Intestinal defense against bacterial toxins : role of LPS binding protein and lipoproteins

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Aan mijn ouders,

Voor Jogchum, Stijn en ...

Contents

Chapter 1	General introduction	
	The natural defense of the intestine against infection; a compass for future strategies in the battle against sepsis	9
	Outline of the thesis	34
Chapter 2	Lipopolysaccharide Binding Protein and Serum Amyloid A secretion by human intestinal epithelial cells during the acute phase response	35
Chapter 3	Lipopolysaccharide Binding Protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice	51
Chapter 4	Lipopolysaccharide Binding Protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction	67
Chapter 5	Lipopolysaccharide Binding Protein mediates LPS detoxification by chylomicrons	85
Chapter 6	Enteral administration of high-fat nutrition before and directly after hemorrhagic shock reduces endotoxemia and bacterial translocation	103
Chapter 7	Phosphatidylcholine enhances apolipoprotein secretion by enterocytes during the acute phase response	119
Chapter 8	Summary and discussion	135
	Samenvatting en discussie	141
	Dankwoord	149
	Curriculum vitae	151
	Publications	153

Chapter 1 General introduction

The natural defense of the intestine against infection; a compass for future strategies in the battle against sepsis

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Introduction

The clinical syndrome following invasion of Gram-positive and Gram-negative bacteria and their toxins, including hypotension and hypoperfusion of tissues, tachycardia, tachypnea, disseminated intravascular coagulation and organ dysfunction often resulting in death, is called the sepsis syndrome. The term "sepsis syndrome" suggests an unequivocal succession of events with well-defined signs and symptoms. However, the individual response initiated upon invasion of bacteria and their toxic metabolites is extremely whimsical and unpredictable. The course of sepsis can be fulminant with a fast clinical deterioration and death within 24 hours in otherwise healthy persons, as seen for instance in meningococcal sepsis. However, the course of sepsis and the systemic inflammatory response syndrome (SIRS) after major trauma and surgery may also occur late and can have a slow evolving course. The precise etiology and decisive factors in these distinct courses of sepsis are poorly understood, although a serious deterioration of the immune system is considered to be the leading cause of the life threatening multi organ failure (MOF). Paradoxically both, the self-destructive effects of an overwhelming immune response as well as immunoparalysis are considered to be fundamental in the sepsis paradigm. After surgical procedures a phase of inflammation and hypo-inflammation do not only occur in sequence to each other but also in parallel, which complicates immunological monitoring and therefore also immunomodulation. Despite advances in intensive care, and decades of research, this notorious complication of surgery still lacks an effective treatment and consequently the mortality of sepsis is still estimated between 20-60% (1). The diversity of responsible pathogens, the complexity of the immunologic response and individual variation in the clinical course complicate the development of therapies. In the past, several interventions aimed at interrupting the inflammatory cascade, were studied and proven not successful. Inhibition of bacterial invasion and prevention of the initiation of this cascade are the ultimate goals of new treatment strategies aimed at intercepting the fatal effects of a deregulated immune response in the earliest stage. Most interestingly, the host is equipped with natural defense mechanisms extremely potent in deactivating bacterial toxin and preventing the initiation of an inflammatory response. In our vision, failure of these host defense mechanisms primarily causes the activation, deregulation and eventually inactivation of the immune system during sepsis. In search for new strategies aimed at reducing the fatal effects of sepsis, understanding the physiologic mechanisms that neutralize and clear bacterial toxin is of considerable importance.

Here the routes of entry for bacteria, the factors predisposing to bacterial invasion, and most important the host response to presence of bacterial compounds are described. In particular the natural defense mechanisms of the gut will be illuminated for two reasons. First, since the gut, being continuously exposed to high amounts of bacteria is an organ optimally equipped to prevent bacterial invasion and to defend against translocated bacteria and toxins. Second, the gut is a predominant source of bacteria and their toxic metabolites under pathologic conditions and is seen as the "engine" that drives sepsis.

Immune activation

After entrance of bacteria and toxins into the host, cellular activation by bacterial toxins is an important initial step in the cascade of events that eventually results in sepsis and subsequent MOF. The principal pathogen-associated molecule of gram-negative bacteria, is lipopolysaccharide (LPS), a constituent of the outer membrane of all Gram-negative bacteria. Lipid A was identified as the biologically active part of LPS (2, 3). Lipoteichoic acid (LTA) and peptidoglycan (PGN) are important pathogenic compounds of gram-positive bacteria (4, 5, 6). Cellular responses induced by these bacterial toxins are the net result of interaction with various plasma components such as complement, soluble CD14, LPS binding protein (LBP) and membrane receptors such as membrane bound CD14, NOD1 and 2 and Toll-like receptors (TLR) (Figure 1). CD 14, a protein found on the surface of monocytes, macrophages and neutrophils (7), plays a crucial role in the cellular response to LPS, LTA and PGN (8, 9). CD14 is also found as a soluble protein in plasma, and complexes of LPS with soluble CD14 participate in responses of endothelial cells (10), epithelial cells (11) and possibly other cell types that do not express membrane CD14. Although CD14 is a glycosylphosphatidylinositol-anchored protein, which implicates that the receptor lacks an intracellular domain, binding of bacterial toxin to CD14 initiates a response of cells (12, 13). Toll like receptors, a family of transmembrane proteins, were recently identified to cooperate with CD14 and function as signal transducing molecules (14).

FIGURE 1. *Cell activation by bacterial toxins.* LPS derived from Gram-negative bacteria binds to CD14-TLR4-MD2 complexes on the cell membrane of macrophages. LPS binding protein (LBP) monomerizes LPS from micelles and catalyzes this binding. Peptidoglycan (PGN) and Lipoteichoic acid (LTA) derived from Gram-positive bacteria binds to TLR2/TLR6 complexes. Binding of bacterial toxin to these cell-membrane receptors activates NF-κB in macrophages, thereby inducing cytokine transcription and release.

LPS is reported to activate cells via binding of TLR4 (15, 16), which requires the presence of the accessory protein MD2 (17). The toxins of gram-positive bacteria, PGN and LTA, activate cells via TLR2 (18). Findings of Bulut et al suggest that there is a functional interaction between TLR2 and TLR6 in the cellular response to these bacterial compounds (19). Other members of the TLR family are involved in the response to bacterial products as well; TLR9 was found to be responsible for mediating the recognition of bacterial DNA (20) and TLR5 is the Toll-like molecule that recognizes flagellin from both Gram-positive and Gram-negative bacteria (21). Binding of bacterial compounds to TLR activates NF- κ B (18, 22) resulting in enhanced expression of cytokines, chemokines and adhesion molecules (Figure 1).

The acute phase protein, LBP, plays an important modulating role in cell activation by bacterial toxins. Although involvement of LBP in cell activation induced by LTA and PGN remains to be further evaluated, its role in LPS induced cell activation is indisputable (23). Once released from bacteria, LPS forms micelles. LBP is considered to monomerize LPS from micelles and subsequently transport LPS to membrane-bound CD14 on target cells (24) (Figure 1). Thereby LPS induced cell activation is strongly enhanced. Constitutive LBP circulates in low concentrations, whereas circulating LBP concentrations rise dramatically during infection for which the liver was held responsible for a long time. Recently, however, LBP secretion at extra-hepatic sites, amongst which the intestinal epithelium (25) and lung (26), was reported. In contrast to low concentrations of LBP, these acute phase concentrations have been shown to decrease endotoxin activity (27) and to protect against septic shock caused by Gram-negative bacteria (23, 27).

The immune response to microbial pathogens is initiated by recognition of pathogen components by host cells not only at the cell surface, also after phagocytosis a cellular response can be initiated in the cytosol. Recently NOD1 and 2 were identified as intracellular sensors of bacterial products. NOD1 and 2 are nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins. This leucine rich repeated region (LRR) is homologues to that found in TLRs and was demonstrated to be required for LPS-induced NF- κ B activation by NOD 1 and 2 (28).

Moreover, bacteria and microbial toxins directly activate the complement and coagulation system, which both play an important part in the host defensive response. The complement system is a multifactorial protein cascade system essentially involved in the early unspecific immune response. Its major function is the activation of cellular defense mechanisms, opsonisation of foreign particles and destruction of target cells.

Cell activation by bacterial toxins leads to secretion of various inflammatory mediators such as cytokines. The biological effects of bacterial toxins are mainly caused by these cytokines. Tumor necrosis factor- α (TNF- α) is considered to be the major mediator of the pathogenesis of infection (29, 30). Chemokines, cytokines with chemoattractive capacity, are secreted in response to bacterial toxins, as well. Together with an increased expression of adhesion molecules, the presence of these chemokines results in chemotactic recruitment of neutrophils that are professional phagocytes, which take up bacteria and kill them by secretion of bactericidal substances and production of oxidative metabolites.

Hyper- and hypo-inflammation

The initial systemic pro-inflammatory response to pathogenic components derived from invading bacteria is in particular caused by TNF- α , interleukin-1 (IL-1), IL-6, and interferon- γ , which act synergistically and are considered to be 'alarm' cytokines. Besides these mediators generated by activated cells, humoral cascades are activated directly in response to bacterial toxin or in response to cytokines, such as the complement- and coagulation-system that also release pro-inflammatory mediators. Although the release of these mediators is induced to promote survival of infection, an overwhelming response during fulminant sepsis becomes self-destructive and is responsible for induction of vascular leakage, hypotension and hypoperfusion of tissues, tachycardia, tachypnea, disseminated intravascular coagulation and organ dysfunction (Figure 2). The initial inflammation also induces a compensatory anti-inflammatory response that prevents ongoing auto-destructive inflammation. The activation of these endogenous counter-regulatory systems may also predominate and cause the so-called immunoparalysis (Figure 2). This state of immunodeficiency is characterized by monocytic deactivation, marked by depressed HLA-DR antigen expression, the altered capacity of monocytes to induce antigen-specific T cell stimulation and to release various cytokines (31). The multiplication and spread of bacteria and the toxic effects of their metabolites then becomes unanswered, resulting in nosocomial infections that cause late sepsis. There is evidence that inflammation and immunoparalysis do not only occur in sequence to each other but also in parallel early after extensive surgical procedures.

FIGURE 2. *Inflammatory responses during sepsis.* 1. Adequate response; an early proinflammatory response adequately copes with circulating bacteria and endotoxin. The anti-inflammatory response initiated by the initial proinflammatory response prevents auto-destructive-hyperinflammation. 2. Early MOF; the early proinflammatory response evolves into hyperinflammation which causes early MOF and shock 3. Late MOF; immediately after the release of inflammatory mediators an anti-inflammatory response is initiated which paralyses the immune system and results in late MOF.

Lipoproteins, the natural antitoxins

Inactivation of bacterial toxins by lipoproteins

Of the known physiologic mechanisms for decreasing the stimulatory potency of bacterial toxins, the binding and detoxification by lipoproteins is most prominent. Already in the late seventies, high density lipoprotein (HDL) was demonstrated by Ulevitch et al to bind LPS as part of a two step process requiring serum (32, 33). After binding to HDL, the ability of LPS to induce a cytokine response, fever, leucocytosis, and hypotension is strongly impaired (32, 33). Whereas Ulevitch et al focused on the interaction between HDL and LPS, nowadays all major lipoprotein classes have been demonstrated to bind LPS and reduce its activity as reflected by a diminished capacity of endotoxin to induce cellular cytokine production (34, 35, 36). It was recently demonstrated that lipoproteins not only prevent LPS binding to cells, hence also promote the release of cell associated LPS thereby reducing cell activation (37) (Figure 3). This LPS detoxifying capacity of lipoproteins may underlie the in vivo observation that enhanced circulating concentrations of LDL (38, 39), VLDL, chylomicrons and HDL (38) reduce the lethal effect of LPS in mice. Also in human, lipoproteins were demonstrated to be effective in attenuating the response to endotoxin (40). Besides binding of LPS, lipoproteins also bind toxins derived from gram-positive bacteria (41) and inhibit the ability of those toxins to activate macrophages (42, 43). Overall, these findings indicate that binding of bacterial toxins to lipoproteins prevents the initiation of the inflammatory cascade resulting from cell activation.

There is no consensus in the literature concerning the class of lipoproteins most relevant for LPS detoxification in vivo. Although several reports describe that LPS in human serum binds preferentially to LDL and VLDL (34, 44, 45), work from other laboratories has shown that LPS binds to HDL (32, 46, 47, 48). One reason for this discrepancy is that rabbits and rodents were used in the majority of the latter studies. The predominant binding of LPS to HDL in plasma from rabbits and rodents can be explained by the high levels of HDL and high HDL:LDL/VLDL ratio in the circulation of these animals as compared to human. The importance of this ratio in the fate of LPS in the circulation is derived from studies using New Zealand white rabbits in which LPS associates predominantly with HDL, while in Watanabe heritable hyperlipidemic rabbits LPS binds predominantly to LDL (34). The relevance of lipoprotein levels is also observed in human since persons with high HDL levels show higher binding of LPS to HDL as compared to persons with low HDL levels (45). HDL, LDL and VLDL might compete for LPS binding while in human serum the high LDL and VLDL concentrations result in predominant binding to these lipoproteins. However the preferential binding of LPS to HDL in humans is also described (49). These different observations are possibly caused by differences in separation techniques or by using EDTA plasma instead of serum to study interaction of LPS with lipoproteins or by presence of EDTA during the separation of lipoproteins. It has been shown that calcium chelation by EDTA increases binding of LPS to HDL (50, 51).

Lipoprotein constituents involved in LPS-lipoprotein interaction

Since endotoxicity can be prevented by cholesterol-rich as well as triacylglycerol rich lipoproteins, it is most likely that a lipid-lipid interaction is involved in the interaction between LPS and lipoproteins. Levine suggested a simple leaflet insertion model for

binding and neutralization of lipopolysaccharide by phospholipids on the surface of lipoproteins (52). However, also apolipoproteins appear to be importantly involved in LPS binding and possibly also inactivation. We recently have shown LPS binding to apoB in a solid phase assay (44). In accordance, Victorov et al demonstrated the association of LPS with predominantly apoB-100 on LDL (53). Emancipator found that both apoA-I and apoB reduce detection of endotoxin in the *Limulus* amoebocyte lysate assay (54). Also, apoE was found to bind LPS (55). In line with this observation apoE-deficient mice display increased susceptibility to endotoxemia (56). In addition, hypertriglyceridemia induced by Intralipid was not effective in protection from endotoxemia (57) underlining the importance of apolipoproteins.

FIGURE 3. Detoxification of endotoxin by lipoproteins. LPS release from LPS-micelles (A) or cell-membranes (B) is catalyzed by circulating LBP and CD14. LPS is then incorporated into the phospholipid layer of lipoproteins (leaflet-insertion model). Also lipoprotein associated LBP enhances this incorporation. Lipid A, the biologic active part of LPS, is masked by lipoprotein binding and is not capable of cell activation. Subsequent lipoprotein bound LPS is cleared from the circulation.

Plasma proteins involved in LPS-lipoprotein interaction

It was demonstrated that plasma constituents other than lipoproteins or lipoprotein associated agents are required for lipoproteins to achieve maximal prevention of endotoxin-induced death in experimental animals (38), suggesting that a constituent of plasma is required for the binding of endotoxin to lipoproteins. Later studies identified various plasma proteins involved in the interaction of bacterial toxins with lipoproteins. The most essential protein in this respect is LBP. Besides its role in the transfer of monomerized LPS to CD14 on cells as described above, it also catalyzes binding of LPS (58) and LTA (42) to lipoproteins (Figure 3 route A). Subsequent, detoxification of these bacterial toxins by lipoproteins is strongly enhanced (43, 44,

58). Recently, using new techniques, we demonstrated that LBP circulates in association with apoB-containing lipoproteins. LBP associated with these lipoproteins effectively catalyzes LPS binding and detoxification (43, 44). These LBP and apoB-containing lipoproteins were also identified as the predominant LPS transporting lipoproteins under healthy and septic conditions (44). Lamping et al demonstrated in a murine model that high levels of LBP in the circulation, as seen during an acute phase response, inhibit LPS effects and prevent mortality induced by endotoxemia (27). The latter observation strongly supports a physiological role for LBP dependent detoxification of LPS in host defense.

Another plasma protein involved in LPS binding to lipoproteins is sCD14. LBP catalyzed neutralization of LPS by lipoproteins is accelerated more than 30-fold in the presence of sCD14 (59). Both LBP and sCD14, also diminish monocyte responses to LPS by transferring cell-bound LPS to lipoproteins (37) (Figure 3 route B). Besides LBP and sCD14, PLTP (phospholipid transfer protein), which shares 24% sequence similarity with LBP, was demonstrated to mediate the transfer of LPS to lipoproteins (60).

FIGURE 4. Hepatic processing and clearance of endotoxin. 1. LPS binds to membrane receptors on Kupffer cells, resulting in cell activation and subsequent cytokine release. LPS is internalized by Kupffer cells, processed and redistributed to hepatocytes and delivered to the bile and faeces. 2. LPS associates with chylomicrons which enhance the plasma clearance and hepatic uptake of LPS. Chylomicrons shunt LPS to hepatocytes and away from hepatic macrophages. The LDL receptor (LR) and LDL receptor related protein (LRP) 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, and excreted with the faeces.

Clearance of bacterial toxins

Besides detoxification of bacterial toxins, removal of toxins from the blood stream is an essential part of coping with bacterial infection. LPS is cleared from the bloodstream in a biphasic way. There is an initial rapid clearance followed by a phase with slower clearance rate (61). The liver is the main organ responsible for uptake of LPS from the circulation (23, 55, 61). LPS internalized by Kupffer cells is redistributed to parenchymal cells and is delivered to the bile and faeces (61) (Figure 4). The TNF secretion by macrophages in response to LPS released by Kupffer cells is much lower than to native LPS, indicating that LPS is processed by Kupffer cells (62). Organ distribution studies of LPS demonstrate that several extra-hepatic binding sites exist.

FIGURE 5. *Chylomicrons and LBP cooperate in LPS clearance by enterocytes.* In vitro differentiated human colonic adenocarcinoma (Caco-2) cells were used as a model of normal human intestinal epithelial cells. Caco-2 cells were grown on collagen-coated membranes inserted in Transwells. After two weeks, Caco-2 monolayers display functional properties similar to those found in normal enterocytes and they form a polarized monolayer with tight junctions. Therefore, the experiments were conducted 14 days after seeding of the cells. At the start of the experiment, maintenance medium was removed, cells were washed and serum free medium containing 100 µg/ml LPS-FITC with or without chylomicrons (CM) isolated from human serum (10% of circulating levels) and with or without 5 µg/ml purified human LBP, was added at the basolateral side of the monolayers. After 12.5, 16.5 and 20.5 hours small amounts of the apical medium were removed and the fluorescence determined. LPS is transported from the basolateral towards the apical side of Caco-2 monolayers. This process is enhanced by presence of chylomicrons. LBP further enhanced the LPS transport across the monolayers in the presence of chylomicrons.

Recently evidence was found for uptake of circulating endotoxin by the intestinal epithelium followed by secretion of endotoxin into the intestinal lumen (63). These data suggest an important role for the intestine in the clearance of bacterial toxins in addition to the liver.

The metabolic fate of LPS was shown to be strongly affected by complex formation with lipoproteins (64, 65, 66). The association of LPS with HDL decreases its clearance from the circulation (64, 65). In contrast chylomicrons enhance the plasma clearance and hepatic uptake of LPS (Figure 4). Chylomicrons shunt LPS to hepatocytes and away from hepatic macrophages (55, 66). Harris et al recently presented data indicating that both the LDL receptor (LR) and LDL receptor related protein (LRP) participate in the rapid internalization of chylomicron-bound LPS by hepatocytes (67). Consequently, LPS is delivered into the bile, where it is inactivated by bile salts (68). In addition, pilot experiments performed recently in our laboratory demonstrated that the clearance of LPS by intestinal epithelial cells is markedly enhanced by presence of both, chylomicrons and LBP (Figure 5).

Lipoprotein metabolism during infection

Lipoprotein metabolism is dramatically altered during infection, which alterations are mediated by cytokines. In human, circulating levels of total cholesterol, LDL-cholesterol and HDL cholesterol decrease, whereas one of the earliest metabolic responses is an hypertriglyceridemia and concomitant enhanced hepatic secretion of the triacylglycerol rich VLDL (69), an effect caused by cytokines, in particular TNF (70). In contrast, in rodents and rabbits, endotoxemia induces hypercholesterolaemia instead of hypocholesterolemia (71, 72). In addition, in man alterations in apolipoprotein levels are observed (69, 73). ApoA-I concentrations drop and HDL becomes depleted in apoA-I (69, 71). ApoB levels are not affected by either viral or bacterial infection (69). The rise in lipoprotein levels is postulated to reflect an endogenous mechanism to limit activity of bacterial toxin and thus is part of the host defense mechanism against bacterial toxin. The physiologic relevance of high lipoprotein concentrations appears from studies demonstrating that a low cholesterol concentration is associated with increased mortality due to a wide variety of chronic and acute disorders (74). Although the high concentration of circulating lipoproteins apparently has beneficial effects during acute infection, during chronic inflammation the enhanced presence of lipoproteins in the circulation is associated with the initiation of cardiovascular disease. Further enhancement of this efficient defense mechanism, i.e. maximizing lipoprotein production in order to overcome the battle of man against sepsis, might from evolutionary point of view be disastrous, since life threatening cardiovascular disease would then be a major complication of chronic inflammation at an early age.

The intestine: the bacterial barrier that fails

The gastrointestinal tract is considered the "motor" of the sepsis syndrome. Disruption of the gut mucosal barrier between the gastrointestinal content containing high amounts of bacteria and the sterile internal milieu of the host and subsequent bacterial translocation has been identified as an integral part of the development of sepsis and MOF in critically ill, burn, trauma, and surgical patients (75, 76). Several studies support the concept that bacteria translocate from the gut to mesenteric lymph nodes, distant organs and systemic blood (77, 78) and are a potential source for lethal infections. Bacterial translocation is predisposed by conditions that 1) disturb the gastrointestinal microflora leading to bacterial overgrowth (75) such as antibiotic therapy and gastrointestinal stasis, 2) disrupt the physical barrier of the gut mucosa

such as a reduced splanchnic blood flow, parenteral nutrition and starvation (78, 79) or 3) compromise the host immune system such as during the hypo-inflammatory phase of sepsis (Figure 6).

The mechanisms that underlie bacterial translocation are not well understood. Whether bacteria migrate transcellular, paracellular or both, remains unclear. In addition, most of the insights concerning bacterial translocation come from animal studies. Despite extensive evidence from animal studies, the number of publications concerning bacterial translocation involving human subjects is small. Human studies evaluating the role of bacterial translocation in pathogenic mechanisms are therefore urgently needed.

FIGURE 6. *The intestinal barrier to bacteria and bacterial toxin.* The physiologic epithelial barrier of the intestine against bacteria and bacterial toxin consist of a mechanical epithelial barrier layered with mucus, the gut flora, anti-microbial peptides secreted by the epithelium and a cellular immunologic defense. Further, lipoproteins secreted by enterocytes are effective in detoxification of bacterial toxin. Under the extreme conditions of hemorrhagic shock, major trauma, severe burns, and complex surgical interventions, the intestinal barrier often fails. Hypoperfusion of the splanchnic circulation and disuse of the gut (during fasting or TPN) results in atrophy of the intestinal mucosa, enhanced permeability and bacterial translocation. In addition, depletion of oral feeding and antibiotics disturbs the microflora resulting in bacterial overgrowth. The conditions for intestinal lipoprotein secretion are strongly impaired. An initial hyperinflammatory response may result in local immunosuppression.

