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Lipopolysaccharide (LPS)-Binding Protein Mediates LPS Detoxification by Chylomicrons

Anita C. E. Vreugdenhil,* Corine H. Rousseau,* Thomas Hartung, † Jan Willem M. Greve,* Cornelis van ‘t Veer,* and Wim A. Buurman2*

Chylomicrons have been shown to protect against endotoxin-induced lethality. LPS-binding protein (LBP) is involved in the inactivation of bacterial toxin by lipoproteins. The current study examined the interaction among LBP, chylomicrons, and bacterial toxin. LBP was demonstrated to associate with chylomicrons and enhance the amount of LPS binding to chylomicrons in a dose-dependent fashion. In addition, LBP accelerated LPS binding to chylomicrons. This LBP-induced interaction of LPS with chylomicrons prevented endotoxin toxicity, as demonstrated by reduced cytokine secretion by PBMC. When postprandial circulating concentrations of chylomicrons were compared with circulating levels of low density lipoprotein, very low density lipoprotein, and high density lipoprotein, chylomicrons exceeded the other lipoproteins in LPS-inactivating capacity. Furthermore, highly purified lipoteichoic acid, an immunostimulatory component of Gram-positive bacteria, was detoxified by incubation with LBP and chylomicrons. In conclusion, our results indicate that LBP associates with chylomicrons and enables chylomicrons to rapidly bind bacterial toxin, thereby preventing cell activation. Besides a role in the detoxification of bacterial toxin present in the circulation, we believe that LBP-chylomicron complexes may be part of a local defense mechanism of the intestine against translocated bacterial toxin. The Journal of Immunology, 2003, 170: 1399–1405.

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esides a crucial role in the transport of dietary lipids, chylomicrons appear to reinforce the host defense against LPS, a major constituent of the outer membrane of Gram-negative bacteria (1, 2). LPS provokes an inflammatory response in the infected host by virtue of its ability to bind membrane factors present on the surface of macrophages and other cells essential to host defense. Once the response to Gram-negative bacteria is initiated, there is a need to inactivate and eliminate LPS to minimize potential deleterious effects of an unregulated response. Circulating chylomicrons were previously demonstrated to be potent inhibitors of LPS activity in vivo and to prevent endotoxin-induced mortality (1, 2). The metabolic fate of LPS was shown to be strongly affected by complex formation with chylomicrons. Whereas the association of LPS with high density lipoprotein (HDL) decreases its clearance from the circulation (3, 4), chylomicrons enhance the plasma clearance and hepatic uptake of LPS, with shunting of LPS to hepatocytes and away from hepatic macrophages (2, 5). Harris et al. (6) recently presented data indicating that both the low density lipoprotein (LDL) receptor and LDL receptor-related protein participate in the rapid internalization of chylomicron-bound LPS by hepatocytes. Consequently, LPS is delivered into the bile, where it is inactivated by bile salts (7). Lipoproteins other than chylomicrons were previously found to prevent a cellular response by binding of LPS (8), and recent studies by Kitchens et al. (9) demonstrated that LPS already bound to monocytes is released in response to lipoproteins, thereby reducing cytokine secretion. Besides an enhanced clearance of LPS from the circulation, such mechanisms may also be applicable to chylomicrons and may account for the chylomicron-induced increase in survival during endotoxemia. It was demonstrated that plasma constituents other than lipoproteins are required for maximal binding of LPS to lipoproteins (10) and for lipoproteins to achieve maximal prevention of endotoxin-induced death (1), suggesting that a constituent of plasma is required for the binding of endotoxin to lipoproteins.

The acute phase protein, LPS-binding protein (LBP), plays an important role in the reduction of LPS activity by catalyzing the transfer of monomerized LPS from micelles into circulating lipoproteins (11). The enhanced concentration of circulating LBP during an acute phase response was recently demonstrated to be crucial, since it diminishes the transfer of LPS to monocytes and reduces cytokine secretion (12). LBP also induces lipoprotein inactivation of lipoteichoic acid (LTA), a component of most Gram-positive bacteria (13). Although initial studies focused on the LPS-detoxifying function of HDL (14), later studies demonstrated that in serum LPS predominantly binds to very low density lipoprotein (VLDL) and LDL, which also results in the inactivation of LPS (15–17). We recently demonstrated that LBP circulates in complex with apolipoprotein B (apoB)-containing lipoproteins, i.e., LDL and VLDL (18). LBP complexed with LDL and VLDL strongly enhances LPS binding to these lipoproteins, which suggests an important role for LBP/apolipoprotein complexes in the scavenging of endotoxin present in the circulation (18). The association of LBP with LDL and VLDL appears to result in part from the high affinity of LBP for apoB. This prompted us to study whether LBP also cooperates...
with the apoB-containing chylomicrons in detoxification of the bacterial toxins LPS and LTA. Consequently, we studied the interaction of LBP with chylomicrons and the effect of this interaction on toxin binding and activity.

