons (1 g triglyceride/kg, $n = 15$), Intralipid (1 g triglyceride/kg, $n = 17$), or normal saline (volume equivalent to that of the chylomicron dose, $n = 15$). Infusions were administered every 4 h for 28 h. Survival was determined up to 96 h. All deaths occurred during the first 72 h after CLP. * $P \leq 0.03$ vs controls at 96 h.

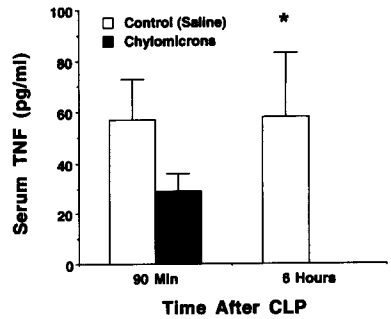

Figure 4. Serum TNF levels after CLP. Rats underwent internal jugular vein catheter placement and CLP as described above. Intravenous infusions of mesenteric lymph containing nascent chylomicrons (1 g triglyceride/kg) or an equivalent volume of normal saline (controls) were administered at the time of CLP and every 4 h thereafter. Animals were killed either 90 min ($n = 16$ in each group) or 6 h ($n = 6$ in each group) after CLP for serum collection. Serum TNF levels were determined by a cytotoxicity assay (10). No TNF was detected in the serum of rats in the chylomicron-treated group killed 6 h after CLP. Data represent mean ± SEM for each group. * $P < 0.05$ vs saline controls.

the cecum to the abdomen. To avoid investigator bias, a single investigator (T. E. Read) performed all CLP procedures. A second investigator assigned animals to the various treatment groups.

Previous work has demonstrated that triglyceride-rich lipoproteins protect animals from endotoxemic death via two complementary mechanisms: (*a*) by binding endotoxin and inhibiting its activity directly; and (*b*) by accelerating endotoxin clearance from plasma. All lipoproteins, including triglyceride-rich VLDL (2) and chylomicrons (4), and cholesterol ester-rich HDL (2, 12–15) and LDL (2, 14, 15), have been shown to bind and inactivate endotoxin in vitro (as measured by the limulus assay) and prevent endotoxin-induced death in animals. Ultrastructural studies of LDL–endotoxin complexes suggest that inhibition of endotoxin activity occurs via the insertion of the toxic lipid A moiety of endotoxin into the phospholipid surface of the lipoprotein particle (16). In addition to inhibiting detection of endotoxin by the limulus assay, lipoproteins also block the ability of endotoxin to induce secretion of the cytokines TNF, IL-1, and IL-6 from macrophages in cell culture (17, 18). During CLP, endotoxin bound to circulating triglyceride-rich particles would be effectively hidden from the reticuloendothelial system, thus reducing endotoxin-induced stimulation of macrophages. The secretion of inflammatory cytokines would therefore be limited, as is reflected by the reduced TNF levels seen with chylomicron treatment after CLP.

Triglyceride-rich lipoproteins may further shield the organism from macrophage activation by redirecting endotoxin metabolism. Previous work in our laboratory has demonstrated that the clearance of chylomicron-bound endotoxin is directed by the chylomicron rather than by the endotoxin molecule. When administered with chylomicrons, the clearance of endotoxin from plasma is accelerated, and uptake of endotoxin by the liver is increased (3). Autoradiographic data indicate that chylomicron-binding alters the cellular distribution of endotoxin uptake within the liver (3). Hepatic macrophages (Kupffer cells) clear the majority of an intravenous load of unbound endotoxin (19–22). However, chylomicron/endotoxin complexes, like chylomicrons alone (23), are rapidly cleared by hepatocytes (3). We have previously shown that the reduction in endotoxin uptake by Kupffer cells correlates with lower peak serum TNF levels (3).

The lower serum endotoxin levels after CLP suggest that chylomicrons also accelerate endotoxin clearance during polymicrobial gram-negative sepsis. Before performing the limulus assay, serum samples were heated to liberate lipoprotein-bound endotoxin. The reduction in endotoxin activity therefore represents a decrease in total circulating endotoxin, rather than inhibition of the activity of endotoxin bound to chylomicrons. The acceleration of endotoxin clearance from plasma may contribute to the reduction in serum TNF levels seen with chylomicron treatment after CLP. Thus, chylomicrons may improve survival during gram-negative sepsis by inhibiting endotoxin activity and enhancing endotoxin clearance, both of which would limit macrophage exposure to endotoxin and thereby blunt an otherwise fatal cytokine response to endotoxemia.

Intralipid particles exhibit many of the same properties as chylomicrons and may prevent septic death by a similar mechanism. Chylomicron and Intralipid particles are both rich in triglyceride and are of similar size. Intralipid particles acquire apolipoproteins once in circulation (24) and, like chylomicrons (23), are rapidly cleared by hepatocytes (25). Synthetic triglyceride-rich emulsions, as well as chylomicrons, have the capacity to inactivate endotoxin in vitro (4) and to prevent death from a lethal dose of endotoxin in animals (2).

Protection from endotoxemia and gram-negative sepsis by the administration of triglyceride-rich lipoproteins could be viewed as an augmentation of a natural host defense against infection. There is now growing evidence that triglyceride-rich lipoproteins are a component of the acute-phase response. When an organism is challenged with infectious agents, the liver increases production of an array of proteins, termed "acute-phase proteins," which are thought to enhance the natural defense against infection and inflammation by a variety of mechanisms (26). The increase in hepatic synthesis and secretion of many of these proteins has been demonstrated to be mediated by inflammatory cytokines, including TNF (27, 28), IL-1 (28), and IL-6 (29, 30). Similarly, various inflammatory mediators, including endotoxin (1), interferons (31, 32), TNF (31, 33, 34), IL-1 (32, 35), and IL-6 (36), have been shown to increase hepatic fatty acid and VLDL synthesis or to decrease lipoprotein lipase and lipid storage by adipocytes. These processes would increase (or maintain) plasma concentrations of triglyceride-rich lipoproteins during infection, which may represent an attempt by the body to neutralize the toxic effects of circulating endotoxin.

The data presented here add to the growing evidence that lipoproteins can act as adjuncts to the immune system by decreasing the toxicity of a variety of harmful biological and chemical agents. Lipoproteins and apoproteins reduce the infectivity of several viruses (37–39) and parasites (40) and decrease the inflammatory response to monosodium urate crystals (41). Thus, lipoproteins have significant antiinflammatory and antiinfectious properties aside from their role in lipid transport. Moreover, this study suggests that there may be a role for lipoproteins or lipid particles in the treatment of endotoxemia or gram-negative sepsis in humans.

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