The self-perpetuating effect of translocated enteric bacteria

The gut is seen as a source of bacteria, toxins and cytokines that initiates, aggravates and perpetuates the systemic inflammatory response in critically ill patients. Circulating bacterial toxins, and most likely inflammatory mediators produced in response to bacterial toxins, are thought to further disturb the integrity of the intestinal epithelium and enhance its permeability (80). This loss of integrity is considered to result in further bacterial translocation (80). In addition, gastrointestinal translocation of bacteria and endotoxin may induce immunosuppression after an initial local or

systemic hyperinflammation. LPS-primed monocytes have a marked decreased HLA-class II antigen expression and were demonstrated to be greatly impaired in their capacity to induce antigen-specific T cell proliferation and cytokine production in vitro (immuno-paralysis) (31, 81). Such an induced immunoparalysis is considered to be an important predisposing factor for further bacterial translocation. This self-perpetuating capability of bacterial toxins, i.e. further destruction of the structure and function of the epithelium and undermining of the immunological defense of the intestinal mucosa, might be essential in the evolution of a simple infection into sepsis and MOF.

The phenomenon of impaired gut barrier function induced by circulating toxins, also suggests a possible role for enteric bacteria in infections initially caused by entrance of bacterial toxins via extra-intestinal sites such as wounds, respiratory airways, drains, arterial/venous catheters and the urinary tract.

Furthermore, PMNs primed in the intestine relocate to other organs where they can cause damage during a 'second hit' such as tissue injury or hemorrhagic shock. This second insult than causes these primed cells to react with an augmented response and cause increased endothelial injury within the target organs (82), in particular in the lungs. This explains the morphological changes observed in early sepsis-induced ARDS, i.e. pulmonary edema, vascular thrombosis and hemorrhages.

Prevention of bacterial invasion by the intestine

The innate immune system of the intestinal mucosa consists of a physical barrier which functions to confine the intestinal microflora within the gut lumen and prevents colonization of the host with enteric bacteria. The first line of intestinal defense is formed by a thick mucus layer covering the epithelium of the intestine that prevents the attachment of microbes and helps to sweep bacteria away by peristalsis. Microbes are trapped in the mucus by sugar chains mimicking epithelial receptors for bacteria. The intestinal epithelium secretes antimicrobial substances in the mucus such as lysozyme (83) and defensins (84) (Figure 6). Furthermore, the presence of phospholipids in the mucus, derived from both the intestinal epithelium and bile, is thought to serve as surfactant preventing attachment of bacteria to the epithelium. Presence of bile in the intestinal lumen was demonstrated to be important in prevention of bacterial translocation (85). The microflora of the gut forms part of the nonspecific defense system of the gut as well, since the secretion of antibacterial substances by these microbes inhibits the growth of pathogenic bacteria (colonization resistance) (86, 87). In addition the microflora induces the intestinal production of bactericidal peptides (88).

Defense against translocated bacteria and toxin

Translocated bacterial toxins attract and activate strategically located macrophages and neutrophils and induce a cytokine response in the intestinal mucosa including production of the pro-inflammatory cytokines IL-1 α (89), IL-8 (90), TNF- α and IL-6 (91, 92). The function of the intestinal mucosa is regulated by epithelial cell-immune cell interactions mediated by these cytokines. Although activated macrophages are thought to be the predominant source for cytokines in the intestine, also endothelial cells, fibroblasts and even the epithelial cells in the intestine are involved in this cytokine response. The intestinal epithelium recognizes and responds to invading

pathogens by secretion of various anti-microbial and pro-inflammatory mediators (93, 94). Recently, CD14 (95), TLR2 and TLR4 (94, 96, 97), the receptors for bacterial toxins, were demonstrated to be present on intestinal epithelial cells. TLR expression by these cells is markedly upregulated under inflammatory conditions (94). Binding of LPS to TLR4 on intestinal epithelial cells activates NF- κ B (96). In addition intestinal epithelial cells were demonstrated to release LBP, which is strongly upregulated by presence of cytokines (25). These data indicate that under non-inflammatory conditions, the low expression of TLR, MD-2 and LBP by intestinal epithelial cells protects against a dysregulated proinflammatory response to bacterial toxins. However, during inflammation, intestinal epithelial cells have all the necessary factors at their disposal to directly respond to bacterial toxin, implying that these cells may play a frontline role in the defense against bacteria and bacterial toxins (Figure 7).

FIGURE 7. *Acute phase response of intestinal epithelial cells.* Receptors for bacterial toxins are upregulated during inflammation on intestinal epithelial cells. Subsequent, these cells are activated by binding of bacterial toxins to membrane receptors; CD14 and TLR's. The secretion of LBP by intestinal cells is enhanced during inflammatory conditions. LBP catalyzes the binding of bacterial toxins to the epithelial cells, and as a result cell activation. The secretion of cytokines, acute phase proteins, defensins and NO is enhanced by cell activation.

Cytokines produced in the intestine exert local effects on the regulation of the mucosal metabolism. The secretion of several anti-microbial agents such as the free radical nitric oxide (NO) (98) and defensins (83) are upregulated in response to cytokines. In addition the presence of cytokines induces the secretion of several acute phase proteins by the intestinal mucosa (25, 99, 100) (Figure 7) that are generally seen as important contributors to host defense.

Pathogenic antigens such as bacterial toxin also induce the activation of the gut-associated lymphoid tissue (GALT) among other things resulting in the luminal secretion of sIgA. sIgA plays an important role in bacterial agglutination thereby preventing attachment and translocation of bacteria (101).

There is strong evidence that the intestine contributes to the lipoprotein induced detoxification and clearance of bacterial toxins. In response to nutrition, enterocytes produce chylomicrons, which bind endotoxins and accelerate their clearance from the

circulation (55, 66). In addition chylomicron bound LPS is not able to elicit a cytokine response in cells (43). As a result, chylomicrons protect against endotoxin induced death and infusion of chylomicrons before or up to 30 min after the administration of endotoxin improved survival in rodents (66, 102). Moreover, LBP enhances the binding and detoxification of bacterial toxin by chylomicrons (43). In this context, the secretion of LBP by the intestinal mucosa is most interesting (25). 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, we hypothesize that this mechanism may be part of a local defense mechanism of the intestine against translocated bacterial toxins (Figure 8). Such a mechanism may occur in the subepithelial space or in the lymphatics and possibly prevents further damage to the intestinal epithelial barrier caused by bacterial toxins and the entrance of biological active toxins into the circulation.

FIGURE 8. *Proposed mechanism of detoxification of bacterial toxin in the gut.* Bacteria translocate across an injured intestinal mucosa. Bacterial toxins released from bacteria induce the secretion of cytokines by intestinal epithelial cells and immunocytes. LBP secretion by the intestinal epithelium is enhanced in response to these cytokines. In response to enteral nutrition, the epithelial cells produce chylomicrons as well. Chylomicrons cooperate with LBP in the detoxification of bacterial toxins. Detoxification may take place in the subepithelial space or lymphatics. Hence, further damage to the intestinal barrier is prevented as well as entrance of biologically active bacterial toxin into the circulation. Moreover clearance of bacterial toxins is enhanced.

Impaired intestinal defense in surgical patients

The fatal effects of bacterial toxins are a rising surgical concern since the incidence of infectious complications, such as sepsis and SIRS, are increasing (103, 104) most probably due to aging of the population and as a result of more complex surgery. Although gram-negative bacteria have long been seen as the principle causative microbes, the incidence of sepsis caused by gram-positive bacteria has risen markedly the last few years (105).

Trauma patients or patients undergoing major surgery have several predisposing factors making them at risk for developing enteric bacteraemia. These patients experience hypotensive periods and subsequent diminished perfusion of the splanchnic circulation resulting in intestinal and liver ischemia/reperfusion injury. The mucosal integrity is also impaired as a result of non- or parenteral feeding, which both cause atrophy of the intestinal mucosa. These injuries lead to a disturbed gut barrier function and are thought to promote the translocation of enteric bacteria and toxins into the portal circulation.

Also various drug treatments, i.e. antibiotics and antacids, disturb the normal ecology of the gut resulting in bacterial overgrowth. A reduction of gastrointestinal peristalsis due to intra-abdominal disease or injury resulting in stasis in the stomach and the intestines, also contributes to disturbance of the normal microbial ecology of the intestinal tract.

In conclusion, in patients undergoing extensive cardiovascular or abdominal surgery, the intestinal defense systems, which protect the host against invasion of bacteria, are severely impaired. Therefore the host depends on mechanisms that detoxify and clear bacterial toxin in an early stage, such as binding of toxin to lipoproteins. Ideally, toxins associate with lipoproteins directly after translocation across the intestinal mucosa by lipoproteins present in the submucosal space or the lymphatics. However, cytokines released in response to these bacterial toxins interact with enterocytes and diminish the secretion of intestinal lipoproteins. In this respect TNF- α and IL-6 were demonstrated to reduce the synthesis of apoB and apoA-I and the secretion of lipids and lipoproteins by intestinal epithelial cells (106, 107, 108). In addition, TNF decreases the enteral absorption of fat (109). An additional effect of these circulating cytokines is anorexia (110). Moreover, critically ill patients admitted to an intensive care unit are in general initially starved or receive total parenteral nutrition (TPN). Hence, the main trigger for intestinal lipoprotein production, i.e. the presence of intraluminal nutrients, is strongly impaired during disease. This lack of enteral nutrition during critical illness also causes a profound reduction of bile flow and biliary phospholipid concentrations (111), which most probably further impedes intestinal lipoprotein secretion since presence of phospholipids in the intestinal lumen is required for secretion of lipoproteins by enterocytes (112, 113, 114). Overall, during the acute phase response the conditions for intestinal lipoprotein secretion are poor, hence the capacity of the intestine to contribute to detoxification and clearance of bacterial toxins by means of lipoprotein secretion is limited. Improvement of the conditions required for intestinal lipoprotein secretion might interrupt this vicious circle and should be target of new strategies.

Modulation of host defense against bacterial toxins

Interception of the inflammatory cascade

There are distinct difficulties in the development of therapies for the prevention and management of sepsis. First of all an important point of dispute in the development of therapeutic strategies for sepsis is to either stimulate the immune system in order to enhance the defense against bacteria and toxin or to downregulate the response to prevent or reduce the self-destructive effects of a pro-inflammatory 'overshoot'. Stimulation of the immune system may be useful during the anti-inflammatory

response, for instance by activation of granulocytes and monocytes by IFN-gamma and colony stimulating factors, which indeed were demonstrated to have potential as adjunctive therapy in the treatment of infectious diseases (115). In contrast, during the hyperinflammatory phase anti-inflammatory therapy theoretically would be more effective. However, clinical trials with antagonists of Il-1 (116) and antibodies against TNF (117) have proved to be disappointing. The latter observation is in line with that of Echtenacher et al who demonstrated that TNF is required for survival of experimental septic peritonitis in a mouse model of cecal ligation and puncture (118). They hypothesize that during the early phase of peritonitis endogenous TNF may stimulate nonlymphoid cells such as granulocytes, macrophages, platelets, and fibroblasts to destruct bacteria and to localize inflammation. Most importantly, the current immunologic analyses are deficient in monitoring the biphasic pattern of hyper- and hypo-inflammation during the course of sepsis. Such analyses are a prerequisite to decide which immuno-therapy to apply, as both hyper and hypo-inflammation are major determinants of an adverse clinical outcome.

Besides the lack of information concerning the immunologic stage of these patients, a major bottleneck concerning interventions is that once the sepsis cascade is initiated the diversity and complexity of the chain of events is enormous, including activation of the cytokine cascade, the complement cascade and the coagulation cascade. The inflammatory mediators produced form a network of interacting signals with overlapping activity. Attenuation of these complex effects by one therapeutic strategy once the acute phase response is initiated obviously is problematic.

Considering the apparent insuperable difficulties of interrupting the inflammatory cascade once it is started, prevention of bacterial translocation and detoxification of bacterial compounds responsible for the induction of an inflammatory response would hypothetically be more effective in preventing sepsis in the earliest stage.

Prevention of bacterial invasion

In the past, strategies to prevent invasion of the host with bacteria and their toxic metabolites were investigated. The intestine has been an important target of these strategies. The absence of nutrients in the intestinal lumen, as occurs during starvation, malnutrition, or TPN, is associated with deleterious consequences to the intestinal-barrier function and, although not proven yet, is thought to predispose to bacterial translocation. Meta analysis have demonstrated that in a large variety of patients, including patients with acute pancreatitis, ulcerative colitis, Crohn's disease, multi-organ failure, and after major surgery and trauma, enteral feeding was associated with a lower risk of infection compared to starvation and parenteral nutrition (119). Early post-operative enteral feeding was found to reduce septic morbidity (120, 121) and hospital stay (122). Several mechanisms are proposed for this beneficial role of enteral feeding. First of all, early postoperative enteral nutrition was demonstrated to improve the maintenance of intestinal integrity as manifested by a reduced permeability (121). Certain nutrients in particular are thought to ameliorate the mucosal architecture by exertion of a trophic effect on the intestinal mucosa, such as glutamine (123), short-chain fatty acids (124) and fiber supplementation (125). However, there is no strong evidence in the literature that supports the association of the ameliorated intestinal permeability with bacterial translocation. Second, evidence was found for an immune

enhancing function of enteral feeding (126) and specific nutrients such as arginine or RNA supplementation (127). Furthermore, enteral nutrition stimulates the enteric nervous system, which enhances GALT and MALT function. In addition, since we demonstrated that intestinal lipoproteins are efficient in detoxifying bacterial toxins (43) we hypothesize that intestinal lipoprotein secretion induced by enteral nutrition may reduce damage to the intestinal epithelium caused by bacterial toxin and thereby prevent further bacterial translocation. There are a number of other factors that might be involved in the beneficial effects of enteral feeding such as alterations in splanchnic bloodflow and gastrointestinal microflora, and stimulation of gastrointestinal protein production, peristalsis, mucus and acid secretion. Since the microflora of the gut is an important factor in the prevention of bacterial translocation, several studies focused on alteration of this microflora by dietary fibers and non-digestible oligosaccharides (pre-biotics), by live micro-organisms (pro-biotics), and by selective gut decontamination using antibiotics.

Prevention of immune activation

In an attempt to intercept active toxins before interacting with responsive cells, antibodies directed against the LPS molecule have been developed, such as human antiserum to heat killed *Escherichia coli* J5 and murine (E5) and humanized (HA1A) antibodies directed against the lipid A component of endotoxin. Although initial results were encouraging in patients with gram-negative sepsis, larger clinical trials showed no benefit (128, 129). Other toxin antagonists were investigated for their clinical relevance in the treatment of sepsis. Polymyxin, the polycationic antibiotic most frequently added to neutralize effects of LPS in vitro, is of limited clinical use due to its high toxicity. Bacterial permeability-increasing protein (BPI) is a constituent of PMN primary granules, which is cytotoxic to gram-negative bacteria, binds endotoxin and inhibits LPS induced cytokine release in vitro. Although, BPI was described not to be effective in baboons (130), others found that administration of rBPI resulted in improved clinical outcome in patients undergoing major liver resection (131). In addition, the results of a recent study using rBPI as adjunctive treatment for children with severe meningococcal sepsis indicate that BPI is beneficial in decreasing complications of meningococcal disease (132). Naturally occurring cationic peptides, as well as synthetic analogues block the ability of LPS to stimulate cytokine secretion by immune reactive cells in vitro (133). Clinical relevance of such peptides still has to be proven in clinical trials. Another strategy studied in detail is the use of glucocorticoids during endotoxemia and bacteremia. Although in animal models large doses of corticoids improved survival (134), in human administration of high doses of glucocorticoids at the onset of sepsis was proven not successful (135). More recently, however, glucocorticoids in lower doses were shown to have a beneficial effect in patients with septic shock (136). Recently a snowball effect in the progress of understanding the mechanisms underlying cell activation was made after recognition of the TLRs as signal transducers of bacterial toxins. This breakthrough raised hope for development of therapeutic interventions based on blocking the binding of toxin to its receptor or inhibiting receptor function. Although further research is required, unfortunately, these new insights in immune activation did as yet not result in new effective therapeutic

strategies in the treatment of sepsis since inactivation of TLRs appeared to sensitize the host for specific bacterial infections (137, 138).

Since lipoproteins bind and inactivate endotoxin and have been shown to increase survival of endotoxemia, several investigators increased circulating lipoprotein concentrations artificially by infusion of exogenous lipoproteins in animal models of infection (38, 66, 102). Modest increases in circulating chylomicron concentrations profoundly enhanced resistance to endotoxemia *in vivo* (38, 66). In contrast to studies using exogenous lipoproteins, Harris et al recently demonstrated that endogenous lipoproteins, produced in response to feeding, reduce the toxic effects of bacterial toxins in human (40). In line with these findings we recently demonstrated that elevation of endogenous lipoproteins by administration of a high-fat enteral nutrition just before and after haemorrhage, reduces endotoxemia and bacterial translocation in a rat model (139). Others found that intravenous infusion of intralipid did not influence inflammatory responses to endotoxin (57), which emphasizes the importance of the structure and composition of endogenous lipoproteins and of apolipoproteins in the detoxification of bacterial toxins. In conclusion these studies suggest that induction of endogenous lipoprotein secretion may have an important potential as a therapy that anticipates on bacterial translocation and infection in case of extensive surgery and for instance in premature neonates. Besides prevention of the initiation of the inflammatory cascade, lipoprotein enhancing strategies may have a role as an adjuvant therapy after the onset of sepsis. Although it is unlikely that once circulating bacterial toxins have initiated the inflammatory cascade lipoproteins will reverse all the effects of this cascade, it was demonstrated that lipoproteins are able to detach LPS from macrophages and thereby prevent cytokine induction. Most interestingly *in vivo* infusion of chylomicrons up to 30 min after the administration of endotoxin improved survival in rodents (66, 102). Moreover, antibiotics given during sepsis, result in the release of enormous amounts of bacterial toxin, often causing an initial deterioration of the clinical condition. Enhancement of endogenous lipoprotein secretion might be effective in diminishing the excessive immune response to the release of those bacterial toxins.

In search for further improvement of the intestinal defense against bacteria we investigated conditions to raise the endogenous intestinal lipoprotein secretion and found evidence for an upregulation induced by oral administration of phosphatidylcholine under normal as well as under acute phase conditions (140). These results are in agreement with earlier observations demonstrating that luminal presence of dietary fat and biliary phospholipids are a prerequisite for intestinal lipoprotein secretion (112, 113, 114). Early post-operative feeding with phosphatidylcholine or phosphatidylcholine enriched enteral nutrition in patients at risk for translocation of enteric bacteria may be of clinical relevance. Enhancement of intestinal lipoprotein secretion by these feeding strategies hypothetically reinforces a biological defense mechanism of the intestine against bacterial toxins by enhanced neutralization and clearance of circulating bacterial toxins and possibly, even more important, by direct neutralization of bacterial toxins translocated across a compromised intestinal mucosa. Harris et al found that preincubation of LPS with postprandial blood was required for attenuation of the immune response by the endogenous lipoproteins secreted in response to feeding (40). We hypothesize that the endogenous lipoproteins secreted by

the intestine have the opportunity to interact and neutralize bacterial toxins already in the intestinal submucosa and lymphatics, hence before active toxins reach the blood stream (Figure 8).

Discussion

Derangements in gut barrier function are in general considered to play a key role in the etiology of sepsis and MOF. However, there is still no hard evidence for the relevance of bacterial translocation across an injured intestinal barrier in the development of sepsis in human. For development of new interventions that prevent invasion of the host with bacteria, it is important to further define the pathophysiology of bacterial translocation.

Significant advances have been made in unraveling defense mechanisms of the intestine against bacteria and their toxic metabolites. This new knowledge will form the foundation of new interventions. We anticipate that future studies will further optimize therapeutic concepts that maintain gut barrier function, for instance by reducing intestinal hypoperfusion and the effects of the subsequent ischaemia-reperfusion damage, by prevention of cell apoptosis and enhanced mucosal permeability, by reducing the harmful effects of pro-inflammatory mediators, by stimulating the secretion of anti-microbial substances, and by controlling the bacterial flora of the gut. Until it is possible to prevent entrance of bacteria into the host, therapies supporting optimal host-microbial homeostasis are needed. Since extensive attempts to modify the inflammatory cascade after its onset has proven to be disappointing, attempts to prevent the initiation of this cascade might be a good alternative. The success of lipoprotein enhancement to overcome endotoxemia in animal studies appears to be promising in the development of adjuvant therapies for the treatment of sepsis. Clinical trials should be initiated to verify that also in human enhanced circulating concentrations of lipoproteins alter the outcome of sepsis. We hypothesize that studying the effect of interrupting cholesterol lowering therapies in patients suffering from cardiovascular disease in the period before cardiovascular or abdominal surgery will in part answer this question. In addition, enhancement of endogenous intestinal lipoprotein production by enteral feeding of fat, in particular phospholipids, thereby reducing the toxic effects of microbial components direct after entering the host, might be an interesting focus for targeting the host response.

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Outline of the thesis

The cellular effects induced by bacterial toxins are modulated significantly by interactions with LPS Binding Protein (LBP) and lipoproteins. Prior studies described beneficial effects of LBP as well as lipoproteins on survival of infection. We hypothesized that local presence of LBP and lipoproteins in the intestine might be involved in the barrier function of this organ, which is continuously exposed to high concentrations of bacteria and bacterial toxin.

The main object of the experimental studies described in the first part of this thesis (Chapter 2 and 3) was to explore the local production and secretion of LBP by intestinal epithelial cells. The effects of proinflammatory cytokines on intestinal LBP production was evaluated as well as the polarization of LBP secretion by the intestinal epithelium. For this purpose both an *in vitro* model with human intestinal epithelial cell lines and an animal model of endotoxemia was used.

Earlier studies found evidence for an association of LBP with lipoproteins in plasma from healthy persons. We considered that the physical association of LBP with lipoproteins is of importance for the cooperation of LBP and lipoproteins in the detoxification of bacterial toxins. Tools were developed to further elucidate the interaction of LBP with lipoproteins and to study the effect of this interaction on binding and detoxification of bacterial toxins. By separation of lipoprotein classes under the mild conditions of agarose electrophoresis followed by westernblot analysis of LBP and apoproteins, we studied the association of LBP as well as LPS with lipoproteins in serum of healthy persons and septic patients. Besides the evaluation of the interaction of LBP and LPS with liver derived lipoproteins, we focussed on LBP association with intestine-derived lipoproteins, i.e. chylomicrons, and on the effect of this association on binding and detoxification of bacterial toxins.