Materials and Methods

Reagents

LPS from *Escherichia coli*, serotype 055:B5, was purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal Ab to human LBP was obtained by immunizing rabbits with purified human LBP. Protein A-purified anti-LBP IgG was biotinylated following standard procedures. Anti-human LBP mAb HM14 was obtained by immunizing mice with LBP following classical procedures (18). BSA (containing <1 ng LPS/mg) was derived from Sigma-Aldrich. Human TNF-α was provided by BASF/Knoll Antigen (Ludwigshafen, Germany). Since LTA preparations available to date are highly contaminated by LPS (19), we used a recently introduced preparation method for LTA (20) that allows study of the effects of LPS-free, biologically active LTA. LTA, 99% pure and containing <30 pg of LPS/mg LTA, was prepared from *Staphylococcus aureus*.

**Blood samples**

Human venous blood was collected from healthy volunteers after an overnight fast and 2.5 h postprandially. To prepare serum, blood was allowed to clot for 1 h at room temperature. Fresh serum was used for isolation of lipoproteins.

**Lipoprotein isolation**

Chylomicrons were isolated from pooled postprandial human serum (six donors) by ultracentrifugation according to the procedure described by Terpstra et al. (21). Briefly, dry potassium bromide (770 mg) and sucrose (50 mg) together with 2 ml of serum were placed in the bottoms of ultracentrifuge tubes (Beckman Instruments, Brea, CA). These components were carefully mixed with a spatula. A discontinuous gradient was made with the serum mixture at a density of 1.250 g/ml, two potassium bromide solutions with densities of 1.225 and 1.100 g/ml, respectively, and, on top, a water density of 0.998 g/ml. The tubes were placed in an SW50.1 rotor and centrifuged in a Beckman XL-80 ultracentrifuge (Beckman Instruments) at 30,000 rpm and 20°C for 40 min. The chylomicrons were collected by aspiration of the top 1-ml fraction and were stored at −80°C until use. HDL, LDL, and VLDL were isolated from pooled fresh human sera (six healthy donors) by a 22-h single spin density gradient ultracentrifugation at 40,000 rpm at 17°C according to the method reported by Terpstra et al. (21). Bacteria on their biologic activity, cytokine induction by toxin incubated with chylomicrons in the presence or the absence of LBP was assessed. PBMC were isolated from buffy coats of healthy volunteers, provided by the local blood bank, by buoyant density centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Cells were washed five times in HBSS to remove LBP and other plasma proteins and then were suspended in macrophase serum-free medium (Life Technologies, Paisley, U.K.) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). LPS and LTA were preincubated with LBP, LBP and lipopolysaccharide lipoproteins alone as indicated in 96-well, flat-bottom tissue culture plates (Costar, Cambridge, MA) for 24 or 0.5 h at 37°C. Cells were added immediately after isolation at 10^5 cells/well. Control cultures were incubated with medium alone. The cell cultures were incubated for 4 h at 37°C in 5% CO₂. Supernatants were collected and stored at −20°C until use.

**TNF-α and IL-8 ELISA**

Cytokine concentrations in the cell culture supernatants were determined using sandwich ELISA for TNF-α (25) and IL-8 (26). In short, 96-well Immunomaxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with cytokine-specific mAb. TNF-α in the supernatants was detected with polyclonal rabbit anti-human TNF-α, followed by peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). IL-8 was detected by biotinylated polyclonal rabbit anti-human IL-8, followed by peroxidase-conjugated streptavidin. TMB was used as a substrate for peroxidase. Photospectrometry was performed at 450 nm.

**Results**

**LBP associates with chylomicrons**

Recently, LBP was shown to be predominantly complexed with apoB-containing lipoprotein particles, i.e., LDL and VLDL. This interaction was found to be at least in part mediated by an interaction of LBP with apoB (18). These findings prompted us to investigate the interaction of LBP with other triacylglyceride-rich apoB-containing lipoprotein, i.e., chylomicrons. To study the binding of LBP to isolated chylomicrons, an in vitro assay was devised.
used that revealed a concentration-dependent binding of biotinylated LBP to chylomicrons (Fig. 1). Binding of LBP to chylomicrons was completely blocked by a pAb against LBP (Fig. 1), indicating the specificity of this interaction. Addition of aspecific pAb did not affect the results. The data strongly suggest that LBP and chylomicrons form a complex.

**LBP associated with chylomicrons enhances the LPS binding capacity of chylomicrons**

LBP was demonstrated to transfer LPS into lipoproteins, thereby enhancing its detoxification (11). To elucidate whether LBP complexed to chylomicrons is able to enhance LPS binding to chylomicrons, an in vitro assay was used. LPS binding to chylomicrons was compared with binding of LPS to LBP-chylomicron complexes. LPS was observed to interact with chylomicrons in the absence of LBP (Fig. 2A). However, LBP associated with chylomicrons strongly enhanced the LPS binding capacity of chylomicrons (Fig. 2A). The LBP-induced up-regulation of LPS binding to chylomicrons was dose dependent. This effect of LBP was blocked by pAb against LBP (Fig. 2B). LPS binding to chylomicrons in the absence of LBP was not affected by the presence of pAb against LBP, which excludes that this binding was caused by small amounts of LBP present in the chylomicron fractions. Overall, the data imply that LBP associated with chylomicrons was functionally active in transferring LPS to chylomicrons.

**LBP accelerates the transfer of BODIPY-LPS into chylomicrons**

To confirm the ability of LBP to mediate transfer of LPS to chylomicrons and to monitor the time kinetics of this interaction, we used BODIPY-LPS donor micelles and chylomicrons as acceptor particles. Addition of chylomicrons to BODIPY-LPS in the absence of LBP resulted in a dequenching of the fluorescence, which is indicative for dissociation of LPS from the micelles and binding to chylomicrons. Incubation with LBP together with chylomicrons resulted in a pronounced enhancement of the rate of dequenching of BODIPY-LPS (Fig. 3). This acceleration of LPS binding to chylomicrons was LBP dose dependent. These findings are in line with our observations described above and indicate that LBP catalyzes LPS binding to chylomicrons and, more importantly, accelerates LPS binding to chylomicrons.

**Chylomicrons detoxify LPS in a LBP dose-dependent fashion**

Next, we studied whether the observed enhanced LPS binding to chylomicrons induced by LBP results in abrogation of the bioactivity of LPS. Therefore, we exposed PBMC to LPS preincubated with chylomicrons and different concentrations of LBP. TNF-α and IL-8 release by PBMC after a 4-h incubation period were measured. Fig. 4 shows that preincubation of LPS with chylomicrons and LBP clearly reduced the LPS-evoked TNF-α and IL-8...
secretion by PBMC. This LPS-detoxifying capacity of chylomicrons was found to depend on the concentration of LBP present. In the absence of chylomicrons, LBP enhanced the LPS-induced cell activation dose-dependently, as expected. In the presence of increasing LBP concentrations (100 ng/ml, 1.7 nM), however, this effect of LBP declined, as described by others (27). A high concentration of LPS (100 ng/ml, 40 nM) was used in these experiments to allow stimulation of cells in the absence of LBP. In addition, pilot experiments showed that the capacity to induce cytokine secretion by 10 ng/ml LPS was completely abrogated in the presence of chylomicrons and 5 ng/ml LBP (data not shown).

To study the effect of the chylomicron concentration on inhibition of LPS-induced cytokine secretion, 100 ng/ml of LPS was preincubated with 100 ng/ml of LBP and different concentrations of chylomicrons (Fig. 5). The LPS-detoxifying function of chylomicrons was concentration dependent. Moreover, 50% of the postprandial circulating level of chylomicrons abrogated the cytokine response induced by 100 ng/ml of LPS, while a profound reduction of LPS activity was also seen at low chylomicron concentrations.

Prior studies described a maximal endotoxin inactivation by lipoproteins after 24 h of preincubation (17). However, using BODIPY-LPS dequenching experiments we found a rapid binding of LPS to chylomicrons in the presence of LBP (Fig. 3). To study whether this rapid LBP-mediated binding of LPS to chylomicrons results in detoxification of LPS, chylomicrons were preincubated with LBP and LPS only for 0.5 h, and PBMC were added. Fig. 6 demonstrates that in the presence of LBP, chylomicrons are capable of reducing LPS activity within 0.5 h.