The results of these studies indicated that LBP is produced by the intestinal epithelium and cooperates efficiently with chylomicrons in the deactivation of bacterial toxins. In addition, there is strong evidence that the conditions for secretion of chylomicrons are poor during infection, which hypothetically impairs this defense mechanism. These considerations have raised our interest in the development of an enteral formula that optimally induces endogenous intestinal lipoprotein secretion. Phospholipids were earlier found to be essential for secretion of intestinal lipoproteins and were demonstrated to be deficient in the intestinal lumen during critical illness. Therefore the effect of phosphatidylcholine on the production of intestinal lipoproteins was studied under quiescent and inflammatory conditions. In addition we studied the effect of a high-fat enteral diet on endogenous lipoprotein production, endotoxemia and bacterial translocation in a rat model of non-lethal hemorrhagic shock. In this model a compromised gut function is thought to result in bacterial translocation and subsequent infectious complications.

With the studies described in this thesis we aimed to gain more insight in the natural defense of the gut against bacterial toxin. Based on the insights derived from these fundamental studies we found a new target for therapeutic intervention aimed at prevention of the deleterious effects of bacterial toxin in the earliest stage of the development of sepsis, under conditions in which the natural defense of the intestine is compromised.

Chapter 2

Lipopolysaccharide Binding Protein and Serum Amyloid A secretion by human intestinal epithelial cells during the acute phase response

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Abstract

The acute phase proteins LPS Binding Protein (LBP) and serum amyloid A (SAA) are produced by the liver and present in the circulation. Both proteins have been shown to participate in the immune response to endotoxins. The intestinal mucosa forms a large surface that is continuously exposed to these microbial products. By secretion of antimicrobial and immunomodulating agents, the intestinal epithelium contributes to the defense against bacteria and their products. The aim of this study was to explore the influence of the inflammatory mediators TNF- α , IL-6 and IL-1 β on the release of LBP and SAA by intestinal epithelial cells (IEC). In addition, the induction of LBP and SAA release by cell lines of intestinal epithelial cells and hepatic cells was compared. The data obtained show that in addition to liver cells, IEC also expressed LBP mRNA and released bioactive LBP and SAA upon stimulation. Regulation of LBP and SAA release by IEC and hepatocytes was typical for class 1 acute phase proteins, although differences in regulation between the cell types were observed. Endotoxin did not induce LBP and SAA release. Glucocorticoids were demonstrated to strongly enhance the cytokine-induced release of LBP and SAA by IEC, corresponding to hepatocytes. The data of this study, which imply that human IEC can produce LBP and SAA, suggest a role for these proteins in the local defense mechanism of the gut to endotoxin. Furthermore the results demonstrate that tissues other than the liver are involved in the acute phase response.

Introduction

Lipopolysaccharide binding protein (LBP) is an acute phase protein that strongly modulates the response to endotoxins, which are present in the outer membrane of Gram-negative bacteria. LBP enhances the recognition of endotoxin and bacterial surfaces by the immune system (1). Other studies demonstrated that an important function of LBP is to mediate the neutralization of endotoxin by lipoproteins (2, 3). Furthermore, it was demonstrated that high concentrations of LBP decrease endotoxin activity and protect against septic shock caused by Gram-negative bacteria (4).

The intestinal lumen contains high amounts of endotoxin, and the intestinal mucosa forms the interface between this potentially harmful material and the interior of the host. The intestinal epithelium contributes to the immunologic defense by secretion of proinflammatory cytokines (5) and microbicidal agents such as defensins (6). Although the liver has been held responsible for the production of circulating acute phase proteins, there is recent evidence for the synthesis of acute phase proteins in the intestinal mucosa as well (7, 8). This prompted us to study whether the release of LBP and the major acute phase protein serum amyloid A (SAA), which is involved in the routing and binding of high density lipoprotein (HDL) to inflammatory cells (9), is a part of the epithelial response to inflammation. In the current study we examined the regulation of LBP and SAA secretion by a number of intestinal epithelial cell lines in detail and compared it to the regulation in hepatocytes. The data obtained show that intestinal epithelial cells can release both LBP and SAA, which strongly implicates a role for these proteins in the local inflammatory processes in the gut.

Materials and methods

Reagents and Abs.

Human recombinant TNF- α was kindly provided by BASF/Knoll (Ludwigshafen, Germany); human recombinant IL-6 was provided by Prof. W. Sebald (Physiologisch-Chemisches Institut de Universität Würzburg, Germany); human rIL-1 β was a gift from Dr. S. Gillis (Immunex, Seattle, WA); dexamethasone was a gift from Merck Sharp & Dohme (Haarlem, The Netherlands); MTT and LPS (from *Escherichia coli*, serotype 055:B5) were purchased from Sigma (St. Louis, MO). Human recombinant LBP was produced by transfected Chinese Hamster Ovary (CHO) cells, kindly provided by Dr. P. Tobias (Research Institute of Scripps Clinic, La Jolla, CA). Polyclonal Abs to human rLBP were obtained by immunizing rabbits with human rLBP. After protein A purification, IgG was biotinylated. A specific anti-human LBP mAb HM14 was obtained by immunizing mice with human rLBP following classical procedures. The selection and properties of the mAb will be discussed in detail elsewhere. In short, this mAb was selected on basis of its reactivity with both free human LBP and human LBP that has formed a complex with LPS. The SAA immunoassay was kindly provided by Dr. P.C. Limburg (Department of Rheumatology, University Groningen, the Netherlands).

Culture and stimulation of cells

The cell lines Caco-2, Colo-205 and T-84 cells were obtained from the American Type Culture Collection (Manassas, VA; ATCC HTB-37, CCL-222, CCL-248 respectively). Human colonic adenocarcinoma Caco-2 cells undergo in vitro differentiation and

express structural characteristics and functional properties typical of small intestinal enterocytes. The Colo-205 and T-84 cell line are human colonic carcinoma cell lines. The small intestinal epithelial cell line Int-407 was obtained from the European Collection of Cell Cultures (Wiltshire, UK, ECACC 85051004). Human hepatoma (HuH-7) cells were a generous gift of Dr. K.C.H. Fearon, department of Surgery, University of Edinburgh (Edinburgh, UK). Cells were cultured at 37°C with 5% CO₂. HuH-7 and Colo-205 cells were cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Hyclone, Logan, UT). Caco-2 cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS (Hyclone), 1.0 mM sodium pyruvate (Life Technologies) and 0.1 mM non-essential amino acids (Life Technologies). Int-407 cells were cultured in MEM with Earle's salts (Life Technologies) supplemented with 10% fetal calf serum (Hyclone) and 0.1 mM non-essential amino acids (Life Technologies). All culture media were supplemented with 100 IU penicillin/ml, 100 µg streptomycin/ml (Life Technologies). Cell lines were maintained in 25 cm² flasks (Costar, Cambridge, MA). Cultures were split when they reached 80% confluence. For experiments, designed to investigate the effect of cytokines on the release of LBP and SAA, cells were plated at a density of 1×10^4 cells/well on 96-well flat bottom tissue culture plates (Costar). Cells were cultured in complete medium as described above. The medium was changed 48 h after plating and then every other day. After 2 wk, Caco-2 monolayers display morphologic and functional properties similar to those found in normal small intestinal enterocytes (10). Therefore, all experiments with Caco-2 cells were conducted between 14-17 days after seeding of the cells. Experiments with Colo-205, T-84, Int-407 and HuH-7 cells were conducted after confluence was established microscopically. At the start of each experiment, culture medium was removed and complete medium, supplemented with IL-1 β , IL-6, TNF- α , LPS or dexamethasone, as single agents or in combination as given in *Results*, was added. Stimulation with LPS was performed in the presence of human serum. At the end of the incubation period, the medium was removed and centrifuged to remove cells and cell debris. Cell number and viability were determined using the MTT-method as described previously (11). In order to determine whether the presence of serum affected the cytokine induced LBP and SAA release, Caco-2 cells were stimulated under serum-free conditions in a protocol similar to stimulations conducted in the presence of serum.

For experiments, designed to determine whether endothelial cells also possess the capacity to release LBP and SAA, HUVEC were isolated from fresh umbilical cords by treatment with collagenase type I (Sigma). Cells were seeded into fibronectin-coated tissue culture flasks. For stimulation experiments cells were seeded in 96-well plates at a density of 1×10^4 cells/well and were cultured for 2-3 days to confluence. Cells were stimulated in a protocol similar to IEC and hepatocytes.

Detection of LBP mRNA

Total cellular RNA was isolated from quiescent and stimulated Caco-2 and HuH-7 cells using a Quick Prep Total RNA Extraction Kit (Pharmacia, Uppsala, Sweden). cDNA was obtained by reverse transcription of total RNA. PCR of β 2-microglobulin was employed for standardization of the different RNA samples as described by Kloppenburg et al. (12). Human LBP-targeted PCR was performed in a 25-µl reaction

mixture containing 100 μ M of each dNTP, 200 nM primers and 0.5 U of Taq DNA polymerase (Perkin Elmer/Cetus, Emeryville, CA). The primers were described by Su et al. (7): sense primer 5'-AGG-GCC-TGA-GTC-TCA-GCA-TCT-3'; and antisense primer 5'-CAG-GCT-GGC-CGT-GTT-GAA-GAC-3'. Thirty-five cycles were run under the following conditions: 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min. The reaction product was analyzed on a 1.2 % agarose gel containing ethidium bromide. A mock PCR (without cDNA) was included to exclude contamination.

Immunoassays.

LBP and SAA release was evaluated using sandwich ELISA's. LBP was determined as described previously (13). In short, plates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) were coated with polyclonal anti-human LBP Abs. Supernatants of the cells and a standard dilution series of rLBP were added to the plate. Detection was performed with a biotinylated polyclonal rabbit anti-human LBP IgG, followed by peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The detection limit was 200 pg/ml.

SAA was quantified as described elsewhere (14). In short, SAA specific mAb Reu 86.5 was used as capture reagent. The amount of SAA bound to the wells was quantified by incubation with a horseradish peroxidase labeled monoclonal anti-SAA Ab (Reu 86.1) followed by TMB substrate. The detection limit for the SAA assay was 100 pg /ml.

Characterization of LBP released by Caco-2 cells

To confirm the identity of LBP released by Caco-2 cells, the protein was isolated from the medium by selective-affinity immunosorption, followed by determination of the molecular weight by Western immunoblotting. For this purpose the newly developed anti-LBP mAb HM14 was cross-linked to NHS-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Conditioned culture medium of Caco-2 cells was applied to the anti-LBP column, and the unbound proteins were washed out with PBS. Glycine-HCL buffer (pH 2.5) was used to elute bound LBP. For comparison, rLBP was isolated from the culture medium of transfected CHO-cells by the same method. Gel electrophoresis of Caco-2 derived LBP, rLBP and a prestained low molecular weight marker was performed using 10-15% gradient polyacrylamide gels (Pharmacia) run in a Pharmacia Phast system followed by electrophoretic transfer onto a immobilon-P membrane (Millipore Corporation, Bedford, MA). LBP bands were detected using a biotin labeled polyclonal antibody to human LBP followed by peroxidase labeled streptavidin and 3,3' diamobenzidine substrate.

The bioactivity of LBP produced by Caco-2 cells was determined by measuring the LPS-binding-capacity. For this purpose Immuno-Maxisorp plates were coated with the monoclonal anti-human LBP Ab HM14. Next, dilution series of LBP produced by Caco-2 cells and rLBP were added, followed by biotinylated LPS. For biotinylation of LPS a hydrazide-biotin reagent (Pierce, Rockford, IL) was used. Detection occurred with peroxidase-conjugated streptavidin and TMB substrate. The ODs obtained represent the LPS binding capacity of the LBP.

Statistical analysis

Data were analyzed using SPSS software (SPSS, Chicago, IL). All values in the figures and text are expressed as mean $\pm$ SD of n observations. Groups were compared by Student's t test. Results of experiments designed to study the effects of cytokines and dexamethasone on the secretion of LBP and SAA by Caco-2 and HuH-7 cells were hierarchically analyzed by four-way-ANOVA, using dummy regression analysis after square root transformation of data. A p value of 0.05 or less was considered statistically significant.

FIGURE 1. Human LBP targeted RT-PCR amplification of RNA from Caco-2 and HuH-7 cells. Cells were stimulated for 24 h with a mix of IL-1 β (5 ng/ml), IL-6 (100 ng/ml) and TNF- α (100 ng/ml). Controls consisted of unstimulated cells. PCR for β -2-microglobulin (β -2M) was employed for standardization of the samples. Lane A, Unstimulated HuH-7 cells; Lane B, HuH-7 cells after stimulation; Lane C, Unstimulated Caco-2 cells; Lane D, Caco-2 cells after stimulation; Lane E, blank

Results

Cytokines induce the synthesis of LBP and SAA by Caco-2 cells

First we investigated whether IEC express LBP mRNA. In vitro differentiated human colonic adenocarcinoma (Caco-2) cells were used as a model of normal human intestinal epithelial cells. The monolayers were stimulated with a combination of IL-1 β (5 ng/ml), IL-6 (100 ng/ml) and TNF- α (100 ng/ml). For comparison, human hepatoma (HuH-7) cells were treated in a protocol similar to that used for Caco-2 cells. LBP mRNA was expressed in quiescent Caco-2 and HuH-7 cells in minor quantities. Subsequently, after culture of cells in the presence of cytokines LBP mRNA expression was up-regulated substantially (Fig.1). The PCR product was of predicted size (565 bp). These data are the first to demonstrate that intestinal epithelial cells express LBP mRNA, which strongly suggests synthesis of LBP by these cells.

Next, the induction pattern of LBP release by Caco-2 cells was studied. After incubation of the cells with cytokines, supernatants were harvested and analyzed. In the absence of cytokines low amounts of LBP were detectable in the supernatants. A significant ($p < 0.001$) up-regulation of LBP release was induced by IL-6 (Fig.2A). IL-1 β and TNF- α alone did not induce a marked up-regulation of the LBP release, although both cytokines strongly enhanced the effect of IL-6 ($p < 0.01$) and each other ($p < 0.001$) on LBP release. The strongest up-regulation was induced by the combination of IL-1 β , IL-6 and TNF- α ($p < 0.01$; Fig.2A).

The capability of Caco-2 cells to express SAA mRNA was demonstrated by others (15). We investigated the induction pattern of SAA release by Caco-2 cells (Fig.2B). The SAA release of the cells was markedly up-regulated by IL-1 β ($p < 0.001$), while treatment with either IL-6 or TNF- α had no significant effect (Fig. 2B). However, IL-6 ($p < 0.01$) enhanced the IL-1 β induced SAA synthesis, while TNF further up-regulated the effect of the combination of IL-1 β and IL-6 ($p < 0.001$). The necessity of IL-1 β for

apparent induction of SAA release by Caco-2 cells was evident and is typical for class 1 acute phase proteins. In conclusion, Caco-2 cells have the capability to release both LBP and SAA in response to cytokines, while each protein has its own specific induction pattern.

In order to investigate whether the secretion of LBP and SAA is a general property of in vitro cultured cells, HUVEC were stimulated with proinflammatory cytokines. LBP and SAA was not detected in the supernatants (data not shown), which implicates that LBP and SAA secretion is not a common feature of cells in general or epithelial cells in particular.

FIGURE 2. Cytokines induce LBP (A) and SAA (B) release by Caco-2 cells. Cells were incubated with IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) or combinations of these agents as indicated. Controls consisted of unstimulated cells. After 72 h incubation, supernatants were collected and LBP and SAA release were determined by ELISA. The data of a representative experiment are shown and expressed as mean $\pm$ SD (n=6). * Significant effect of individual cytokines ($p < 0.01$); ** significant effect of individual cytokines ($p < 0.001$); † significant positive interaction between cytokines ($p < 0.01$); †† significant positive interaction between cytokines ($p < 0.001$).

Next, the concentration-dependency of IL-6 and IL-1 β on the induction of LBP and SAA release was studied (Fig. 3). A clear up-regulation of LBP release is mediated in Caco-2 cells by 100 ng/ml IL-6. However, when cells were treated with IL-6 plus IL-1 β simultaneously, the enhancement of LBP release was evident at concentrations as low as 1 ng/ml IL-6 (Fig. 3A). The data demonstrate that 0.5 ng/ml IL-1 β is enough to enhance SAA release. The synergistic effect of IL-6 on IL-1 β mediated SAA release was clear at 5 ng/ml (Fig. 3B). Furthermore, synthesis of both proteins increased when concentrations of IL-1 β and IL-6 were raised. The data of this experiment indicate that cytokine concentrations that has been previously reported in actively inflamed intestinal mucosa (16, 17) as well as in the mesenteric blood (18) induce LBP and SAA secretion by IEC.

The data obtained in the stimulation experiments were not caused by an effect of cytokines on cell numbers as determined by the MTT-method (data not shown). In addition, in absence of serum, identical induction patterns of LBP and SAA release were observed, although total amounts of LBP and SAA released were lower in serum deprived conditions (data not shown).

FIGURE 3. Cytokine induced enhancement of LBP and SAA release by Caco-2 cells is concentration dependent. Caco-2 cells were incubated with increasing concentrations of IL-1 β (0 - 5 ng/ml) or IL-6 (0 - 100 ng/ml), or a combination of both. Controls consisted of unstimulated cells. Supernatants were collected after 72 h of incubation, and LBP and SAA release were determined by ELISA. The data from a representative experiment are shown and expressed as the increase of LBP released (ng/ml) compared to controls.

FIGURE 4. Time course of LBP and SAA release by Caco-2 cells after treatment with a combination of IL-1 β (5 ng/ml), IL-6 (100 ng/ml) TNF- α (100 ng/ml) and dexamethasone (1 μ M). Supernatants (n=6) were collected after 24, 48, 72 and 96 h incubation, and LBP and SAA release was determined by ELISA.

Time kinetics of LBP and SAA release by Caco-2 cells

Fig. 4 shows the time kinetics of LBP and SAA release. After an incubation period of 24 h LBP and SAA concentrations in the supernatants were modest. A substantial increase was seen after 48 h, and at 72 and 96 h LBP and SAA release was further increased. These data are in agreement with the kinetics of LBP and SAA concentrations in the circulation during an acute phase response in man (19, 20).

LBP and SAA release by Caco-2 cells is not induced by endotoxin

The gastrointestinal tract contains high amounts of endotoxins that are important inducers of the acute phase response in vivo. To determine whether endotoxins can directly induce the release of LBP and SAA by IEC, endotoxin derived from *Escherichia coli* was added to Caco-2 cells cultured in the presence of human serum containing human LBP and soluble CD14, both necessary for non-immune cells to respond to endotoxins. Concentrations up to 10 $\mu\text{g/ml}$ endotoxin did not lead to enhanced release of LBP or SAA by Caco-2 cells (data not shown).

To elucidate whether endotoxins are able to enhance the cytokine-induced release of LBP and SAA, Caco-2 cells were incubated with a combination of different concentrations of endotoxin, IL-1 β and IL-6. For all concentrations tested endotoxin did not affect the LBP and SAA release induced by IL-6 or IL-1 β (data not shown). In conclusion these data indicate that LBP and SAA release by IEC is not influenced by endotoxins.

FIGURE 5. Cytokines induce LBP (A) and SAA (B) release by HuH-7 cells. Cells were incubated with IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml), or combinations of these agents as indicated. Controls consisted of unstimulated cells. After 72-h incubation, supernatants were collected and LBP and SAA release was determined by ELISA. The data from a representative experiment are shown and expressed as the mean $\pm$ SD ($n=6$). * Significant effect of individual cytokines ($p < 0.001$); † significant negative interaction between cytokines ($p < 0.01$); †† significant negative interaction between cytokines ($p < 0.001$)

Regulation of LBP and SAA release by Caco-2 cells is different from HuH-7 cells

Acute phase proteins, such as LBP and SAA, are thought to be predominant liver

derived. Therefore, the induction of LBP and SAA release in liver cells was investigated for comparison. To this end, HuH-7 cells were stimulated in a protocol similar to that used for Caco-2 cells (Fig. 5). These experiments revealed that with HuH-7 cells, as with Caco-2 cells, LBP release is induced by IL-6 ($p < 0.001$), whereas SAA release is induced by IL-1 β ($p < 0.001$). Despite this conformity, several differences in the induction pattern of both proteins were notable between both cell types. The data demonstrate that IL-1 β (5 ng/ml) significantly ($p < 0.001$) up-regulates the LBP release by HuH-7 cells, whereas no effect on LBP release was observed in Caco-2 cells. Furthermore, TNF- α potentiated the LBP and SAA synthesis induced by the combination of IL-6 and IL-1 β in Caco-2 cells ($p < 0.01$), whereas a down regulation of this induction was observed in HuH-7 cells ($p < 0.05$; Fig. 5). In addition, the amount of SAA released by Caco-2 cells exceeded the LBP release by these cells, whereas the opposite was found in HuH-7 cells. As in Caco-2 cells, endotoxin did not evoke a significant LBP and SAA release by HuH-7 cells (data not shown).

FIGURE 6. Dexamethasone enhances cytokine-induced LBP (A) and SAA (B) release by Caco-2 cells. Cells were stimulated for 72 h with IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) or combinations of these agents in the presence (open bars) or the absence (filled bars) of 1 μ M dexamethasone. Controls consisted of unstimulated cells. After 72 h incubation, supernatants were collected and LBP and SAA release was determined by ELISA. The data from a representative experiment are shown and expressed as mean $\pm$ SD ($n=6$). * Significant positive effect of the individual parameter dexamethasone ($p < 0.01$); † significant positive interaction between dexamethasone and cytokines ($p < 0.05$); †† significant positive interaction between dexamethasone and cytokines ($p < 0.001$); ‡ significant negative interaction between dexamethasone and cytokines ($p < 0.001$).

Dexamethasone enhances cytokine-induced LBP and SAA release by Caco-2 cells

An important feature of the systemic acute phase response is the involvement of glucocorticoids. These glucocorticoids enhance the IL-1, TNF and IL-6 mediated induction of the acute phase response in the liver. To elucidate the effect of glucocorticoids on cytokine-mediated release of LBP and SAA by IEC, Caco-2 cells were stimulated in the presence of 1 μ M dexamethasone, a synthetic glucocorticoid.

Dexamethasone enhanced the LBP synthesis induced by IL-6 ($p < 0.05$) and markedly enhanced the induction by the combination of IL-6, IL-1 β and TNF- α ($p < 0.01$; Fig. 6A). Furthermore, spontaneous SAA release and SAA release induced by the combination of IL-1 β and IL-6 were strongly enhanced by dexamethasone (Fig. 6B). The enhancement of LBP and SAA release by glucocorticoids was concentration dependent (data not shown). A negative interaction between dexamethasone and the combination of IL-1 β , IL-6 and TNF- α was found ($p < 0.001$). These data indicate a role for glucocorticoids in the local acute phase response by regulation of the cytokine mediated LBP and SAA release in the gut.

Characterization of LBP released by Caco-2 cells

To characterize LBP produced by Caco-2 cells the M_r of the isolated protein was determined by western immunoblotting, and compared with that of rLBP. LBP produced by Caco-2 cells migrated as a single band with a M_r of approximately 60 kDa, identical with that of rLBP (Fig. 7).

FIGURE 7. Westernblot of the Caco-2 derived LBP and rLBP. LBP was isolated from the supernatant of stimulated Caco-2 cells and CHO-cells. Gel electrophoresis of LBP was followed by electrophoretic transfer onto an Immobilon-P membrane. LBP was detected by a polyclonal Ab to human LBP. Lane 1, rLBP; lane 2, LBP purified from supernatant of stimulated Caco-2 cells; lane M, molecular mass marker (kDa).