Postprandial chylomicron levels exceed the LPS-neutralizing capacity of circulating levels of HDL, LDL, and VLDL

It was demonstrated that lipoproteins have the ability to neutralize LPS in an LBP-dependent fashion (11). To determine to which extent postprandial chylomicron levels contribute to LPS neutralization by lipoproteins, the LPS-detoxifying capacity of lipoproteins was compared at concentrations equated with their physiologic plasma ratio. For chylomicrons, this is in regard to postprandial levels. All lipoproteins were diluted to 12.5% of their plasma concentration and preincubated with LPS and LBP. The ability of the remaining bioactive LPS to induce TNF-α was determined. In the absence of lipoproteins, 100 ng/ml of LPS together with 50 ng/ml of LBP led to a strong increase in TNF-α induction (Fig. 7). This TNF-α secretion was reduced by all tested lipoproteins. However, the most pronounced reduction of TNF-α secretion was observed when LPS was incubated with chylomicrons and LBP.

Chylomicrons reduce LTA-induced TNF secretion

The incidence of septic shock caused by Gram-positive bacteria has risen markedly in the last decade (28, 29). LTA, a component of the membrane of Gram-positive bacteria, can elicit the release of proinflammatory cytokines from immune cells (30). We determined whether chylomicrons in cooperation with LBP are also able to inhibit the stimulation of PBMC by LTA. Fig. 8 demonstrates that LBP induces detoxification of LTA by chylomicrons dose-dependently. These data suggest that besides a role in combating Gram-negative bacteria, LBP-chylomicron complexes may be of importance in the host defense against Gram-positive bacteria.
Discussion

In the present study we obtained evidence for a functional interaction of LBP with chylomicrons. The association of LBP with chylomicrons was shown to enhance the LPS binding capacity of chylomicrons. Most importantly, chylomicrons and LBP cooperate in reducing LPS toxicity, resulting in decreased cytokine and chemokine secretion by PBMC. Moreover, chylomicron-LBP complexes were demonstrated to prevent cell activation by LTA, a cell wall component of Gram-positive micro-organisms. These data imply that LBP is an important cofactor for chylomicrons to execute their function as scavengers of bacterial toxin.

Consistent with others (31), we found that isolated chylomicrons bind LPS in the absence of LBP (Figs. 2 and 3). ApoB (18), and apoE (5), present in chylomicrons, were previously demonstrated to bind LPS and may account for LPS binding to chylomicrons. Binding of LPS to the phospholipids in the membrane of the chylomicrons under these conditions is not likely, since plasma proteins, such as phospholipid transfer protein (32), cholesteryl ester transfer protein, and LBP, were demonstrated to be required for intercalation of LPS into phospholipid membranes (33). In line with these findings, plasma factors were reported to be necessary for enhancement of the LPS binding capacity of chylomicrons and maximal prevention of LPS-induced death by i.v. administrated...
lipoproteins (1). We now describe that LBP binds to chylomicrons and enhances the LPS-chylomicron interaction dose-dependently. Moreover, LBP was observed to accelerate LPS binding to chylomicrons. These data indicate that LBP is an important plasma factor involved in LPS binding to chylomicrons.

Harris et al. (2) reported that infusion of LPS together with chylomicrons compared with infusion of LPS alone resulted in lower levels of circulating TNF, the cytokine known to mediate much of the LPS toxicity in vivo. Moreover, they suggested that an enhanced uptake of LPS by hepatocytes and clearance of LPS from the circulation contribute to the reduced cytokine levels in such treated animals. In line with the reduction of TNF levels by chylomicrons observed in vivo by these investigators, we now found evidence for a direct LBP-mediated inhibiting effect of chylomicrons on LPS-induced cytokine secretion by PBMC in vitro. On the basis of our findings we postulate that the LPS-LBP-chylomicron interaction may participate in the reduced TNF levels in vivo by both enhanced detoxification of LPS and enhanced clearance of LPS complexed to chylomicrons from the circulation. Although in this study we determined the effect of preincubated LPS-chylomicron complexes on cellular activation, the capability of chylomicrons to bind LPS may also enable these lipoproteins to release LPS already bound to monocytes, as described for other lipoproteins (9). The finding that infusion of chylomicrons up to 30 min after the administration of endotoxin improved survival in rodents (2, 34) makes such a mechanism plausible.