A characteristic of functional LBP is its capacity to bind LPS. Therefore, the capacity of Caco-2 derived LBP to bind LPS was established. The LBP produced by Caco-2 cells showed a dose dependent binding of LPS similar to that of rLBP (Fig. 8). These results ascertain the functional capacity of LBP produced by intestinal epithelial cells.

LBP and SAA release by the intestinal cell lines Colo-205, T-84 and Int-407

To elucidate whether the release of LBP and SAA is restricted to Caco-2 cells or is a common feature of IEC, the capacities of three other intestinal epithelial cell lines to release LBP and SAA were evaluated. For this purpose Colo-205 and T-84 cells, both derived from the human colon, and Int-407 cells, derived from human ileum/jejunum, were cultured in the presence of IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) and 1 μ M dexamethasone. LBP release was inducible by cytokines in all tested cell

lines. Non-stimulated cells produced only minute amounts of LBP. SAA was only detectable in the supernatant of stimulated Int-407 cells (Table I). These data confirm the capacity of human intestinal epithelial cells to synthesize and release LBP.

FIGURE 8. LPS binding capacity of Caco-2 derived LBP (●) and rLBP (■). Concentration range of Caco-2 derived LBP and rLBP were added to plates coated with anti-LBP mAb HM14. Binding of biotinylated LPS to the immobilized LBP was determined. Data are expressed as OD 450 nm.

Discussion

In the present study we demonstrated the synthesis and release of the endotoxin binding protein LBP by human intestinal epithelial cells. Furthermore, the regulation of LBP and SAA release by IEC was studied in detail and compared to the regulation in liver cells. The results support the role of the intestinal mucosa as an active participant in the inflammatory response.

The results reveal that Caco-2, Colo-205, T-84 and Int-407 cells produce LBP. To our knowledge, this is the first evidence for the production of LBP by human IEC. Investigation of the induction of LBP release revealed that IL-6 induced LBP release by Caco-2 cells, while IL-1 β and TNF- α showed a synergistic effect when used together with IL-6. Similarly, LBP synthesis was induced in liver cells by IL-6, whereas the effects of IL-1 β and TNF- α on IL-6 induced LBP release were additive. TNF- α down regulated the LBP synthesis induced by a combination of IL-1 β and IL-6 in liver cells, whereas the opposite effect was found for Caco-2 cells. The data concerning the release of LBP by liver cells are supported by others (1, 21, 22).

SAA synthesis by Caco-2 cells was predominantly regulated by IL-1 β and this induction was enhanced by IL-6. This regulation is consistent with the SAA synthesis in liver cells, which is also primarily regulated by IL-1 β and shows strong synergism with IL-6 (23). The regulation of SAA synthesis by TNF- α in Caco-2 cells was shown to differ substantially from the regulation of SAA release by liver cells. TNF- α downregulated the IL-1 β induced SAA release by HuH-7 cells, while in Caco-2 cells IL-1 β and TNF- α showed an additive effect on SAA release. Subsequently, parallel to the differences seen for LBP induction between these cell types, TNF- α suppressed the SAA release induced by the combination of IL-6 and IL-1 β in liver cells, while a

synergistic effect was seen in Caco-2 cells. In summary, both proteins behave like secretory class 1 acute phase proteins in IEC, similar to their regulation in hepatocytes (19, 21, 23, 24), although differences in the regulation of LBP and SAA release between both cell types were discernible. These differences possibly reflect a distinction between the regulation of systemic levels, controlled by the liver, and the regulation of local tissue levels in the intestine, for which epithelial cells are responsible. The high levels of cytokines found in the intestine during intestinal inflammation (25, 26) seem in line with our hypothesis of a localized regulation of acute phase protein synthesis in the gut. Furthermore, the ratio of SAA to LBP secreted by Caco-2 cells is high compared to HuH-7 cells. It is not possible to conclude from the data that the absolute amount of SAA secreted by IEC exceeds the secretion of SAA by liver cells, because the number of Caco-2 and HuH-7 cells per well was not identical during experiments. However, these data clearly show a dramatic increase in SAA synthesis by Caco-2 cells in response to cytokines, which suggests that the intestinal mucosa is an important extrahepatic production site of SAA and which may reflect an immediate local requirement for this apolipoprotein during inflammation.

Table I: *LBP and SAA release by intestinal epithelial cell lines¹*

		LBP ng/ml	SAA ng/ml
Colo-205	Unstimulated	1.05 ± 0.06	ND
	Stimulated	3.69 ± 0.10*	ND
T-84	Unstimulated	0.09 ± 0.09	ND
	Stimulated	0.69 ± 0.13*	ND
Int-407	Unstimulated	0.24 ± 0.38	ND
	Stimulated	0.85 ± 0.04*	410.6 ± 167.5 *

¹Cells were stimulated in the presence of IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) and 1 μ M dexamethasone for 72 h. Data are expressed as mean $\pm$ SD. ND; not detectable.

* Significantly different from unstimulated cells ($p < 0.05$).

In contrast to cytokines, endotoxin induced neither LBP nor SAA release by Caco-2 cells in the presence of soluble CD14. The lack of response to endotoxin in Caco-2 cells is described for other acute phase proteins as well (8). Hypothetically this absence of response protects the host from an ongoing stimulation by endotoxins present in the gut lumen. Our data indicate that an inflammatory reaction leading to cytokine release is a necessary step for the induction of LBP and SAA release by IEC. Endotoxin enters under pathological circumstances the lamina propria, where it causes inflammation and the release of cytokines such as IL-6, IL-1 and TNF. We presume that these cytokines induce the release of acute phase proteins by the neighboring epithelium. However, it has been described that IEC themselves can generate cytokines in response to an endotoxin or bacterial challenge (27), although no consensus is achieved in the literature concerning this property of IEC (8).

Glucocorticoids were demonstrated to enhance the cytokine mediated induction of LBP and SAA release by IEC. This result is consistent with the enhancing effect of glucocorticoids on cytokine induced SAA (24) and LBP (19, 21) synthesis by liver

cells, as reported earlier and confirmed in this study (data not shown). The data indicate that the local synthesis of acute phase proteins in the gut is reinforced by glucocorticoids, which are synthesized by the adrenal glands in high amounts during a systemic acute phase response.

We hypothesize that the release of LBP by human intestinal epithelial cells is involved in the defense against endotoxins, which is pre-eminently important in the mucosa. Besides its role in neutralization and clearance of endotoxin, a basal concentration of LBP is known to enhance the sensitivity of the immune system to endotoxin by catalyzing the binding of endotoxin to macrophages. LBP produced by IEC may therefore also have a function in sensitizing the mucosal immune system to translocated endotoxins. However, high concentrations of LBP have been shown to decrease endotoxin activity (4). The ratio of LBP and endotoxin is reported to determine a number of essential processes; the monocytic response (4), the binding of LBP to HDL (28) and the inactivation of endotoxin by HDL (29). We suggest that a local production of LBP by epithelial cells during inflammation in the intestine may result in high local concentrations of LBP, and consequently to a reduced toxicity of endotoxin. The proposed importance of LBP in local defense against gram negative bacteria is supported by recent studies which demonstrate that intraperitoneal (i.p.) administration of bacteria is tolerated by wild type mice, although it results in an uncontrolled multiplication and spread of bacteria in LBP knockout mice (30). Moreover, i.p. injection of LBP can reduce mortality mediated by endotoxin and bacteria in mice (4).

Secretion of SAA by the intestinal epithelium refers to a role for SAA in the local inflammatory processes in the intestine. Although the primary function of SAA in the acute phase response is not fully understood, several immunological functions have been proposed. There is evidence that SAA enhances the binding of HDL, which possesses substantial endotoxin binding capacity (2), to macrophages (9). Furthermore, it was demonstrated recently that SAA has cytokine-like properties (31). Further studies are necessary to determine the biological role of SAA secretion by the intestinal mucosa.

In summary, we have demonstrated the release of LBP and SAA by intestinal epithelial cells in response to specific sets of cytokines and glucocorticoids. Endotoxin is shown not to induce the release of LBP and SAA by IEC, indicating that cytokine release in the proximity of the epithelial cells is necessary for the induction of LBP and SAA release by these cells. The capability of IEC to release LBP and SAA suggests a role for the intestinal epithelium in the acute phase response and in the local defense of the gut against bacteria and endotoxin.

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Chapter 3

Lipopolysaccharide Binding Protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice

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Abstract

Lipopolysaccharide-Binding Protein (LBP) is an important modulator of the host's response to endotoxin. In a previous study, we found evidence for the synthesis of LBP by intestinal epithelial cells. In this study, we explored the polarity of LBP secretion by these cells. Polarized monolayers of Caco-2 cells were used as intestinal mucosa model. Cells were stimulated apically or basally with cytokines, and LBP secretion was analyzed. Furthermore, the presence of LBP in intestinal mucus of healthy and endotoxemic mice was studied using a mucus-sampling technique. The constitutive unipolar LBP secretion from the apical cell surface was markedly enhanced when cells were exposed to cytokines at their apical surface. However, bioactive LBP was secreted from both cell surfaces after basolateral stimulation of cells. Cytokines also influenced the secretion of the acute phase proteins serum amyloid A, apoA-I and apoB from both surfaces of Caco-2 cells. Furthermore, transport of exogenous LBP from the basolateral to the apical cell surface was demonstrated. In line with these *in vitro* data, the presence of LBP in intestinal mucus was strongly enhanced in mice after a challenge with endotoxin. The results indicate that LBP is present at the mucosal surface of the intestine, a phenomenon for which secretion and transport of LBP by intestinal epithelial cells may be responsible.

Introduction

The acute phase protein LPS-binding protein (LBP) strongly modulates the response to endotoxins, which are present in the outer membrane of Gram-negative bacteria. LBP has the paradoxical dual function of sensitizing the immune system to endotoxin (1), and on the other hand enhancing the neutralization of endotoxin by high density lipoprotein (HDL) (2, 3). Furthermore, high concentrations of LBP have recently been shown to decrease endotoxin activity (4) and to protect against septic shock caused by Gram-negative bacteria (4, 5). The LBP concentration in the circulation increases enormously during inflammation, for which the liver has been held responsible. Previous *in vitro* studies from our laboratory, however, provided evidence for the extrahepatic synthesis of LBP by human intestinal epithelial cells (6). The intestinal mucosa serves as a boundary between the sterile interior of the host and the bacterial contents of the gut lumen. Therefore, the local release of LBP in the gut seems particularly important.

LBP secretion by intestinal epithelial cells was demonstrated to be dramatically up-regulated by exposure to proinflammatory cytokines such as IL-1, IL-6 and TNF (6). Enhanced concentrations of these cytokines are present in the subepithelial tissue during inflammatory bowel diseases (7) and during systemic inflammation (8, 9). This implicates that epithelial cells are exposed to cytokines at their basolateral cell surface. However, inflammatory processes in the intestine take place at both poles of the epithelium, that is in the subepithelial tissue and at the luminal surface of the epithelium. Active inflammation of the intestine is histologically characterized by transmigration of neutrophils across the epithelial monolayer and subsequent collection of these cells in dilated crypts. Furthermore, there is evidence for the enhanced presence of cytokines at the luminal surface of the mucosa during infection and inflammation of the gut (10, 11, 12). Therefore, the intestinal epithelium may be exposed to cytokines at the apical pole as well. Moreover, receptors for IL-1 and IL-6 were described to be present at both the basolateral and the apical surface of intestinal epithelial cells (13). This prompted us to study whether intestinal epithelial cells synthesize LBP in response to cytokines administered to either the basolateral or the apical side of the cells. Furthermore, we studied from which surface of the cells LBP is secreted under various conditions.

Material & methods

Reagents and Abs.

Human rTNF- α was kindly provided by BASF/Knoll (Ludwigshafen, FRG); human recombinant IL-6 by Prof. W. Sebald (Physiologisch-Chemisches Institut der Universität Würzburg, Germany); human rIL-1 β was a gift from Immunex (Seattle, WA); dexamethasone was a gift from Merck Sharp & Dohme (Haarlem, The Netherlands). Human rLBP was produced by transfected Chinese hamster ovary (CHO) cells, kindly provided by Dr. P. Tobias, Research Institute of Scripps Clinic (La Jolla, CA). Polyclonal Abs to human rLBP were obtained by immunizing rabbits with human rLBP. Murine LBP (mLBP) was produced by transfected *Spodoptera frugiperda* (SF9) cells. The baculovirus used for transfection as well as the rat anti-murine LBP mAb clone 43 and a polyclonal rabbit anti-murine LBP Ab (14) was kindly provided by Dr. D. Heumann, department of Medicine, Lausanne, Centre

Hospitaier Universitaire Vaudois. The Serum Amyloid A (SAA) immunoassay was kindly provided by Dr. P.C. Limburg, department of Rheumatology, University of Groningen, the Netherlands. The apoA-I and apoB immunoassay were a gift from Dr. Gavilondo, Center for Genetic Engineering and Biotechnology (Havana, Cuba).

Culture and stimulation of Caco-2 cells

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA; ATCC HTB 37). Caco-2 cells were cultured at 37°C with 5% CO₂ in DMEM (Life Technologies, Paisley, UK), supplemented with 10% FCS (HyClone), 1.0 mM sodium pyruvate (Life Technologies), 0.1 mM non-essential amino acids (Life Technologies), 100 IU penicillin/ml and 100 µg streptomycin/ml (Life Technologies), further defined as complete medium. Cells were maintained in 25-cm² or 75-cm² flasks (Costar, Cambridge, MA). Cultures were split when they reached 80% confluence.

For experiments, 5 x 10⁵ Caco-2 cells/cm² were seeded onto a 1 cm² or 4.7 cm² collagen-coated membrane (3.0 µm pore size) inserted in Transwells (Costar). Cells were cultured in complete medium, which was changed 48 h after seeding and every other day. After two weeks, Caco-2 monolayers display functional properties similar to those found in normal enterocytes (15) and they form a polarized monolayer with tight junctions and brush border microvilli at their apical surfaces (16). Therefore, in this study all experiments were conducted between 14 and 17 days after seeding of the cells. The formation and maintenance of a sealed monolayer of Caco-2 cells was monitored by measurement of the passage of the permeability probe phenol red (500 Da). Monolayers permeable for phenol red were not used (except for studies in which EDTA was added to disturb monolayer integrity).

For experiments designed to study the polarized release of acute phase proteins by Caco-2 cells, 1 cm² filters were used. At the start of experiments, culture medium was removed from both compartments and complete medium, supplemented with IL-1β, IL-6, TNF-α and dexamethasone, was added to the apical or basolateral compartment as given in the results. At the end of an 84 h incubation period, the apical and basolateral medium was removed, and apical derived medium was centrifuged to remove cells and cell debris. Acute phase protein concentrations in the medium were determined by ELISA.

To study the transport of LBP across Caco-2 monolayers, mLBP was used to prevent interference of human LBP synthesized by the Caco-2 cells in the analysis. At the start of experiments, medium was removed from both compartments. Complete culture medium containing LBP-deficient human serum instead of FCS and containing mLBP was added to the basolateral or apical compartment. In many species, including humans, LBP is present in normal serum at a concentration of 0.1-5 µg/ml (17). Two concentrations in this range, 150 and 1500 ng/ml, were used. After 72 h incubation, the mLBP concentration in the contralateral compartments was measured by a mLBP ELISA that lacks cross reactivity for human LBP.

Immunoassays.

Acute phase protein release by Caco-2 cells was evaluated using sandwich ELISA's. Human LBP was detected as described previously (18). In short, plates (Nunc-Immuno

Plate Maxisorp, Roskilde, Denmark) were coated with polyclonal anti-human LBP Abs. The washing and reagent dilution buffer contained 40 mM MgCl₂ to prevent disturbance of LBP recovery by LPS in the ELISA. LBP was detected using a biotinylated polyclonal rabbit anti-human LBP IgG, followed by peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

SAA was quantified as described elsewhere (19). In short, SAA specific mAb Reu 86.5 was used as capture reagent. The amount of SAA bound to the wells was quantified by incubation with a horseradish peroxidase labeled monoclonal anti-SAA Ab (Reu 86.1), followed by TMB substrate.

For analysis of human apoA-I and apoB concentrations plates were coated with specific monoclonal Abs for apoA-I and apoB, respectively. Peroxidase labeled mAbs were used for detection of immobilized apoA-I and apoB. Peroxidase activity was determined by addition of TMB substrate.

IL-6 concentrations were determined using sandwich ELISA for IL-6. In short, plates were coated with mAb 5E1, specific for human IL-6. Immobilized IL-6 was detected using a biotinylated polyclonal rabbit anti-human IL-6 Ab, followed by peroxidase-conjugated streptavidin and TMB.

mLBP was quantified using sandwich ELISA. Rat mAb clone 43 was used as capture reagent. For detection of mLBP a polyclonal rabbit anti-murine LBP Ab was followed by peroxidase-conjugated polyclonal goat-anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and TMB. Human LBP is not detected by the Abs used in this assay.

For measuring murine IgG, plates were coated with goat anti-murine IgG (Jackson ImmunoResearch). Peroxidase-conjugated polyclonal goat anti-murine IgG (Jackson ImmunoResearch), followed by TMB substrate, was used for detection of mIgG.

Bioassay for LBP

An important feature of LBP is its capacity to enhance the stimulation of PBMC by endotoxin. Therefore, the biologic activity of LBP secreted from either the apical or the basolateral cell surface was determined by assessing its effect on the endotoxin-induced stimulation of PBMC. For this purpose Caco-2 monolayers were cultured on 4.7 cm² filters and basally stimulated as described before in the presence of complete medium containing 5% LBP-depleted human serum instead of FCS. This LBP-depleted serum was produced by passing human serum (15 µg LBP/ml) over an anti-LBP column. To this end anti-hLBP mAb HM-14 was cross-linked to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. LBP depleted serum, contained less then 3.5 ng LBP/ml as assessed by LBP-ELISA. After an 85 h incubation period, the apical and basolateral supernatants were harvested and the concentration of LBP in the supernatants was determined by ELISA. To prevent LBP independent stimulation of PBMC caused by cytokines or other agents in the supernatants, a special method was developed. The apical and basolateral supernatants of stimulated cells, containing 66 and 33 ng/ml LBP, respectively, and of unstimulated cells, containing 2.5 and 0 ng/ml LBP, were added to a 96-well plate coated with a polyclonal Ab against LBP. This Ab binds LBP without affecting its biological function. As a positive control complete stimulation medium containing 35 ng rLBP/ml was added to the plates. Negative controls consisted of complete stimulation medium

without LBP. After 1.5 hour incubation the plates were washed extensively. PBMC were isolated from buffy coats of healthy volunteers, kindly provided by the local blood bank by density-gradient centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Cells were added to the plates at a density of 1×10^5 cells/well and stimulated with 0.5 ng/ml LPS (from *Escherichia coli*, serotype 055:B5, Sigma) for 20 h. The supernatants were harvested, and IL-6 concentrations in the culture supernatants were determined using ELISA. Enhanced IL-6 release was used as an indicator of biological activity of LBP.

LBP content of the intestinal mucus layer in mice

Swiss mice were injected i.p. with 100 μ g LPS. Controls consisted of untreated mice. Mice were killed 24h after injection of LPS and the small intestine was removed and spread open. The mucus on the luminal surface, was absorbed during 30 s using 0.5 x 1 cm filter paper (Whatmann no. 42; Maidstone, UK), a modified method described by Hendel et al. (12). Sections containing Peyer's patches were excluded. The filter papers were immediately transferred to a vial containing buffer solution (300 μ l Tris buffer 0.1 M, pH 7.4 in normal saline containing 0.3% BSA, 0.01% azide and 0.002% Tween). Vials were placed on a rocker and agitated gently overnight at 4°C. mLBP in the buffer was assessed using ELISA. To exclude the possibility that the LBP detected resulted from increased permeability of the mucosal membrane or contamination with blood, also the amount of IgG present in the buffer was analyzed.

Statistical analysis

Data were analyzed using SPSS (Chicago, IL) software. All values in the figures and text are expressed as mean $\pm$ SD of n observations.

Comparison of acute phase protein levels in the supernatants of Caco-2 monolayers after different treatments was performed by the Mann-Whitney U test. Data derived from the bioassay for LBP were analyzed by ANOVA with Dunnett's method as post hoc test, after $\log_{10}$ transformation of the data. The LBP and IgG concentration in the blood of healthy and endotoxemic mice was compared using the Mann-Whitney U test. The data concerning LBP content of mucus in healthy and endotoxemic mice were corrected for IgG content of mucus. For this purpose $\log_{10}$ transformation of mucus:serum ratios was performed. $u = \text{Log} (\text{LBP ratio}/\text{IgG ratio})$ was counted for the untreated and LPS treated mice and Student's t -test for unpaired values was performed to compare the means ($\bar{u}$) of both groups. $10^{\bar{u}_{(\text{untreated})} - \bar{u}_{(\text{LPS-treated})}}$ was counted to determine the relation of LBP in mucus of untreated and LPS treated mice. A probability of 0.05 or less was considered statistically significant.

Results

Vectorial secretion of LBP by Caco-2 cells.

First, it was studied whether LBP is secreted from the apical or the basolateral plasma membrane surface of quiescent and apically or basally stimulated Caco-2 cells. To this end, Caco-2 cells were cultured on permeable filters which provide separation of apical and basolateral compartments. For stimulation of the monolayers, a mix of IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) and 1 μ M Dexamethasone was administered to the differentiated cells at either the apical or the basolateral side. In a previous study, this cytokine mixture was proven optimal for the induction of LBP secretion (6). After 84 h incubation the culture medium was harvested from both compartments and the LBP concentration was determined (Table I). It was found that LBP was secreted in minor amounts (0.37 ng/ml absolute 0.19 ± 0.12 ng) exclusively from the apical surface of unstimulated cells. When cytokines were added to the apical compartment, the secretion of LBP into the apical medium was significantly enhanced (2.23 ng/ml absolute 1.12 ± 0.57 ng LBP, $p < 0.05$), while no LBP was detectable in the basolateral medium. Control experiments demonstrated that recombinant LBP traversed the naked filters and appeared in both compartments (not shown).

Table I: Vectorial secretion of LBP by intestinal epithelial cells¹

Stimulation	Apical LBP	Basolateral LBP
None	0.19 ± 0.12	ND [‡]
Apical	$1.12 \pm 0.57^*$	ND [‡]
Basolateral	$2.03 \pm 0.42^{*†}$	$7.22 \pm 2.27^{*†‡}$

¹Cells were stimulated apically or basally with a combination of IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) and 1 μ M dexamethasone for 84 h. Data are expressed as mean of total released ng LBP $\pm$ SD per compartment (n=7). ND; not detectable. * Significant difference from unstimulated cells ($p < 0.05$); [†] significant difference from apical stimulated cells ($p < 0.05$); [‡] significant difference from the concentration in the contralateral compartment ($p < 0.05$).

This observation eliminates the possibility that the absence of LBP in the basolateral compartment results from its selective adsorption onto the filters supporting the monolayers.

In contrast to the unipolar secretion under neutral and apical stimulated conditions, basolateral administration of cytokines resulted in the secretion of LBP from both cell surfaces; LBP was secreted into the basolateral medium (4.81 ng/ml absolute 7.22 ± 2.27 ng) and the amount of LBP in the apical compartment increased markedly (4.06 ng/ml absolute 2.03 ± 0.42 ng LBP). The permeability for phenol red during these experiments was not disturbed, indicating intact monolayer integrity. Taken together, these data indicate that both surfaces of Caco-2 cells interact with cytokines and that cells are able to secrete LBP from both surfaces. The intracellular targeting or delivery of LBP to either the apical or the basal cell surface, however, is influenced by the presence of cytokines at these surfaces.

Table II: Vectorial secretion of SAA and apolipoproteins by intestinal epithelial cells¹

Stimulation	SAA		apoA-I		apoB	
	Apical	Basolateral	Apical	Basolateral	Apical	Basolateral
None	ND	ND	1.1±0.2	3.3±1.3 [‡]	3.6±1.7	13.9±4.0 [‡]
Apical	184.2±69.8*	48.3±15.3* [‡]	0.9±0.1*	2.1±1.1 [‡]	2.2±0.7	8.5±3.4 [‡]
Basolateral	217.5±74.4*	583.3±132.3* [‡]	0.9±0.2	1.3±0.5*	1.8±0.2*	5.7±3.0* [‡]

¹Cells were stimulated apically or basally with a combination of IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) and 1 μ M dexamethasone for 84 h. Data are expressed as the mean of total released ng SAA, μ g apoA-I and μ g apoB $\pm$ SD per compartment (n=7). ND; not detectable. * Significant difference from unstimulated cells ($p < 0.05$); [‡] significant difference from apical stimulated cells ($p < 0.05$), [‡] significant difference from the concentration in the contralateral compartment ($p < 0.05$).

Vectorial secretion of SAA and apolipoproteins by Caco-2 cells

During inflammation the concentration of several plasma proteins other than LBP are increased, such as SAA, which is one of the major positive acute phase proteins. Another important aspect of the acute phase response is the reduced presence of negative acute phase proteins in the circulation, such as the apolipoproteins apoA-I and apoB (20). The intestinal mucosa is a known producer of the apolipoproteins SAA, apoA-I and apoB. Previous work on the acute phase response in the intestine *in vivo* indicates that apoA-I decreases only in the duodenum, while SAA increases throughout the intestine (21). A common feature of these proteins and LBP is their association with lipoproteins. These lipoproteins contribute to the neutralization of endotoxin (2, 3). To study whether the polarized distribution, as observed for LBP, is a common feature of acute phase proteins or apolipoproteins in general, we investigated the polarized distribution of SAA, apoA-I and apoB by Caco-2 monolayers in parallel to LBP (Table II). SAA was not detectable in the supernatant of quiescent monolayers. After cytokine stimulation however, Caco-2 monolayers produced high amounts of SAA. In contrast to LBP, SAA was detectable at both surfaces in response to the addition of cytokines to either side of the monolayer. However, similar to LBP secretion, the secretion of SAA predominated in the compartment corresponding to the side of cytokine stimulation.

The negative acute phase proteins apoA-I and apoB are produced in high amounts under normal culture conditions and are secreted from both cell surfaces, although the secretion into the basolateral compartment predominates. As expected, the total secreted amount of apoA-I and apoB decreased in response to cytokines. Apical addition of cytokines resulted in a 15% decline in apical and a 35% decline in basolateral apoA-I concentration. Under these conditions, the apoB concentration decreased almost 40% in the apical and basolateral compartment. When cytokines were administered basolaterally the amount of apoA-I secreted into the apical compartment reduced with 20% and basolateral concentrations declined 60%. Apical and basolateral apoB concentrations declined approximately 50% and 60% respectively under these conditions.

These results indicate that the secretion of SAA, apoA-I and apoB from both surfaces of Caco-2 cells can be influenced by addition of cytokines to either pole of the cells. These results are consistent with those found for LBP secretion. However, the specific pattern of polarized LBP secretion under different conditions was not observed for the other tested acute phase proteins.

FIGURE 1. Bioactivity of apical and basolateral secreted LBP. Caco-2 cells were grown on Transwell-Col and stimulated basally with IL-1B (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) and 1 μ M Dexamethasone for 85 h. The concentration of LBP in the supernatants was determined by ELISA. Apical and basolateral supernatants of stimulated cells, containing 66 and 33 ng LBP/ml respectively, and unstimulated cells, containing 2.5 and 0 ng LBP/ml, were added to 96 wells ELISA plates coated with a polyclonal Ab against human LBP. A positive control containing 35 ng rLBP/ml and a negative control without LBP were included. After extensive washing of the plates, 1×10^5 PBMC and 0.5 ng LPS/ml were added to the wells. Supernatants were harvested after 20h and IL-6 released into the supernatant was analyzed. Results shown represent percentage IL-6 secreted compared to negative controls, and are presented as mean $\pm$ SD of 3 wells. * Significant difference from negative control ($p < 0.05$).

Apical and basolateral secreted LBP enhances the endotoxin induced cell activation.

Next, the functional capacity of apical and basolateral secreted LBP was studied. Several functions of LBP have been described, among which its capacity to strongly enhance endotoxin induced cell activation (22). It was demonstrated that LBP binds to LPS and facilitates the interaction of endotoxin with membrane CD14 on monocytes, resulting in cytokine release (22). The capacity of the apical and basolateral released LBP to up-regulate the endotoxin induced IL-6 secretion by PBMC was studied. Using

a new developed bio-assay, LBP in both apical and basolateral medium, was demonstrated to enhance the endotoxin-induced cell activation compared to LBP deprived conditions ($p < 0.05$) (Fig.1). Apical and basolateral media from untreated monolayers did not significantly affect the endotoxin induced cytokine release.

FIGURE 2. Transport of LBP across *Caco-2* monolayers is directed towards the apical surface. 150 or 1500 ng/ml mLBP was added to the basolateral compartment of cells that were grown on Transwell-Col™ filters. mLBP was measured after 72h incubation in the apical compartments. Presented are the means $\pm$ SD of the mLBP concentration in the apical compartment of 3 wells.

LBP is transported across Caco-2 monolayers from the basolateral to the apical side. The data obtained in this study demonstrate that *Caco-2* cells secrete LBP from their apical surface under all tested conditions. These findings suggest a putative importance of the presence of LBP at the apical surface of the intestinal epithelium. Therefore it was studied next whether besides the secretion of endogenous LBP towards the apical surface, *Caco-2* cells are also able to transport exogenous LBP from their basolateral to their apical surface. For this purpose, physiologic concentrations (150 and 1500 ng/ml) of murine LBP (mLBP) were added to the basolateral compartments. After 72 h incubation the amount of mLBP was measured in the apical compartments using an ELISA highly specific for mLBP. The data show that after administration of physiologic amounts of mLBP to the basolateral side of the cells, substantial amounts of mLBP were detected in the apical compartment (Fig.2). The passage of LBP across the monolayers was dose-dependent. To elucidate whether the passage of LBP across the monolayer was specific for the basolateral-apical direction, mLBP was also added to the apical compartment. No passage of mLBP across the *Caco-2* monolayers could be detected in the apical-basolateral direction (data not shown). This restriction of passage required the integrity of the monolayers, since EDTA induced disruption of the integrity resulted in the traversing of LBP through the filter and cell layer (data not shown). These results are in line with experiments, which demonstrated the presence of LBP in the apical and not the basolateral medium after apical stimulation of the cells and in quiescent cells.

FIGURE 3. LBP presence in mucus of mice is enhanced during endotoxemia. Swiss mice were injected i.p. with 100 μ g LPS. Controls consisted of untreated mice. Mice were killed 24 h after injection of LPS. The mucus of the small intestine was absorbed using filter paper. Histological examination of the intestinal segment showed no damage to the epithelium attributable to the procedure in controls (A) and endotoxin-challenged mice (B).

(C) Absorbed LBP and serum concentrations of LBP were assessed using ELISA. Absorbed IgG was used as a marker of plasma leakage. Results are presented as the mean $\pm$ SD mucus:serum ratio of LBP and IgG of 4 and 3 mice, respectively. * Significant enhanced compared to controls after correction for IgG leakage ($p < 0.05$).

The LBP content of mucus is enhanced during endotoxemia

The data obtained in this study so far suggest that cytokines enhance the constitutive secretion of LBP towards the luminal surface of intestinal epithelial cells. Subsequent, the data implicate that transport of LBP toward the apical surface of the epithelium occurs. Therefore, we next studied whether the amount of LBP in the mucus overlaying the intestinal mucosa is enhanced during inflammation. In this model, mice were challenged with endotoxin. Endotoxemia induces inflammatory processes in the intestine, including intestinal production of cytokines (8, 9).

I.p. administration of endotoxin to mice resulted in a significantly increased serum concentration of LBP (20.9 ± 7.3 μ g/ml, $p < 0.05$) as compared with the controls (5.4 ± 2.6 μ g/ml). The amount of LBP absorbed from 0.5 cm^2 mucus increased from 1.4 ng in controls to 30.6 ng in the LPS treated mice. Histological examination of the intestinal segment showed no damage to the epithelial lining (Fig.3A/B). The integrity of the intestinal mucosa was further confirmed by investigating the leakage of IgG across the epithelium. The IgG concentration in serum was not significantly altered. The mean ratio of IgG in mucus to serum, was 6×10^{-5} and 1×10^{-4} in untreated and LPS treated mice respectively, indicating a slightly enhanced degree of leakage from the intestinal mucosa (Fig.3C). The ratio of LBP absorbed from 0.5 cm^2 mucus to the concentration of LBP in serum of untreated mice was 2.6×10^{-4} and of mice injected intraperitoneally with endotoxin 1.4×10^{-3} (Fig.3C). In view of the mucus:serum ratios for IgG and LBP,

the possibility that the detected LBP was a result of a disturbed intestinal integrity is not likely. In endotoxemic mice the ratio LBP in mucus to serum corrected for IgG leakage was significantly enhanced 5 times ($p < 0.05$) compared to controls.

Discussion

In previous studies we demonstrated that Caco-2 and other intestinal epithelial cell lines, synthesize and secrete the endotoxin modulating protein LBP, and that this secretion is dramatically up-regulated by exposure to proinflammatory cytokines and dexamethasone (6). The data of this study demonstrate that the synthesis of LBP and other acute phase proteins by Caco-2 cells is affected by the presence of cytokines at both the subepithelial and the luminal surface of the cells. These data are in line with the presence of IL-1 and IL-6 receptors on the basolateral and the apical surface of these cells as described by others (13). Local high concentrations of these cytokines can be found in both the subepithelium of the gut (7) and at the luminal surface of the mucosa during infection and inflammation of the gut (10, 11, 12), which implies that both, the apical and the basolateral pole of enterocytes can be exposed to cytokines.

Constitutive LBP was secreted exclusively from the apical surface of Caco-2 cells and the amount of apical secreted LBP was strongly up-regulated by proinflammatory cytokines if present at either the apical or the basolateral surface of the cells. Remarkably, apical exposure of cells to cytokines resulted in unipolar secretion of LBP from the apical surface, whereas bipolar secretion of LBP from the basolateral and the apical surfaces of the cells occurred in response to cytokines present at the basolateral side of cells. The monolayer integrity was shown not to be disturbed and LBP was demonstrated not to pass the monolayers in the apical to basolateral direction. Therefore, it is not likely that the presence of LBP in the basolateral compartment resulted from a redistribution of cytokines or from LBP that was originally secreted from the apical surface. The data indicate that signals, induced by contact of cytokines with the basal cell surface, are necessary to direct LBP to the basolateral surface.

The data further indicate that LBP secretion is directed primarily toward the side of cytokine administration. This observation implies that the secretory response of intestinal epithelial cells is predominantly directed towards the cell pole at which inflammatory processes take place. These data are strongly supported by the vectorial secretion of cytokines by Caco-2 cells and the ileal mucosa in response to a bacterial challenge (11, 23). Luminal secretion of cytokines will predominate when infection takes place at the apical surface of the mucosa such as during bacterial overgrowth (23). Cytokines are also present in high amounts at the apical surface of the mucosa during cryptabscesses, characteristic for inflammatory bowel disease. Therefore enhanced apical secretion of LBP may be of physiologic relevance during these conditions.

Surprisingly, Caco-2 cells were also able to secrete the acute phase proteins SAA, apoA-I and apoB from both surfaces. SAA was only secreted upon stimulation by cytokines. The secretion of the negative acute phase proteins apoA-I and apoB by Caco-2 cells was reduced in response to cytokines, congruent with observations by others (21, 24, 25). Although the reduction was most prominent when cytokines were added to the basolateral side, protein production was also diminished after apical

cytokine administration. The reduced production of these negative acute phase proteins and the up-regulation of positive acute phase protein synthesis are consistent with the reprioritization of protein release described for hepatocytes during the acute phase response (26). The potential function of SAA, apoA-I and apoB at the apical surface of the intestinal mucosa is currently being explored.

In addition to the secretion of endogenous LBP from the apical surface, transport of exogenous LBP from the basolateral to the apical side of Caco-2 cells was observed. Remarkably, the passage of LBP in reversed direction did not occur. These data imply that LBP derived from the circulation can be transported toward the intestinal lumen and that the enhanced concentrations of LBP as found in the circulation during the acute phase response may result in increased transport of LBP.

The *in vitro* data obtained in this study were supported by *in vivo* data, which demonstrated the presence of LBP in mucus overlaying the small intestine, which was significantly enhanced during endotoxemia. The measured amounts of LBP in the mucus exceeded the levels that could be expected to result from passive leakage from the circulation. Based on the results obtained in this study we consider that the enhanced LBP concentration in mucus during systemic inflammation can be a result of both, enhanced local production of LBP induced by cytokines and enhanced transport of LBP from the circulation towards the lumen across the epithelial cells. The presence of LBP at the luminal surface of the intestinal mucosa is consistent with the presence of other acute phase reactants in the intestinal lumen during inflammation, such as cytokines (10, 11, 12) and complement components (27).

The data of this study suggest that the intestinal epithelium can participate in inflammatory processes, which take place at the mucosal surface, by secretion of locally produced LBP and SAA and by transport of circulating LBP toward the lumen. It is tempting to speculate that the vectorial secretion and transport of LBP direct this endotoxin modulating protein to a location where its function can contribute to the defense of the gut, comparable to other proteins which contribute to host immunity at mucosal surfaces, such as IgA (28) and the microbicidal molecules lysozyme (29) and defensins (30). Several functions of LBP have been described, which can be applicable to processes taking place at the apical surface of normal and diseased epithelium. The secreted LBP may regulate the function of other cells in the local micro-environment. LBP is known to enhance the sensitivity of the immune system to endotoxin by catalyzing the binding of endotoxin to macrophages (22). High concentrations of LBP, however, have been shown to decrease endotoxin activity (4). In addition, evidence for a role for LBP in the sequestration of Gram-negative bacteria was supported by a recent study which demonstrated that an intraperitoneal administration of bacteria is tolerated by wild type mice, where it results in an uncontrolled multiplication and spread of bacteria in LBP knockout mice (5). Moreover, *i.p.* injection of LBP can reduce mortality mediated by endotoxin and bacteria in mice (4). In view of these functions apical secreted LBP possibly contributes to the intestinal barrier against bacteria and their toxin by enforcement of the ability of mucus to impair bacterial translocation.

The observation that exposure of cytokines to the basolateral cell pole is necessary for LBP secretion from this cell surface, implies that *in vivo* basolateral LBP secretion occurs only when inflammatory processes take place at the subepithelium or during

systemic inflammation. Basolateral secreted LBP may contribute to the handling of endotoxins that have penetrated into the subepithelium during conditions in which a disturbed mucosal integrity results in an enhanced permeability. This handling possibly concerns catalyzing the neutralization of endotoxin by lipoproteins, which are secreted in high amounts by the intestinal epithelium. Neutralization of bacterial toxin by LBP in the intestine might be important for the defense against gram-negative and gram-positive bacteria, since LBP was demonstrated to catalyzes the lipoprotein neutralization of LPS and lipoteichoic acid, a component of the cell membrane of gram-positive bacteria (31). Low non-harmful amounts of endotoxin are thought to leak continuously across the intact intestinal epithelium. LBP has a role in both, sensitizing the immunesystem for endotoxin and in the neutralization of endotoxin. Since this balance is concentration dependent, we hypothesize that the restriction of low concentrations of basolateral LBP in the absence of inflammatory processes taking place at the subepithelium may prevent hypersensitivity to endotoxin.

In conclusion, the results described in this paper provide evidence for the vectorial secretion of LBP and other acute phase proteins by the intestinal epithelium, a phenomenon that is not described before. Because LBP plays a significant role in the handling of endotoxin, the enhanced local presence of LBP at the apical surface of the intestinal mucosa and in the subepithelium, may be critical in host defense. The function of this intriguing phenomenon in events such as cryptabcesses, cellular infiltrates within the bowel wall and bacterial translocation demands further investigation.

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Chapter 4

Lipopolysaccharide Binding Protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction

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Abstract

Lipopolysaccharide (LPS) Binding Protein (LBP) and serum lipoproteins cooperate in reducing the toxic properties of LPS. In the present study we demonstrate that LBP circulates associated with LDL and VLDL in healthy persons. ApoB was found to account at least in part for the interaction of LBP with LDL and VLDL. Although LBP interacted with purified apoA-I in vitro, no association of LBP with apoA-I or HDL was found in serum. Consistent with the observed association of LBP with LDL and VLDL, these lipoproteins were demonstrated to be also the predominant LPS binding lipoproteins. Most interestingly, the association of LBP with LDL and VLDL strongly enhanced the capacity of these lipoproteins to bind LPS. Since this function of LBP is of utmost importance during infection, the association of LBP and LPS with lipoproteins was also studied in serum from septic patients. In septic serum containing high LBP levels and a markedly altered lipoprotein spectrum, most of the LBP is associated with LDL and VLDL, although some LBP appeared to circulate free from lipoproteins. Also in serum from septic patients LPS was found to predominantly bind to LDL and VLDL. The observed binding of LBP and LPS to LDL and VLDL as well as the LBP dependent incorporation of LPS into these lipoproteins emphasize a crucial role for circulating LBP-LDL/VLDL complexes in the scavenging of LPS.

Introduction

Lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria, evokes an inflammatory response by activation of monocytes and endothelial cells. LPS-induced cellular responses are the net result of the interaction of LPS with various plasma components such as soluble CD14, LPS binding protein (LBP) and membrane receptors such as membrane bound CD14 and Toll-like receptors. This initiation of cellular responses is essential for the host defense against bacterial infections. However, if large amounts of endotoxin are present in the circulation, an excessive cellular response can be deleterious for the host and therefore endotoxin-inactivating processes are of extreme importance.

LPS is detoxified in the circulation by incorporation into lipoproteins (reviewed in reference 1). Physiologic levels of lipoproteins protect against endotoxicity *in vitro* and *in vivo* (2, 3). Early studies have demonstrated an interaction of LPS with high density lipoproteins (HDL) (4), albeit later also very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were found to bind and inactivate LPS (5, 6, 7). Consistent with this, LDL, VLDL, chylomicrons and HDL all have been observed to reduce the lethal effect of endotoxin in mice (8, 9, 10).

Evidence for a physiologic role for LBP in inflammation is supported by studies which demonstrate enhanced mortality and uncontrolled multiplication and spread of bacteria in LBP knockout mice compared to wild type mice after intraperitoneal administration of bacteria (11). The results of these studies indicate that LBP is required to induce a rapid inflammatory response, which is essential for the resistance to bacteria. However, LBP has the paradoxical dual function of sensitizing the immune system to endotoxin and on the other hand enhancing detoxification of endotoxin. LBP catalyzes the transfer of LPS into lipoproteins, thereby enhancing LPS detoxification (12). Likewise, LBP catalyzes the lipoprotein neutralization of lipoteichoic acid, a component of the cell membrane of Gram-positive bacteria (13). Lamping et al demonstrated in a murine model that high levels of LBP in the circulation, as seen during an acute phase response, inhibit LPS effects and prevent mortality induced by endotoxemia (14). The latter observation strongly supports a physiological role for LBP dependent detoxification of LPS in the host defense.

Endotoxemia induces an acute phase response characterized by multiple physiologic adaptations. This response appears to play a role in host defense mechanisms, although its physiologic relevance needs further elucidation. One aspect of the acute phase response is a dramatic rise in circulating levels of LBP (15). Concomitantly, large changes in serum lipid and lipoprotein concentrations occur. Circulating levels of total cholesterol, LDL-cholesterol and HDL cholesterol decrease, while serum triglycerides and VLDL levels increase (16). In addition, alterations in apolipoprotein levels are observed (16, 17). ApoA-I concentrations drop and HDL becomes depleted in apoA-I (16, 18). In contrast, apoB levels are not affected by either viral or bacterial infection (16).

Others found evidence for an association of LBP with apoA-I containing lipoproteins in plasma from healthy persons (12). We consider that the physical association of LBP with these lipoproteins may be important for the cooperation of LBP and lipoproteins in the detoxification of endotoxin. However, the strong reduction of apoA-I and HDL levels that coincides with the dramatic raise in LBP levels during endotoxemia seems

in contrast with this cooperative function. Because it is firmly established that LDL and VLDL are critical in the survival of infection with Gram-negative bacteria (19) and that circulating levels of these lipoproteins are relative high during inflammation compared to HDL levels, the present study was undertaken to investigate whether LBP associates with LDL and VLDL. To this end the distribution of LBP among lipoproteins was studied in serum of healthy and septic persons. Subsequent, we investigated the effect of the association of LBP with lipoproteins and apolipoproteins on the LPS binding capacity.

Material & methods

Reagents

LPS from *Escherichia coli*, serotype 055:B5 was purchased from Sigma (St.Louis, MO). Purified apoA-I and apoB were derived from Calbiochem (La Jolla, CA) and ICN (Irvine, CA) respectively. Polyclonal Abs to human LBP were 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. Horseradish peroxidase labeled monoclonal Ab against apoA-I and apoB were gifts from Dr. L. Sorell, Center for Genetic Engineering and Biotechnology (Havana, Cuba).

Blood samples

Blood was collected from healthy donors and from septic patients. Informed consent was obtained from healthy donors and relatives of septic patients. To prepare serum, blood was allowed to clot for 2 hours at room temperature. Serum was separated by centrifugation and stored at -80°C until use. Fresh serum was used for isolation of lipoprotein fractions.