To date, studies of LPS-inactivating properties of chylomicrons have been focused on circulating chylomicrons, and therapeutic application of chylomicrons was performed by systemic infusion of chylomicrons isolated from plasma or lymph (1, 2, 5, 7). Chylomicrons present in the circulation may cooperate with both hepatic and gut-derived LBP and detoxify circulating bacterial toxin. Besides detoxification of toxin in the circulation by LBP-chylomicron complexes, it can be hypothesized that this mechanism may be part of a local defense mechanism of the intestine against translocated bacterial toxin. Chylomicrons are produced by enterocytes, and interestingly, recent evidence was found for the basolateral secretion of LBP by the intestinal epithelium in response to cytokines (35). It is tempting to speculate from the data presented here that LBP and chylomicrons, both secreted by the intestinal mucosa, cooperate in local neutralization of LPS. Such a mechanism may occur in the subepithelial space or in the lymph and possibly prevents further damage of bacterial toxin to the intestinal epithelial barrier and entrance of biological active toxin into the circulation. A local function is only relevant when LPS detoxification by chylomicron-LBP complexes occurs rapidly. The data showed that LBP strongly accelerated the transfer of LPS to chylomicrons (Fig. 3), and in PBMC stimulation experiments we observed that LPS detoxification by chylomicrons was pronounced after 0.5 h of preincubation (Fig. 6). These observations support the potential relevance of a local role for chylomicrons and LBP in the prevention of LPS-induced damage. Such a mechanism is of special interest in the intestine, since this organ is loaded with high amounts of bacteria and their toxic products that under pathologic conditions are thought to translocate and then play an important role in inflammatory and infectious complications, such as multiorgan failure and sepsis (36).

Employing highly purified, essentially LPS-free LTA, we show that the phenomenon also extends to Gram-positive bacteria. LBP was not required for activation of PBMC by 20 μg/ml of LTA, although LTA binds to this acute phase protein (37). Our data suggest that LBP and chylomicrons cooperate in the detoxification of LTA.

Enteral feeding is required for chylomicron secretion, and our findings may therefore explain at least in part the reported beneficial effects of oral feeding in critically ill patients (38). The physiological advantages of enteral nutrition as opposed to both starvation and parenteral nutrition are more and more accepted. Meta analysis has demonstrated that in a large variety of patients, including patients suffering from pancreatitis, ulcerative colitis, Crohn’s disease, surgery, trauma, or multiorgan failure, enteral feeding was associated with a lower risk of infection compared with starvation and parenteral nutrition (38). Early postoperative enteral feeding was found to reduce septic morbidity (39) and hospital stay (40). However, in most centers patients are restricted from early postoperative enteral feeding and from enteral feeding during their stay in the intensive care unit. Such abstention of enteral feeding may cause a decline in the capacity of the body to detoxify bacterial toxin. We consider that enteral feeding of critically ill patients exploits a physiologic mechanism to enhance the endogenous chylomicron production, potentially leading to an enhanced presence of LPS-inactivating LBP-chylomicron complexes in the circulation and, possibly more important, in the intestinal subepithelial space and lymph. The chylomicron levels attained by oral feeding seem to be of relevance, since postprandial levels of chylomicrons are more potent in LPS neutralization than physiologic levels of other lipoprotein fractions (HDL, LDL, and VLDL) in vitro. We are not able to conclude from the data whether this greater inhibiting capacity of chylomicrons is due to a higher relative affinity for LPS, higher LBP or LPS binding capacity, or a molar excess of circulating chylomicrons compared with the other lipoprotein classes. Since even low concentrations of chylomicrons exert a significant effect on LPS activity, and postprandial chylomicron levels rise acutely and drop slowly over hours thereafter (41), the effect of feeding is most likely to persist for several hours.

Overall, in this study we obtained evidence for a cooperative function between LBP and chylomicrons in the detoxification of toxin from both Gram-positive and Gram-negative microorganisms. Enhanced binding of LPS to LBP-chylomicron complexes in the circulation may prevent cytokine production and most likely enhances their clearance from the circulation. We further speculated that the production of LBP-chylomicron complexes in the gut may represent a natural defense mechanism against endotoxia of enteric origin. Enteral feeding and subsequent chylomicron production may be a preventive or even therapeutic intervention in patients who undergo abdominal surgery or major cardiovascular surgery, since in those patients bacterial translocation across the epithelial barrier of the gut is thought to play an important role in postoperative complications (36).

References