Lipid electrophoresis and Western blot analysis

Agarose electrophoresis was performed in barbital buffer pH 8.6 using the Paragon Lipoprotein Electrophoresis kit P/N (Beckman Instruments, Brea, CA) according to the manufacturer's instructions. Agarose gel electrophoresis was followed by electrophoretic transfer onto an Immobilon-P membrane (Millipore, Belford, MA) in blottingbuffer (25mM TRIS, 192 mM Glycine, 10% methanol) using the Phast system (Pharmacia, Uppsala, Sweden). After transfer, the membranes were blocked with 1% BSA in PBS for 1 hour at 37°C and washed with 0.1% BSA 0.5% Tween in PBS. For detection of LBP, membranes were incubated with biotin-labeled polyclonal Ab specific for human LBP, washed and incubated with peroxidase-labeled streptavidin. After detection of LBP, apoA-I was probed on the same blot after elution of the LBP Abs by incubation with 0.1 M Glycine-HCl buffer (pH 2.5) for 15 minutes at 37°C . Localization of apoA-I was followed by detection of apoB in a similar protocol. For immunodetection of apoA-I and apoB, peroxidase labeled monoclonal Abs were used. Interactions of LPS with serum lipoproteins were studied by pre-incubation of biotin labeled LPS with human serum overnight at 37°C . After pre-incubation, serum was subjected to electrophoresis, blotted and membranes were incubated with peroxidase-labeled streptavidin. For studying LPS binding to isolated (apo)lipoproteins human serum was first subjected to electrophoresis, blotted and membranes were incubated

with biotinylated LPS, followed by incubation with peroxidase-labeled streptavidin (Zymed Laboratories Inc., San Francisco, CA). Peroxidase activity was detected by chemiluminescent substrates (SuperSignal Substrate, Pierce, Rockford, IL) according to the manufacturer's recommendation.

Lipoprotein fractionation

Lipoproteins were isolated from pooled fresh normal human serum (6 healthy donors) by a 22 hours single spin density gradient ultracentrifugation at 40.000 rpm and 17°C according to Terpstra et al. (20), using a Beckman XL-80 ultra centrifuge with a SW40 rotor and ultra clear centrifuge tubes (Beckman Instruments). A step gradient was constructed from 2 ml serum adjusted to density 1.250 g/ml with KBr and sucrose, two NaCl/KBr solutions with density 1.225 and 1.100 g/ml respectively, and endotoxin free water density 0.998 g/ml. All solutions contained 0.1 mg/ml EDTA. The lipoprotein fractions VLDL, LDL, HDL2 and HDL3 were collected by aspiration and dialyzed extensively against PBS at 4°C and stored at -80°C until use. Cholesterol concentrations in the isolated lipoprotein subfractions were determined using an enzymatic colorimetric test from Roche (Basel, Switzerland). The lipoprotein fractions used contained no detectable LBP as assessed by LBP specific ELISA (15).

Purification of LBP and production of LBP depleted human serum.

LBP was isolated from human plasma by selective affinity immunosorption as described before (21). Plasma from healthy volunteers was kindly provided by the local blood bank. In short, anti-hLBP mAb HM-14 was cross-linked to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Human plasma was applied to the anti-LBP column and unbound proteins were washed out with 0.5 M MgCl. Bound LBP was eluted with 0.1 M Glycine-HCl buffer (pH 2.5). LBP depleted serum was produced by passing serum over the anti-LBP column. LBP depleted serum, contained less LBP then 0.05% of normal serum as assessed by LBP-ELISA.

Preparation of biotin labeled LBP and LPS.

LPS and LBP were biotinylated using hydrazide-biotin (Pierce). Briefly, LBP and LPS were oxidized with sodium periodate to generate aldehydes from vicinal diols present on the saccharide units. The products were then made to react with the hydrazide functionalities present on the biotin, forming stable hydrazone linkages. Linking biotin to the saccharide units in the LBP glycoprotein using hydrazide-biotin did not alter the biologic activity of LBP (data not shown).

Assays for evaluating the association of LBP and LPS to (apo)lipoprotein.

For detection of complexes of LBP and apoB containing lipoproteins in serum an assay was developed. 96 wells plates were coated with a monoclonal Ab against LBP (HM14). As controls non-coated plates or plates coated with an aspecific Ab (rat anti-murine TNF-R75) were used. Aspecific binding sites were blocked for 1 h with 1% BSA in PBS. Dilution series of human serum were applied and incubated for 1 hour, followed by extensive washing of the plates. Captured apoB was detected using a peroxidase labeled monoclonal Ab against apoB followed by tetramethylbenzidine

(TMB) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The absorbance at 450 nm was determined using an ELISA reader.

For studying the quantitative binding of LBP and LPS to lipoproteins an *in vitro* assay was used. Lipoprotein fractions isolated from pooled human serum were standardized for cholesterol concentration. 96 well plates were coated with lipoproteins or apolipoproteins for 2 hours at 37°C. Aspecific binding sites were blocked for 1 h with 1% BSA in PBS. Plates were washed with PBS. Concentration ranges of biotin labeled LBP or LPS diluted in 0.1% BSA in PBS, were incubated for 1 hour at 37°C with the immobilized lipoproteins or apolipoproteins. Bound LBP or LPS were detected using peroxidase-conjugated streptavidin and TMB.

To determine the effect of LBP association with LDL and VLDL on its capacity to interact with LPS, isolated LDL and VLDL were coated to plates in concentrations of 2 and 14 µg cholesterol/ml respectively. After washing of plates and blocking of aspecific bindingsites with 1% BSA in PBS, a concentration range of LBP was incubated and allowed to associate with the lipoproteins overnight at 37°C. Unbound LBP was washed out and biotin labeled LPS was incubated for 1 hour at 37°C. After washing of plates bound LPS was detected by addition of streptavidin-peroxidase and TMB. The LPS binding capacity of the LBP-lipoprotein complexes was compared with that of lipoproteins free of LBP.

Results

LBP circulates associated with apoB containing lipoproteins in healthy persons.

The distribution of LBP among lipoproteins in human serum derived from healthy volunteers was evaluated using agarose gel electrophoresis in combination with Western blot analysis. Agarose gelelectrophoresis avoids the harsh conditions of several other techniques for separation of lipoproteins such as high salt concentrations and high gravitational forces encountered in ultracentrifugation or perturbation from polyanion precipitation, which cause loss of associated proteins (22). Accordingly, LBP was not detectable by ELISA (detection limit 200 pg/ml) in the lipoprotein fractions separated by ultracentrifugation. In addition this technique enables to study interactions of serum constituents proportional to serum levels.

FIGURE 1. Distribution profile of LBP among lipoproteins in serum of healthy persons. Serum from three healthy persons (lane 1-3) and LBP depleted serum (lane 4) were subjected to agarose gel electrophoresis, blotted and probed with specific Abs to human LBP, apoA-I and apoB respectively. LBP co-localizes with apoB in the β-mobility region and not with apoA-I.

Surprisingly, Western blot analysis with an LBP specific Ab shows that in all subjects investigated LBP is located only at the β (LDL/VLDL) mobility region (Fig. 1). As a control, LBP depleted serum derived by selective affinity immunosorption was used,

which lacked reaction with the anti-LBP Ab (Fig. 1). Immunoblotting for apoB demonstrates co-localization of LBP with apoB (Fig. 1). LBP was not detected in the α mobility region and did not co-localize with apoA-I (Fig. 1).

Free LBP, purified from human plasma and devoid of lipoproteins, migrates between the β and α -region on agarose gel electrophoresis (Fig. 2). We studied whether the β -mobility of LBP in serum, is due to association of LBP with lipoproteins. To this end lipoproteins were fractionated by ultracentrifugation. The isolated lipoproteins were pre-incubated at approximately 50% of their plasma concentration overnight at 37°C with LBP (30 μ g/ml), subjected to electrophoresis, and Western blot analysis of LBP was performed. Pre-incubation of purified LBP with VLDL or LDL, restored the β mobility of LBP (Fig. 2). This conversion in electrophoretic migration did not occur when LBP was pre-incubated with HDL. These results show that in serum the association of LBP with LDL and VLDL is responsible for its β electrophoretic mobility.

FIGURE 2. Effect of LDL and VLDL on the electrophoretic mobility of purified LBP. Purified LBP was preincubated with PBS and isolated LBP-free HDL, VLDL or LDL (0.1, 0.035 and 0.5 mg cholesterol/ml respectively). Agarose gel electrophoresis of purified LBP and the preincubated fractions was followed by Western blot analysis of LBP using a specific Ab. Purified LBP migrates in between the β and α mobility region. Pre-incubation of LBP with isolated VLDL or LDL shifts the migration of LBP to the β mobility region.

Further evidence for the association between LBP and apoB containing lipoproteins in human serum was found using an assay that specifically and quantitatively detects complexes of LBP and apoB containing lipoproteins in serum. In this assay LBP containing lipoproteins are captured from serum by solid phase anti-LBP Abs, followed by detection of apoB in the captured lipoproteins by peroxidase labeled anti-apoB Abs. Consistent with our prior experiments, this assay demonstrates the association of LBP with apoB containing lipoproteins in serum (Fig. 3).

To characterize the association between LBP and the different lipoproteins in a more quantitative manner a binding assay was used. Binding of purified LBP to isolated lipoproteins standardized for cholesterol concentration was studied using this assay. Consistent with the results described above, a concentration dependent binding of LBP to LDL and VLDL is observed (Fig. 4). In contrast, the association of purified LBP with HDL is only minor.

FIGURE 3. *Presence of apoB in LBP-containing lipoproteins.* LBP containing lipoproteins were captured from serum by applying serum to 96-well plates coated with an mAb against LBP (diamonds) followed by extensive washing of the plates. As a control serum was also applied to non-coated plates (squares) or plates coated with an aspecific Ab (rat anti-murine TNF-R75) (triangles). Presence of apoB in the captured lipoproteins was detected by addition of a peroxidase labeled monoclonal Ab against apoB and is expressed as mean $\pm$ sd of the OD 450 nm of four wells.

In conclusion we now demonstrate that isolated LDL and VLDL display higher LBP binding capacity compared to HDL. This is fully in line with our observation that LBP circulates predominantly in association with apoB containing lipoproteins.

LPS interacts predominantly with apoB containing lipoproteins.

LBP binds LPS and catalyzes the transfer of LPS from micelles into lipoproteins (12). In the context of this function of LBP and our data demonstrating that LBP circulates as a complex with apoB containing lipoproteins we studied the relation between LBP-lipoprotein interaction and LPS binding to lipoproteins. To this end, biotin labeled LPS was pre-incubated with human serum from healthy volunteers followed by separation of serum lipoproteins on agarose gels and Western blot analysis. Figure 5 reveals that LPS incubated in serum is recovered in the β mobility region. This pattern is consistent with a predominant interaction of LBP with apoB containing lipoproteins. To test whether LPS binding to apoB containing lipoproteins is LBP-dependent, biotin labeled LPS was incubated with LBP depleted serum. Figure 5 demonstrates that LPS also binds to β -lipoprotein in the absence of LBP.

To study whether non-lipoprotein associated serum factors are responsible for the interaction of LS with LDL and VLDL, serum lipoproteins were separated by agarose gel electrophoresis followed by Western blot analysis. Biotinylated LPS was allowed to associate with the (apo)lipoproteins on the blot membrane. The distribution profile obtained was similar to the profile obtained as when LPS was pre-incubated with serum (data not shown). This suggests that β -lipoprotein associated factors account for the LPS binding and that other serum factors are not essentially affecting the distribution.

The binding of LPS to lipoproteins was further characterized using an assay similar to that used above for studying LBP-lipoprotein interaction. Binding of LPS to isolated lipoproteins standardized for cholesterol concentration was studied. Also in this assay and fully in line with the results found by Western blot analysis, LDL and VLDL display high LPS binding capacity under LBP free conditions, whereas LPS binding to purified HDL is minor (Fig.6).

FIGURE 4. Association of LBP with different lipoprotein classes. Lipoproteins isolated from human serum by ultracentrifugation, free of LBP and standardized for cholesterol concentration were immobilized to 96 well plates and incubated with biotinylated LBP. Bound LBP was detected by peroxidase-conjugated streptavidin and TMB. Binding of LBP to the lipoproteins is expressed as mean $\pm$ SD of the OD 450 nm of four wells after correction for background. LDL and VLDL display high LBP binding capacity in contrast to HDL.

FIGURE 5. Distribution profile of LPS among lipoproteins in serum of healthy persons. Serum of three healthy donors and LBP depleted serum were preincubated with biotinylated LPS. Agarose gel electrophoresis of the sera was followed by Western blotting. Biotinylated LPS was detected using peroxidase-conjugated streptavidin and a chemiluminescent substrate. LPS incubated with serum is recovered in the β mobility region.

FIGURE 6. Association of LPS with different lipoprotein classes. Lipoproteins isolated from human serum by ultracentrifugation, free of LBP, and standardized for cholesterol concentration were immobilized to 96 well plates followed by incubation with biotinylated LPS. Binding of LPS to the lipoproteins was detected by peroxidase-conjugated streptavidin and TMB and expressed as mean $\pm$ SD of the OD 450 nm of four wells after correction for background. LDL and to a lesser extent VLDL display high LPS binding capacity in contrast to HDL.

LBP bound to lipoproteins enhances the interaction of LPS with lipoproteins.

To elucidate whether LBP associated to LDL and VLDL is functionally active in transferring LPS to lipoproteins, we compared LPS binding to lipoproteins with binding of LPS to LBP-lipoprotein complexes. To this end LDL and VLDL were immobilized to 96 wells plates and a concentration range of LBP was allowed to bind to the lipoproteins for 18 hours at 37°C. Plates were washed to remove unbound LBP before biotinylated-LPS was added to the preformed LBP-lipoprotein complexes. LBP associated with LDL and VLDL enhanced the binding of LPS to these lipoproteins dose dependently (Fig. 7). These data strongly indicate that LBP bound to LDL and VLDL displays functional properties and enhances the LPS binding capacity of LDL and VLDL.

Both, LBP and LPS associate with apolipoprotein B.

To elucidate whether binding of LBP and LPS to LDL and VLDL depends on interaction with apoB, present in both LDL and VLDL, we studied the binding of LBP and LPS to purified apoB. Contemporaneously, binding of LBP and LPS to purified apoA-I, the predominant apolipoprotein in HDL, was evaluated. Both LBP and LPS bind to apoB dose dependently (Fig. 8A,B). Binding of LPS to apoA-I was however not significant (Fig. 8A), which is in accordance with the minor binding of LPS to HDL. Surprisingly, LBP forms a complex with purified apoA-I (Fig. 8B).

Because circulating LBP is predominantly associated with lipoproteins containing apoB and not apoA-I (Fig.1), we compared the relative affinities of LBP for apoB and apoA-I. To this end, plates were coated with apoB and a dilution series of apoA-I and apoB was added to the plates together with biotinylated LBP (Fig. 8C). The concentration necessary for 50% reduction of the signal was 50 nM for apoB and 500

nM for apoA-I. These results can be due either to a difference in affinity for LBP or to the possibility that apoA-I and apoB have affinity for a different site of LBP. However, when plates were coated with apoA-I, comparable results were found (data not shown); apoA-I concentrations necessary for 50% reduction of the signal were tenfold higher than apoB concentrations. These data strongly suggest that apoA-I and apoB compete for the same binding site on LBP and that the affinity of LBP for apoB is tenfold higher than for apoA-I.

FIGURE 7. LBP associated with LDL and VLDL enhances the interaction of LPS with lipoproteins. Plates were coated with isolated LDL (2 mg cholesterol/ml) or VLDL (14 mg cholesterol/ml). The immobilized lipoproteins were preincubated with a concentration range of LBP overnight at 37°C. Unbound LBP was removed by washing the plates and biotinylated LPS was added to the LBP-lipoprotein complexes. Plates were washed to remove unbound LPS and bound LPS was detected using peroxidase-conjugated streptavidin and TMB. Binding of LPS to the lipoproteins is expressed as mean $\pm$ SD of the OD 450 nm of four wells after correction for background. LBP associated to LDL and VLDL enhances the binding of LPS dose-dependently.

Since lipoprotein associated LBP enhances the binding of LPS to apoB containing lipoproteins, we evaluated whether LBP also catalyzes the binding of LPS to the purified apolipoproteins. To this end plates were coated with apoB and apoA-I and biotinylated LPS was added to the immobilized apolipoproteins together with 1.7 nM LBP. This LBP concentration was demonstrated to enhance the binding of LPS to LDL and VLDL (Fig. 7). LBP enhanced the binding of LPS to apoA-I, whereas it decreased the binding of LPS to apoB (Fig. 9). These results suggest that the interaction of LBP with apoA-I enables the binding of LPS to this apolipoprotein, whereas LBP competes with LPS for binding to apoB.

Overall these data suggest that apoB contains a binding site for both LBP and LPS and it appears that this binding site account at least in part for the above described association of LBP and LPS with LDL and VLDL in serum.

LBP and LPS associate with LDL and VLDL in serum of septic patients.

Lipoprotein metabolism as well as the composition of lipoproteins is altered during the acute phase response. In addition, serum levels of LBP rise dramatically during sepsis (15). Because the data so far indicate that the association of LBP with lipoproteins affect the functional properties of LBP and lipoproteins which is of utmost interest during endotoxemia, we evaluated in which form the plentitude of LBP produced

FIGURE 8. Association of LBP and LPS with apoB and apoA-I. A) Binding of biotin-labeled LPS to apolipoproteins was evaluated. To this end plates were coated with a concentration range of apoB and apoA-I and biotin labeled LPS was added. Bound LPS was detected by peroxidase-conjugated streptavidin and TMB and expressed as mean $\pm$ SD of the OD 450 nm of four wells after correction for background. B) Binding of biotin labeled LBP to immobilized apoB (25 nM) or apoA-I (100 nM) was detected by peroxidase-conjugated streptavidin and TMB and expressed as mean $\pm$ SD of the OD 450 nm of four wells after correction for background. C) The relative affinities of LBP for apoB and apoA-I were evaluated. Biotinylated LBP was added to plates coated with apoB (25 nM). Inhibition of this interaction of LBP with apoB by apoA-I and apoB was studied by adding concentration ranges of apoA-I and apoB together with LBP. Bound LBP was detected by peroxidase-conjugated streptavidin and TMB and expressed as mean $\pm$ SD of the OD 450 nm of three wells after correction for background.

during sepsis circulates. Serum derived from four septic patients (LBP serum concentration 121-589 $\mu\text{g/ml}$ compared to 13-16 $\mu\text{g/ml}$ in the healthy controls) were subjected to agarose gel electrophoresis followed by Western blot analysis. As observed for LBP in serum from healthy persons, LBP circulates during septicemia predominantly associated with LDL and VLDL (Fig.10). However, an additional LBP band is observed between the β and the α region, indicative for the presence of free LBP. As expected, apoA-I levels dropped markedly in the septic patients (Fig. 10). Also in septic patients no association of LBP with HDL was observed.

In addition, we investigated whether the alterations in the composition of lipoproteins during infection and the presence of free LBP did affect the distribution of LPS among lipoproteins. Biotinylated-LPS pre-incubated with serum from septic patients was found to migrate with β electrophoretic mobility and to co-migrate with apoB. These findings are in accordance to the data obtained from healthy persons and suggest that also during an acute phase response LPS binds predominantly to LDL and VLDL (Fig. 10D).

FIGURE 9. LBP enhances binding of LPS to apoA-I and reduces binding of LPS to apoB. Plates were coated with apoA-I (25 nM) or apoB (100 nM). A total of 1.7 nM of LBP was added together with biotinylated LPS, followed by peroxidase-conjugated streptavidin and TMB. Binding of LBP to the apolipoproteins is expressed as mean $\pm$ SD of the OD 450 nm of four wells after correction for background. The binding of LPS to apoA-I is markedly enhanced by LBP, whereas LBP reduces the binding of LPS to apoB.

Discussion

The structure and function of LBP is extensively studied, however knowledge concerning the *in vivo* forms or associations with other serum components and the effect of these associations on the function of LBP is limited. In the present study, we obtained evidence that LBP circulates associated with apoB containing lipoproteins in healthy persons and septic patients. This association is functional, since LBP bound to LDL and VLDL was observed to enhance the LPS binding capacity of these

lipoproteins, a process known to result in protection from the deleterious effects of LPS-toxicity.

FIGURE 10. Distribution profile of LBP and LPS in serum of septic patients. Agarose gel electrophoresis of serum from four septic patients (lanes 1-4) was followed by Western blot analyses using specific Abs for LBP, and apoB. Agarose gel electrophoresis of serum from three septic patients (lanes 1-3) and a serumpool of healthy persons (lane 4) was followed by Western blot analyses using specific Abs for apoA-I. LBP predominantly co-localizes with apoB. An additional band compared to normal serum is observed in all subjects between the β and α regions, and is most explicit in subject 3. Preincubation of LPS with the sera followed by Western blot analyses demonstrates the distribution of LPS among lipoproteins during an acute phase response. LPS colocalizes with LBP and apoB in the β region under these conditions.

In search for the factors involved in the association of LBP with apoB containing lipoproteins in serum we found that this interaction is at least in part mediated by an interaction of LBP with apoB. Although LBP was not associated with apoA-I or HDL in serum, our experiments demonstrate that LBP does bind to purified apoA-I. Evidence for a specific interaction of LBP with apoA-I is supported by studies by Massamiri et al. who demonstrated that binding of LBP to reconstituted HDL is partially blocked by Abs against apoA-I (23). In the present study we observed a tenfold higher affinity of LBP for apoB compared to apoA-I (Fig. 8C). The amount of apoA-I molecules in serum however, is about thirty times higher than apoB under healthy conditions, and with the present knowledge it is not possible to subscribe the predominant interaction of LBP with LDL and VLDL to the higher affinity of LBP for apoB. It is possible that LBP interacts with domains of apoA-I that are masked by incorporation in native HDL. Furthermore, other constituents than apoB may contribute to the high affinity of LBP for LDL and VLDL and consequently increase the competition for binding of LBP to HDL.

The LBP transporting lipoproteins LDL and VLDL were also found to be the predominant LPS binding lipoproteins in normal human serum, an observation which is supported by others (5, 24). Studies performed in rodents report the preferential binding of LPS to HDL (4, 25, 26, 27). Relative high HDL levels in rodents seem to account for the predominant binding of LPS to HDL, since high LDL levels in Watanabe heritable hyperlipidemic rabbits and high VLDL levels in apoE knockout mice result in the predominant binding of LPS to LDL (5, 28) and VLDL (Kuiper personal communication) respectively. In contrast to rodents, the HDL:LDL/VLDL ratio is low in humans, which most likely causes the differences observed for the distribution of LPS among lipoproteins in humans and rodents.

Our data further demonstrate that LBP associated with LDL and VLDL strongly enhances binding of LPS to these lipoproteins in a dose dependent fashion. However, presence of LBP is not required for the interaction of LPS with LDL and VLDL.

ApoB, present in LDL and VLDL, was demonstrated to have high affinity for LPS and therefore may account for LPS binding under LBP-free conditions. This interaction of LPS with apoB is inhibited by presence of LBP, implicating competition of LBP and LPS for apoB. It can be hypothesized from these data that apoB functions as an anchor for LBP on LDL and VLDL. This bound LBP may catalyze the binding of LPS to other lipoprotein components and thereby enhance the total LPS binding capacity. Since others found that a lipid-lipid interaction accounts for the association of LPS with lipoproteins (5) and have demonstrated that LBP enhances binding of LPS to phospholipid membranes (29, 30), it is likely that LBP linked to apoB enhances intercalation of LPS into the phospholipid membrane of the lipoproteins. Other proteins present in LDL and VLDL may however also contribute to the LPS binding capacity of these lipoproteins. ApoE, present in VLDL, for instance was demonstrated to bind LPS (31). Most interestingly, apoE-deficient mice display increased susceptibility to endotoxemia (32), the latter supporting a possible role for apoE in the scavenging of LPS.

During infection, lipoproteins are proven to be fundamental for the survival of the host (1, 2, 9, 10, 19). Under these conditions lipoprotein levels and composition are known to be altered profoundly (33). These changes prompted us to evaluate the interaction of lipoproteins with LBP and endotoxins in septic serum. The changes in lipoprotein composition and the more than tenfold enhanced LBP levels did not significantly affect the distribution profile of LBP. In serum of septic patients LBP is also co-located predominantly with apoB (Fig. 10). These observations indicate that during sepsis approximately tenfold more LBP is associated with LDL and VLDL. This is of particular interest since we observed that the LBP induced up-regulation of LPS binding to these lipoproteins is dose dependent. Only a small fraction of the total LBP in septic sera migrates as free LBP. Whether there is a difference in biologic activity between lipoprotein associated and non-lipoprotein associated LBP in the host response to endotoxin is under current investigation. The predominant incorporation of LPS in LDL and VLDL is also observed in septic serum and does not seem to be influenced by the alterations in lipoprotein composition and the presence of free LBP. Our results which indicate that LPS and LBP both predominantly bind to LDL and VLDL under septic conditions and that apoB forms a binding site for LBP and LPS, is in line with the findings of others (16) that in contrast to apoA-I, apoB levels stay high during infection. The remaining high concentration of apoB in LDL and VLDL seems functional since it may contribute to the enhanced binding of LBP, and as a consequence LPS, to these lipoproteins during infection.

It is firmly established that binding of LPS to lipoproteins reduces LPS toxicity (3). In accordance, hypolipidemia results in enhanced LPS-induced lethality in animals (2) and in human low levels of cholesterol predict an increased risk of death from infection (34), which emphasizes the significance of lipoproteins in protection from bacteria and their toxins. The protective function of lipoproteins is considered to be due to an increase in the clearance of LPS by formation of LPS-lipoprotein complexes and to prevention of its binding to cells. In addition, it was recently demonstrated that lipoproteins, including LDL, also promote the release of cell associated LPS, which was proven to be dependent on LBP (35). This inhibition of endotoxin binding reduces activation of monocytes and thereby the secretion of cytokines (3, 35, 36). This

protective property of lipoproteins is not only described for Gram-negative bacteria, also the toxic effects of fragments of Gram positive bacteria are inhibited by LDL, a process catalyzed by the presence of LBP (13). The beneficial role for LDL in the host defense against bacteria is supported by a study, which demonstrates that LDL receptor deficient mice, with elevated circulating LDL concentrations, are protected against lethal endotoxemia and severe infections with Gram-negative micro-organisms (19).

The uptake of LPS into LDL, which we consider beneficial during acute infection, should be considered as potentially harmful during chronic inflammation. Since we studied LPS-binding to LDL and VLDL and not toxicity, we cannot exclude that LPS bind to LDL and VLDL in a manner by which it retains some toxic activity. In the long term these complexes may play a pathogenic role in the development of atherosclerosis. Although LDL protects endothelial cells from acute LPS toxicity by formation of LPS-LDL complexes, these complexes migrate across the endothelium and via unknown pathways increase the secretion of monocyte chemotactic activity by endothelial cells (28). As a consequence transport of LDL-LPS complexes into the artery wall may initiate an inflammatory response and provoke an atherosclerotic reaction.

In conclusion, we found strong evidence for an association of LBP with apoB containing lipoproteins in the circulation of healthy persons. Also in septic patients with extremely high circulating LBP concentrations, LBP is predominantly bound to apoB containing lipoproteins. Most interestingly, LBP associated with LDL and VLDL enhances the LPS binding capacity of these lipoproteins in a dose dependent manner. Accordingly, in serum from healthy persons and from septic patients LDL and VLDL are the predominant LPS binding lipoproteins. Overall, the data of this study suggest that LBP is a cofactor of circulating LDL and VLDL, which facilitates the uptake of endotoxin by these lipoproteins and imply an important role for LBP/LDL and VLDL complexes in the defense against bacteria and endotoxin.

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Chapter 5

Lipopolysaccharide Binding Protein mediates LPS detoxification by chylomicrons

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Introduction

Besides a crucial role in the transport of dietary lipids, chylomicrons appear to reinforce the host defense against lipopolysaccharide (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 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. recently presented data indicating that both the LDL receptor and LDL receptor related protein (LRP) participate in the rapid internalization of chylomicron-bound LPS by hepatocytes (6). 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 of Kitchens et al demonstrated that LPS already bound to monocytes is released in response to lipoproteins thereby reducing cytokine secretion (9). 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 of 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 VLDL and LDL, which binding also results in inactivation of LPS (15, 16, 17). We recently demonstrated that LBP circulates in complex with apoB-containing lipoproteins, i.e., low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) (18). LBP, complexed with LDL and VLDL strongly enhances the LPS binding to these lipoproteins, which suggests an important role for LBP/lipoprotein 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 the 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.

Material & methods

Reagents

LPS from *Escherichia coli*, serotype 055:B5 was purchased from Sigma (St.Louis, MO). Polyclonal Abs (pAb) to human LBP were 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 less than 1 ng LPS/mg) was derived from Sigma. Human rTNF- α was kindly provided by BASF/Knoll Ag (Ludwigshafen, Germany). Since lipoteichoic acid (LTA) preparations available sofar are highly contaminated by LPS (19), we have used a recently introduced preparation method for LTA (20) which allows now to study effects of LPS-free, biologically active LTA. LTA 99% pure and containing less then 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 hours postprandially. To prepare serum, blood was allowed to clot for 1 hour at room temperature. Fresh serum was used for isolation of lipoproteins.

Lipoprotein isolation

Chylomicrons were isolated from pooled postprandial human serum (6 donors) by ultracentrifugation according to Terpstra et al (21). Briefly, dry KBr (770 mg) and sucrose (50 mg) together with 2 ml of serum were placed in the bottoms of ultra clear centrifuge tubes (Beckman Instruments, Brea, CA). These components were carefully mixed with a spatula. A discontinuous gradient was made with the serum mixture of density 1.250 g/ml, two KBr solutions with density 1.225 and 1.100 g/ml respectively, and on top water density 0.998 g/ml. The tubes were placed in a SW41 rotor and centrifuged in a Beckman XL-80 ultra-centrifuge (Beckman Instruments) at 30,000 rpm and 20°C for 40 minutes. The chylomicrons were collected by aspiration of the top 1 ml fraction and stored at -80°C until use. HDL, LDL and VLDL were isolated from pooled fresh human serum (6 healthy donors) by a 22 hours single spin density gradient ultracentrifugation at 40.000 rpm and 17°C according to Terpstra et al. (22), using a Beckman XL-80 ultra centrifuge with a SW40 rotor. A discontinuous gradient was constructed from 2 ml serum adjusted to density 1.250 g/ml with KBr and sucrose, two NaCl/KBr solutions with density 1.225 and 1.100 g/ml respectively, and water density 0.998 g/ml. All solutions contained 0.1 mg/ml EDTA. The VLDL, LDL and HDL fractions were collected by aspiration and dialyzed extensively against PBS at 4°C and stored at -80°C until use. The lipoprotein fractions used contained less then 4 ng/ml LBP as assessed by LBP specific ELISA (23).

Purification of LBP

LBP was isolated from human plasma by selective affinity immunosorption as described before (18). In short, anti-hLBP mAb HM-14 was cross-linked to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Normal human plasma kindly provided by the local blood bank was applied to the anti-LBP

column and unbound proteins were washed out with 0.5 M MgCl. Bound LBP was eluted with 0.1 M Glycine-HCl buffer (pH 2.5).

Preparation of biotin labeled LBP and LPS.

LPS and LBP were biotinylated using hydrazide-biotin (Pierce) as described before (18). Briefly, LBP and LPS were oxidized with sodium periodate to generate aldehydes from vicinal diols present on saccharide units. The products were then made to react with the hydrazide functionalities present on the biotin, forming stable hydrazone linkages. Linking biotin to the saccharide units in the LBP glycoprotein using hydrazide-biotin did not alter the biologic activity of LBP (data not shown).

Assays for evaluating the association of LBP and LPS to chylomicrons.

To study binding of LBP and LPS to chylomicrons an *in vitro* assay was used. 96 well plates were coated with chylomicrons for 2 hours at 37°C. Non-specific binding sites were blocked by 2 h incubation with 5% BSA in PBS. Plates were washed with PBS. A concentration range of biotin labeled LBP diluted in 0.1% BSA in PBS, was incubated with the immobilized chylomicrons for 1.5 hour at 37°C. In control experiments 10 µg/ml anti-human LBP pAb or aspecific pAb were added together with LBP. Bound LBP was detected using peroxidase-conjugated streptavidin and tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

The effect of LBP association with chylomicrons on the capacity of these lipoproteins to interact with LPS was evaluated. To this end, chylomicrons were coated to plates and non-specific binding sites were blocked with 5% BSA in PBS. A concentration range of LBP was incubated for 1.5 h and allowed to associate with the chylomicrons at 37°C. Unbound LBP was washed away and biotin labeled LPS was incubated with the chylomicron-LBP complexes for 1 hour at 37°C. In control experiments 10 µg/ml anti-human LBP pAb was incubated for 1 hour at 37°C with the LBP-chylomicron complexes and biotin labeled LPS was added together with anti-LBP Ab. After washing of plates bound LPS was detected using streptavidin-peroxidase and TMB. The LPS binding capacity of the LBP-chylomicron complexes was compared with that of chylomicrons free of LBP.

Labeling of LPS with BODIPY and measurement of fluorescence

LPS from *Escherichia coli* serotype 055:B5 was labeled with the fluorophore BODIPY FL SE (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Fluorescent labeled LPS can be used to study binding and transfer of LPS in real time (24). Time dependent changes in fluorescence caused by addition of LBP and chylomicrons were measured with a Spectra Max Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA) with emission at 518 nm and excitation at 485 nm.

Cell isolation and stimulation.

For experiments designed to determine the effect of chylomicron binding of toxin derived from Gram-negative bacteria as well as from Gram-positive bacteria on their biologic activity, cytokine induction by toxin incubated with chylomicrons in the

presence or absence of LBP was assessed. PBMC were isolated from buffy coats of healthy volunteers, kindly 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 suspended in Macrophage Serum Free Medium (Life Technologies, Paisley, UK) supplemented with 100 U/ml penicillin 100 ug/ml streptomycin (Life Technologies). LPS and LTA were pre-incubated with LBP, LBP and lipoproteins or lipoproteins alone as indicated in 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA) for 24 h 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 and 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, Westgrove, 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.

FIGURE 1. *LBP associates with chylomicrons.* Chylomicrons isolated from postprandial human serum by ultracentrifugation were immobilized to 96 well plates and incubated with biotinylated LBP (●). As a control a pAb against human LBP was added together with LBP (■). Bound LBP was detected by peroxidase-conjugated streptavidin and TMB. Binding of LBP to the lipoproteins was expressed as mean $\pm$ SD of the OD 450 nm of four wells of a representative experiment repeated twice after correction for background binding of biotin labeled LBP to coated plates in absence of chylomicrons. A dose-dependent association of LBP with chylomicrons was observed.

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 another triacylglyceride-rich apoB-containing lipoprotein, i.e. chylomicrons. To study binding of LBP to isolated chylomicrons an *in vitro* assay was 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.

FIGURE 2. *LBP associated with chylomicrons enhances the interaction with LPS.* A) Immobilized chylomicrons were preincubated with a concentration range of LBP overnight at 37°C, unbound LBP was removed by washing the plates, followed by addition of biotinylated LPS to the LBP-chylomicron complexes. Bound LPS was detected using peroxidase-conjugated streptavidin. Binding of LPS to chylomicrons is expressed as mean $\pm$ SD of the OD 450 nm of four wells of a representative experiment repeated twice after correction for background binding of biotin labeled LPS to coated plates in absence of chylomicrons. LBP complexed to the chylomicrons enhanced the LPS binding dose-dependently. B) Immobilized chylomicrons were preincubated either with or without 1 μ g/ml LBP per ml overnight at 37°C (open bars). Unbound LBP was removed. In controls 10 μ g/ml anti-human LBP pAb was added for 1 hour (filled bars). Biotinylated LPS was added to the LBP-chylomicron complexes. Binding of LPS was assessed and values expressed as in A. The anti-human LBP pAb completely blocked the LBP mediated binding of LPS to chylomicrons.

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 the LPS binding to chylomicrons, an *in vitro* assay was used. LPS binding to

Abstract

Chylomicrons have been shown to protect against endotoxin-induced lethality. LPS Binding Protein (LBP) is involved in inactivation of bacterial toxin by lipoproteins. The current study examined the interaction between 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 LDL, VLDL, and HDL, chylomicrons exceeded the other lipoproteins in LPS inactivating capacity. Furthermore, highly purified lipoteichoic acid (LTA), 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 consider that LBP-chylomicron complexes may be part of a local defense mechanism of the intestine against translocated bacterial toxin.

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 found to be dose dependent. This effect of LBP was blocked by pAb against LBP (Fig.2b). The 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.

FIGURE 3. *LBP accelerates the binding of LPS to chylomicrons* BODIPY-LPS was incubated with PBS alone (curve A), with 1 and 10 μg LBP/ml (curve B and curve C), with chylomicrons (curve D), with chylomicrons and 1 μg LBP/ml (curve E) and with chylomicrons and 10 μg LBP/ml (curve F). The fluorescence at 518 nm was recorded over time. The figure is representative of two separate experiments. LBP was found to dose dependently accelerate the binding of 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 time kinetics of this interaction we used BODIPY-LPS donor micelles and chylomicrons as acceptor particles. Addition of chylomicrons to BODIPY-LPS in absence of LBP resulted in a dequenching of the fluorescence, 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 found to be LBP dose dependent. These findings are in line with our observations described above and implicate that LBP catalyzes LPS binding to chylomicrons and more importantly accelerate LPS binding to chylomicrons.

FIGURE 4. Chylomicrons reduce LPS toxicity in an LBP-dose dependent fashion. LPS 100 ng/ml was preincubated with 0, 10, 50 and 100 ng/ml LBP in the presence (●) or absence (■) of isolated chylomicrons (12.5% of circulating levels) for 24h at 37°C. The remaining biological activity of LPS was determined by incubation with PBMC for 4h and measurement of TNF- α (A) and IL-8 (B) secretion in the supernatants. Values represent the mean $\pm$ SD of three wells. The experiment shown is representative of three separate experiments. LBP catalyzed the LPS neutralization by 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.

FIGURE 5. Chylomicrons neutralize LPS in a dose-dependent fashion. A range of chylomicron concentrations and 100 ng/ml LPS were incubated in the presence of 100 ng/ml LBP. The remaining biological activity of LPS was assessed by incubation with PBMC for 4 h and measurement of IL-8 (●) and TNF- α (■) secretion. Values represent the mean $\pm$ SD of three wells. The experiment shown is representative of three separate experiments. Chylomicrons dose-dependently reduced LPS activity in the presence of LBP.

FIGURE 6. *LPS is rapidly neutralized by chylomicron-LBP complexes.* LPS 100 ng/ml was preincubated with (filled bars) or without (open bars) chylomicrons (12.5% of circulating levels) and 100 ng/ml of LBP for a 0.5 h period. Biologically active LPS remaining after 0.5 h was assessed by addition of PBMC and measuring cytokine secretion. Values represent the mean $\pm$ SD of three wells. The experiment shown is representative of three separate experiments. LPS neutralization by chylomicron-LBP complexes was evident after 0.5 h incubation.

To study the effect of the chylomicron concentration on inhibition of LPS-induced cytokine secretion, 100 ng/ml LPS was preincubated with 100 ng/ml LBP and different concentrations of chylomicrons (Fig. 5). The LPS detoxifying function of chylomicrons was found to be concentration dependent. Moreover, 50% of the postprandial circulating level of chylomicrons abrogated the cytokine response induced by 100 ng/ml 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 hour and PBMC were added. Figure 6 demonstrates that in the presence of LBP chylomicrons are capable of reducing LPS activity within 0.5 hour.

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 a LBP dependent fashion (11). To determine to which extend postprandial chylomicron levels contribute to the LPS neutralization by lipoproteins, the LPS detoxifying capacity of lipoproteins was compared at concentrations equated to their physiologic plasma ratio. For chylomicrons that regards 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 LPS together with 50 ng/ml LBP led to a strong increase of 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.

FIGURE 7. LPS neutralizing capacity of chylomicrons compared to other circulating lipoproteins. Lipoproteins were isolated from human serum by ultracentrifugation. HDL, LDL, VLDL and chylomicrons were diluted to 12.5% of their plasma concentration and incubated with 100 ng/ml LPS and 50 ng/ml LBP for 24h at 37°C. PBMC were added and the remaining LPS activity was assessed by measuring the cytokine secretion of the cells after 4h. Values represent the mean $\pm$ SD of three wells. The experiment shown is representative of three separate experiments. The LPS neutralizing capacity of the chylomicrons exceeded the capacity of LDL, VLDL and HDL to neutralize LPS.

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 stimulation of PBMC by LTA. Figure 8 demonstrates that LBP also induces detoxification of LTA by chylomicrons dose dependently. These data suggest that besides a role in combating gram-negative bacteria, LBP-chylomicron complexes may also be of importance in the host defense against Gram-positive bacteria.

FIGURE 8. LBP induces detoxification of LTA by chylomicrons. LTA (20 μ g/ml) was preincubated with (filled bars) or without (open bars) chylomicrons (5 % of circulating level) and 0, 10, 50 and 100 ng/ml LBP for 24 h. Biologically active LTA remaining was assessed by addition of PBMC and measuring TNF secretion. Values represent the mean $\pm$ SD of three wells. The experiment shown is representative of three separate experiments. LBP and chylomicrons cooperate in detoxification of LTA.

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 microorganisms. These data imply that LBP is an important co-factor 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 (Fig 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 (PLTP) (32), cholesteryl ester transfer protein (CETP) 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 intravenous 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 implicate that LBP is an important plasma factor involved in LPS binding to chylomicrons.

Harris et al described that infusion of LPS together with chylomicrons compared to infusion of LPS alone resulted in lower levels of circulating TNF, the cytokine known to mediate much of the LPS toxicity *in vivo* (2). 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 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.

Thus far, studies on LPS inactivating properties of chylomicrons were 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 most 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 multi organ failure and sepsis (36).

Employing highly-purified essentially LPS-free LTA, we show that the phenomenon extends also to Gram-positive bacteria. Noteworthy, LBP was not required for activation of PBMC by 20 $\mu\text{g/ml}$ 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, is more and more accepted. Meta analysis have demonstrated that in a large variety of patients, including patients suffering from pancreatitis, ulcerative colitis, Crohn disease, surgery, trauma or multi-organ failure, enteral feeding was associated with a lower risk of infection compared to starvation and parenteral nutrition (38). Early post-operative enteral feeding was found to reduce septic morbidity (39) and hospital stay (40). However, in most centers patients are restricted from early post-operative enteral feeding and from enteral feeding during their stay in the intensive care unit. Such abstinence 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 as compared to the other lipoprotein classes. Since even low concentrations of chylomicrons exert a significant effect on LPS activity and the fact that 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 enhance their clearance from the circulation. We further speculate that production of LBP-chylomicron complexes in the gut may represent a natural defense mechanism against endotoxemia of enteric origin. Enteral feeding, and subsequent chylomicron production, may be a preventive or even therapeutic intervention in patients that 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 post-operative complications (36).

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Chapter 6

Enteral administration of high-fat nutrition before and directly after hemorrhagic shock reduces endotoxemia and bacterial translocation

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Abstract

Endotoxin and bacterial translocation due to gut barrier failure are important initiating events in the pathogenesis of sepsis after haemorrhage. Systemic inhibition of endotoxin activity attenuates bacterial translocation and distant organ damage. Triacylglycerol-rich lipoproteins constitute a physiological means of binding and neutralizing endotoxin effectively. We hypothesized that enhancement of triacylglycerol-rich lipoproteins via high-fat enteral nutrition would reduce endotoxemia and prevent bacterial translocation. The objective of this study was to determine whether potential enhancement of endotoxin neutralization via high-fat enteral nutrition affects endotoxemia and bacterial translocation after haemorrhage.

A rat model of non-lethal hemorrhagic shock was used. Hemorrhagic shock (HS) rats were divided into three groups: rats starved overnight (HS-S); rats fed with a low-fat enteral diet (HS-LF) and rats receiving a high-fat enteral diet (HS-HF). Circulating triacylglycerol and apolipoprotein B, reflecting the amount of triacylglycerol-rich lipoproteins, were elevated in HS-HF rats compared with both HS-S rats ($p<0.005$ and $p<0.05$, respectively) and HS-LF rats ($p<0.005$ and $p<0.05$). Circulating endotoxin was lower in HS-HF rats (7.2 ± 10.2 pg/ml) compared with both HS-S rats (29.1 ± 13.4 pg/ml, $p\leq 0.005$) and HS-LF rats (29.9 ± 5.2 pg/ml, $p<0.005$). In line, bacterial translocation was lower in HS-HF rats (incidence 4/8 rats; median 3 (range 0-144) cfu/gram) compared with both HS-S rats (8/8; 212 (60-483) cfu/gram; $p=0.006$) and HS-LF rats (8/8; 86 (30-209) cfu/gram; $p=0.002$).

This study is the first to show that high-fat enteral nutrition, leading to increased plasma triacylglycerol and apolipoprotein B levels, significantly decreases endotoxemia and bacterial translocation after haemorrhage.

Introduction

Lipopolysaccharide (LPS) or endotoxin, a constituent of the outer membrane of Gram-negative bacteria, is an important mediator in the pathogenesis of the sepsis syndrome after major trauma, surgery and haemorrhage (1, 2). The incidence of sepsis has increased over the years and a further increase is expected due to aging of the population and more complex surgery (3). Although the pathogenesis of the (late) sepsis syndrome after haemorrhage is not clear, gut barrier failure is considered to play a key role (4, 5). Several animal studies clearly show that hemorrhagic shock results in gut barrier failure leading to translocation of endotoxin and bacteria (5, 6, 7, 8, 9). Bacterial toxins such as endotoxin can lead to local activation of the inflammatory system and subsequent production (locally) of inflammatory cytokines leading to a further deterioration of the gut barrier and bacterial translocation (10). Moreover, an increase of systemic endotoxin levels after haemorrhage plays an important role in the development of acute lung injury (8). This vicious circle of endotoxemia and bacterial translocation and subsequent acute lung injury can be interrupted by interventions that neutralize circulating endotoxin (8, 9). Several physiological defence mechanisms protect against endotoxemia such as the complement system, the coagulation cascade, the cytokine response and lipoproteins. Lipoproteins bind and incorporate both Gram-positive and Gram-negative bacterial toxins rapidly, a process which is mediated by lipopolysaccharide binding protein (LBP) and apolipoproteins (11, 12). Detoxification of endotoxin by lipoproteins prevents endotoxin from initiating an inflammatory response. Triacylglycerol-rich lipoproteins in particular are very potent inhibitors of the bioactivity of endotoxin and protect animals against endotoxin-induced lethality (13, 14, 15, 16). Elevation of triacylglycerol-rich lipoproteins, like chylomicrons and very low density lipoproteins (VLDL) would thus induce an increased capacity to inhibit the bioactivity of endotoxin. Physiological elevation of triacylglycerol levels occurs after a fat meal. Chylomicrons, formed in the gut and transported along mesenteric lymphatics, are present locally in the gut in the early postprandial phase. VLDL circulates systemically and is also elevated after enteral feeding (17, 18). Therefore, high-fat enteral nutrition would theoretically be very effective in reducing the bioactivity of enteric derived endotoxin both locally and systemically after disruption of the gut barrier as occurs following hemorrhagic shock in an early stage. Interestingly, fasting is common in surgical patients most at risk for endotoxemia of enteric origin even though a recent meta-analysis indicates that a "nil by mouth" regime is not beneficial in gastro-intestinal surgery (19). In animal studies investigating the pathogenesis of the sepsis syndrome, animals are generally fasted overnight before trauma or haemorrhage (5, 6, 8). Bark et al. (21) reported in rats that brief fasting was associated with significantly increased bacterial translocation following hemorrhagic shock compared with fed animals, indicating the importance of enteral nutrition.

The aim of this study was to induce an increase of triacylglycerol-rich lipoproteins via high-fat enteral nutrition in order to enhance the natural defence mechanism against endotoxin, thereby reducing endotoxemia and bacterial translocation after haemorrhage. In our experiments, we measured circulating triacylglycerol and apoB as indicators of triacylglycerol-rich lipoproteins. Circulating endotoxin and bacterial translocation to mesenteric lymph nodes, spleen and liver were measured as endpoints.

Material and Methods

Animals

The present study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht and this committee approved the protocol. Healthy male Sprague-Dawley rats, weighing 301 – 410 grams (average, 342 grams) purchased from Charles River (Maastricht, the Netherlands) were housed under controlled conditions of temperature and humidity. Before the beginning of the experiments, rats were fed water and chow ad libitum.

Experimental Design

Animals were divided into five groups ($n=8$ per group). Control rats (C) were starved for 18 hours and sacrificed to assess the effect of fasting alone. Sham shock (SS) rats were starved and the femoral artery was cannulated, but no shock was induced. The haemorrhagic shock groups were either starved 18 hours before the procedure (HS-S), or enterally fed with a low-fat liquid enteral diet (HS-LF) or a high-fat liquid enteral diet (HS-HF) via oral gavage. The exact set up of the procedure is displayed in Figure 1. Blood and tissue samples were taken at 24 hours after onset of shock. The low-fat diet contained: 6.9% (energy-percent) proteins, 75.4% carbohydrates and 16.7% fat. The amount of fat in the low-fat diet was isocalorical to that present in standard rodent chow. The high-fat liquid enteral diet was isocaloric and isonitrogenous to the low-fat diet, but contained 6.9% proteins, 40.9% carbohydrates and 52.2% fat. The types of carbohydrates and fat used in both diets were identical. The amount of omega-3 and omega-6 fatty acids in the high-fat nutrition was less than 5% of the total fat content.

FIGURE 1. Experimental procedure and feeding schedule. At -18 hours, rats were starved overnight (1), 45 minutes before withdrawal of blood anaesthesia was given and a femoral artery catheter inserted (2), at $t=0$ hemorrhagic shock was induced (3); after 50 minutes the femoral artery catheter was removed and the wound was closed (4); after six hours all shock groups were allowed standard chow ad libitum (5); At 24 hours after (sham) shock ($t=24h$) rats were sacrificed (6). A liquid enteral nutrition (low-fat or high-fat) was administered via gavage in the fed groups (HS-LF and HS-HF), at -18 hours (3 ml), -2 hours (0.75 ml), -45 minutes (0.75 ml), +3 hours (0.75 ml) and +6 hours (0.75 ml).

Hemorrhagic shock procedure

Rats were anaesthetized with intraperitoneally injected sodium pentobarbital (40 mg/kg). The skin over the left femoral area was shaved and disinfected with povidone iodine solution. The animals were placed in the supine position and allowed to breathe

spontaneously. During surgery and throughout the experiment, body temperature was maintained at 37°C with an infrared heating lamp controlled by a thermo analyser system (Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a rectal probe. The femoral artery was dissected using aseptic technique and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/ml). Arterial blood pressure was continuously measured (Uniflow^{tm} external pressure transducer; Baxter^{TM}, Utrecht, the Netherlands) and recorded as Mean Arterial Pressure (MAP). Heart rate (HR) was continuously assessed from the instantaneous pressure signal. To keep the arterial catheter patent, it was constantly perfused with physiological saline (3ml/h) via the Uniflow^{tm} system; no heparin was used. After an acclimatisation period of 30 minutes, rats were subjected to haemorrhage by withdrawing blood in quantities of 2.1 ml/100 gram of body weight (representing approximately 30-40% of the circulating volume) at a rate of 1 ml/minute. At 50 minutes after the induction of shock, the catheter was removed and the femoral artery ligated. Six hours after haemorrhage, the rats were allowed access to standard chow ad libitum. Rats in the sham-shock group were anaesthetized and the left femoral artery was cannulated. Sham shock rats were monitored similar as the hemorrhagic shock group, however no blood was withdrawn.

Twenty-four hours after induction of shock, the rats were anaesthetized with sodium pentobarbital (60 mg/kg). The skin over the abdomen was shaved and disinfected with povidone iodine. The abdomen was opened via a midline incision, blood samples were taken and mesenteric lymph nodes, the midsection of the spleen and segment IV of the liver were aseptically removed for bacteriological examination.

Plasma samples

Arterial blood samples were collected in heparinized pyrogen-free glass tubes at the time of induction of shock ($t=0$) and twenty-four hours later. Plasma was separated by centrifugation, frozen immediately and stored (-20°C) until the time of the assay. Hematocrit values were directly measured at the time of shock ($t=0$) and twenty-four hours later.

Triacylglycerol and apoB

Circulating triacylglycerol was determined using a standard enzymatic assay (Sigma Diagnostics, St. Louis, MO). Levels of apoB were determined using Sandwich-ELISA with a pAb against rat apoB, kindly provided by Dr. G. Gibbons, University of Oxford, UK. Briefly, 96-well immunomaxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with rabbit anti-rat apoB in a concentration of 1 µg/ml. ApoB in plasma was detected with biotin-conjugated rabbit anti-rat apoB followed by peroxidase-conjugated streptavidin. TMB was used as substrate for peroxidase. Plates were read in a microplate reader at 450 nm. As no standard was available, apoB levels were expressed as percentage of pooled plasma of normal healthy male Sprague-Dawley rats with the same weight (300 – 400 gram).

Endotoxin and Bacterial Translocation

Total circulating endotoxin was determined by a chromogenic Limulus Amoebocyte Lysate (LAL) assay (Endosafe, Charles River, Charleston, SC). In short, after thawing

heparinized plasma was directly diluted two-fold in pyrogen-free water and subsequently heated for 5 minutes at 75°C, to inactivate LPS inhibitors in plasma. LAL-reagent and plasma were incubated for 45 minutes at room temperature. After blocking the reaction with H₂SO₄ an endpoint measurement was used. This assay has an effective range from 0.001-1 ng/ml.

Mesenteric lymph nodes (MLN), the mid-section of the spleen and a segment of the liver were collected aseptically in 2 ml pre-weighed thioglycolate broth tubes (Becton Dickinson (BBL) Microbiology Europe, Maylan, France). After weighing, the tissue specimens were homogenized with sterile grinding rods (Potter S, B.Braun Melsungen, Melsungen, Germany). Subsequently, 500 µl-volumes were transferred onto the following agar plates: Columbia III blood agar base supplemented with 5% vol/vol sheep blood (BBL) (duplicate plates), Chocolate PolyviteX agar (BioMérieux, Marcy L'Etoile, France), and Schaedler Kanamycin-Vancomycin agar supplemented with 5% sheep blood (BBL). Aliquots were spread over the entire surface of the agar. All agar plates were incubated for 48h, in a 5% CO₂-enriched atmosphere or under anaerobic conditions (Schaedler agar plates). After incubation, the colonies were counted on the non-selective Columbia sheep blood agar plates. For determination of the number of colony forming units (CFU) per gram tissue, the number of colonies was counted on all aerobic plates and next adjusted to the weight of the grounded tissue. All different colony types were identified to the species level using conventional techniques.

FIGURE 2. Mean Arterial Pressure (MAP) of shock rats during the observation period. MAP pressure is represented as median with 25th- 75th percentile. MAP pressure before shock (pre) was 100 (90 – 110) mmHg and decreased to 26 (23 -31) mmHg directly after haemorrhage (t = 0). There was no significant difference in MAP between the groups during the observation period.

Statistical analysis

Bacterial translocation data are represented as median and range; Mean arterial pressure and heart rate as median with 25th - 75th percentile; other data are represented as mean ± SD. A Mann-Whitney U test was used for between-group comparisons. The

Chi-square test was used to compare incidence of translocation. A non-parametric Spearman correlation test was used for bivariate correlations. $P \leq 0.05$ was considered statistically significant.

FIGURE 3. High-fat enteral nutrition leads to enhanced triacylglycerol and apoB concentrations. **A:** Triacylglycerol concentrations in plasma were significantly increased in HS-HF rats at both t=0 (open bars) and 24 hours later (filled bars) compared with both HS-S and HS-LF groups. Values are expressed as mean $\pm$ SD. * $p < 0.005$ vs HS-S group at t=0. ** $p < 0.001$ vs HS-S group at t=24 hours. † $p < 0.005$ vs HS-LF group at t=0. ‡ $p < 0.001$ vs HS-LF group at t=24 hours. **B:** ApoB in the HS-HF group at t=0 was significantly higher compared with the HS-LF group, * $p = 0.006$. At t=24 hours apoB concentrations in the HS-HF group were significantly higher compared with the HS-S group, † $p = 0.036$. Values are expressed as percentage of the apoB concentration in pooled plasma of healthy rats. Concentration of apoB in Control, Sham-Shock, fasted Hemorrhagic Shock (HS) groups and the HS group fed with a low-fat enteral diet did not significantly differ.

Results

Hemorrhagic shock procedure

The severity of the hemorrhagic shock insult as reflected by changes in mean arterial pressure (MAP), heart rate (HR) and hematocrit was similar for all three shock groups, i.e. HS-S, HS-LF and HS-HF (figure 2). Directly after induction of shock ($t=0$) mean MAP-values decreased from 100 (90 – 110) mmHg to 26 (23 – 31) mmHg and the HR decreased from 395 (369 – 415) beats per minute (bpm) to 200 (167 – 232) bpm in all three shock groups. Hematocrit was reduced from $42 \pm 2.3\%$ at $t=0$ to $29 \pm 2.3\%$ at 24 hours after shock ($t=24$ hours), $p<0.001$. After 50 minutes both MAP and HR recovered to respectively 68 (59 – 77) mmHg and 339 (313 – 368) bpm. All rats recovered spontaneously after the haemorrhage and no deaths occurred. These data are comparable with those reported by other groups using a similar model of non-lethal hemorrhagic shock (20, 22).

FIGURE 4. Circulating endotoxin at 24 hours after shock. Circulating endotoxin is significantly lower in the HS-HF group compared to both the HS-S group (* $p=0.005$) and the HS-LF group ($\dagger p=0.002$). Each individual measurement is presented with the 5th and 95th percentile and mean $\pm$ SD.

Triacylglycerol and apoB

As expected circulating triacylglycerol levels at the time of shock ($t=0$) were significantly higher in HS-HF rats (184.5 ± 96.2 mg/dL), compared to both HS-LF rats (18.8 ± 14.8 mg/dL, $p<0.005$) and HS-S rats (23 ± 40.7 mg/dL, $p<0.005$) (Figure 3A). There was no statistical difference between the HS-S group and the HS-LF group. After 24 hours ($t=24$ hours) plasma triacylglycerol was still elevated in the HS-HF rats (87.8 ± 23.6 mg/dL), whereas plasma triacylglycerol levels were below detection level in both the HS-LF rats ($p<0.005$) and the HS-F rats ($p<0.005$). As shown in Figure 3B, plasma concentrations of apoB in HS-HF rats at $t=0$ were higher compared with both HS-LF and HS-S rats. However, statistical significance was observed only between the HS-HF and HS-LF rats ($p=0.006$). After 24 hours circulating apoB levels in plasma were still significantly elevated in the HS-HF group compared with the HS-S group ($p<0.05$), but not with the HS-LF group. As expected, circulating

triacylglycerol and apoB levels were significantly correlated at both $t = 0$ ($r = 0.471$, $p < 0.01$) and $t = 24$ hours ($r = 0.314$, $p < 0.05$).

Table 1. Bacterial translocation at 24 hours after shock

Groups (n=8)	Translocation incidence ¹	Mesenteric lymph nodes ²	Spleen ²	Liver ²	Total ²
Control	0/8	0	0	0	0
Sham Shock	3/8	0 (0-33)	0 (0-17)	0 (0-95)	0
HS-S	8/8	212 (60-483)	12 (0-998)	44 (0-249)	327
HS-LF	8/8	86 (30-209)	66 (7-2543)	28 (0-1416)	261
HS-HF	4/8* [†]	3 (0-144)* [‡]	0 (0-91)**	0 (0-115)* [†]	25*** [‡]

1 Number of rats with bacterial translocation vs total number of rats in the group. * $p < 0.05$ compared with hemorrhagic shock + low-fat enteral diet (HS-LF). [†] $p < 0.05$ compared to the starved hemorrhagic shock group (HS-S).

2 Results are presented as median colony forming units (cfu) /gram tissue (range). * $p < 0.05$, ** $p < 0.01$ compared to HS-LF. [†] $p < 0.05$, [‡] $p < 0.01$ compared to HS-S.

Circulating endotoxin and bacterial translocation

The induced hypertriglyceridemia would potentially increase capacity to inhibit bacterial toxins such as endotoxin and thus preserve gut barrier function. In the systemic circulation endotoxin levels remained near the detection levels in the control groups (C and SS), Figure 4. Circulating endotoxin after 24 hours in the HS-HF group (7.2 ± 10.2 pg/ml) was significantly lower compared with both the HS-S group (29.1 ± 13.4 pg/ml, $p = 0.005$) and the HS-LF group (29.9 ± 5.2 pg/ml, $p = 0.002$). There was no statistical difference between the HS-S group and the HS-LF group. The bacterial translocation data are represented in Table 1. As expected, cultures from tissues taken from the control group were sterile. In the sham-shock rats, three animals had positive cultures, with low numbers of bacteria. After hemorrhagic shock, bacterial translocation was demonstrated in all animals. The number of bacteria found in mesenteric lymph nodes (MLN), spleen and liver was significantly higher in the HS-S group compared with the sham-shock rats ($p = 0.001$). The median of colony forming units found in MLN of the HS-LF group was not significantly different from the amount of bacteria found in MLN of the HS-S group (212 vs. 86 cfu/gram, $p = 0.059$). In contrast, after high-fat enteral nutrition (HS-HF), sterile cultures were found in 4/8 rats. In addition, the amount of bacteria found in MLN, spleen and liver in the whole group was considerably reduced and significantly lower compared with the HS-S and the HS-LF groups. Overall, the bacteria most frequently found in the cultures were *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*. Additionally, *Proteus* and *Lactobacillus spp* were sporadically identified. Bacteria were more often found in MLN, than spleen or liver and *Enterobacteriaceae* translocated more frequently than *Lactobacillus spp*. Both the species of bacteria and the frequency of translocation to the MLN are comparable with other rat studies investigating bacterial translocation

after hemorrhagic shock (20, 23). In line with our hypothesis, levels of circulating triacylglycerol were inversely related with the total bacterial translocation, $r = -0.346$, $p < 0.05$ (Figure 5A) and circulating endotoxin levels, $r = -0.598$, $p \leq 0.01$ (Figure 5B) in each hemorrhagic shock animal.

FIGURE 5. Circulating triglycerides are inversely correlated with total bacterial translocation and circulating endotoxin in all hemorrhagic shock rats. **A:** Circulating triglycerides are inversely correlated with total bacterial translocation. Total bacterial translocation expressed as colony forming units per gram is plotted against circulating triglycerides (mg/dL), $r = -0.346$, $* p \leq 0.05$. **B:** Circulating triglycerides are inversely correlated with the circulating endotoxin level at $t = 24$ hours. Circulating endotoxin (pg/ml) is plotted against circulating triglycerides (mg/dL), $r = -0.598$, $** p \leq 0.01$.

Discussion

In the present study, we show that administration of high-fat enteral nutrition before and directly after haemorrhage induces an increase in circulating triacylglycerol and apoB concentrations. Interestingly, this study is the first to show that administration of high-fat enteral nutrition was accompanied by reduced plasma endotoxin levels and bacterial translocation. Ulevitch et al. (24) and Tobias et al. (25) proposed in the early 1980's a possible role for high-density lipoproteins (HDL) to bind and inactivate endotoxin. Later, others discovered that hypertriglyceridemia as a result of *de novo* synthesis in the liver is part of the early response to low-dose endotoxin (26). This endotoxin-mediated increase in circulating triacylglycerol-rich lipoproteins is considered to have a protective function. *In vitro* studies showed that triacylglycerol-rich lipoproteins such as VLDL and chylomicrons are potent inhibitors of endotoxin activity (13, 27). In addition, *in vivo* studies showed that preincubation of endotoxin with triacylglycerol-rich lipoproteins or repeated intravenous infusions with chylomicrons protect animals against endotoxin-induced death (13, 14). The proposed mechanism for these protective properties of triacylglycerol-rich lipoproteins may be twofold. Firstly, VLDL and chylomicrons can directly inhibit the bioactivity of endotoxin by uptake of endotoxin into these lipoproteins. Secondly, clearance of circulating endotoxin in plasma is enhanced by increased hepatocellular uptake of endotoxin associated with chylomicrons (16). Triacylglycerol-rich lipoproteins shunt endotoxin away from Kupffer cells towards hepatocytes, thereby decreasing cytokine release and TNF-mediated inflammation. Neutralization of the bioactivity of endotoxin by lipoproteins is mediated in part by LBP, apoA-I and apoB (12, 28, 29). LBP functions as a lipid transfer molecule, catalyzing the detoxification of endotoxin. ApoB, the main apolipoprotein of triacylglycerol-rich lipoproteins, is considered to function as a binding site for both endotoxin and LBP.

In our study an increase of triacylglycerols was observed only in the HS-HF rats at both $t=0$ and $t=24$ hours. Chylomicrons are primarily triacylglycerol particles and chylomicron formation is a characteristic property of the enterocytes during the postprandial state (30). Therefore, the majority of triacylglycerol measured at $t=0$ is probably found in chylomicrons. As chylomicrons are rapidly cleared from the circulation (31), the prolonged hypertriglyceridemia found at $t=24$ hours may be due to elevated VLDL levels. At the same time, also apoB levels in plasma were enhanced in the HS-HF group. Since the apoB concentration in plasma is known to be strongly correlated with circulating triacylglycerol levels (32) we expected a higher apoB concentration in plasma of HS-HF rats compared to that of HS-S and HS-LF rats. Statistical significance was obtained with the HS-LF group at $t=0$ and the HS-S group after 24 hours. The absence of a postprandial increase in both triacylglycerols and apoB in the HS-LF group confirms earlier results that the postlipemic response depends on the amount of fat in the administered diet (33). The hypertriglyceridemia and the elevated apoB levels in the HS-HF rats, which reflect an elevation of triacylglycerol-rich lipoproteins, were accompanied with lower circulating endotoxin levels and reduced bacterial translocation. This effect was specific for the high-fat nutrition, since in the HS-LF group both endotoxin levels and bacterial translocation to MLN, spleen and liver were not significantly different compared with the HS-S group.

A transient endotoxemia, attributed to direct endotoxin translocation, has been described in a different model of hemorrhagic shock with a peak concentration at 150 minutes after onset of shock (10). The endotoxemia that we observed at 24 hours after shock may be either the result of direct leakage due to a reduced gut barrier function or derived from translocated bacteria. At this stage, our data do not allow to distinguish between these potential sources of endotoxin. A common hypothesis proposes that endotoxin triggers an inflammatory process locally in the gut resulting in tissue damage, leading to a deterioration of the gut barrier function (9, 10, 34, 35). Neutralization of the bioactivity of endotoxin in an early stage would thus prevent this loss of barrier function. The importance of endotoxin neutralization after hemorrhagic shock in preserving gut barrier integrity is illustrated by animal studies showing that systemically administered endotoxin inhibitors, i.e. recombinant bactericidal/permeability increasing protein (rBPI) and the endotoxin neutralizing monoclonal Ab, WN1 2225, decrease bacterial invasion into the intestinal wall (8, 9). We propose that in our study triacylglycerol-rich lipoproteins play a role in neutralizing endotoxin and that this explains the protective effect of high-fat enteral nutrition regarding bacterial translocation. This is supported by the significant negative correlation between circulating triacylglycerol and both total bacterial translocation and circulating endotoxin levels in all hemorrhagic shock rats. At present, it is unclear whether triacylglycerol-rich lipoproteins function locally in the gut or in the systemic circulation. The fact that chylomicrons, apoB and LBP, all essential in endotoxin neutralization, are produced by enterocytes (12, 36, 37, 38), is in favor of local endotoxin neutralization by high-fat enteral nutrition.

Opposed to data found in rodents, there is still controversy about the effect of triacylglycerol-rich lipoproteins on endotoxin responsiveness in humans. Van der Poll et al. (39) showed that the *in vivo* response to endotoxin in humans is not inhibited by hypertriglyceridemia. However, Harris et al. (40) showed that preincubation of endotoxin with triacylglycerol-rich lipoproteins attenuated the inflammatory response evolving from this toxic compound. Moreover, a recent human study from our group showed that postprandial chylomicrons are very potent in neutralizing both endotoxin and lipoteichoic acid in a lipopolysaccharide binding protein (LBP) depending fashion, resulting in reduced cytokine and chemokine secretion (41). The fact that van der Poll et al. used a bolus injection of endotoxin, creating a sudden increase in endotoxin levels might explain these results, because activation of leukocytes by endotoxin may be more rapid than binding of endotoxin by triacylglycerol-rich lipoproteins (13, 42). In contrast, our model creates a situation in which endotoxin gradually translocates from the gut lumen into the systemic circulation, resembling the clinical situation. In this setting, exposure of endotoxin to triacylglycerol-rich lipoproteins leading to neutralization may precede the exposure to leukocytes.

In comparison with studies on triacylglycerol-rich lipoproteins and endotoxin neutralization in humans, data on the inhibitory effect of HDL on endotoxin responsiveness are more apparent (43). As HDL can be up regulated by triacylglycerol-rich lipoproteins via cholesteryl ester transfer protein (CETP) or phospholipid transfer protein (PLTP)(44), triacylglycerol-rich lipoproteins may also indirectly contribute to protection against endotoxin in humans.

In surgery preoperative fasting is currently still a common routine(45), however a recent meta-analysis of controlled clinical trials concludes that there is no clear advantage of keeping patients on a "nil by mouth" regime (19). Short-term fasting increases the number of coliform bacteria and promotes bacterial adherence to the intestinal mucosa in rats and in a situation of gut barrier failure, these phenomena are thought to promote bacterial translocation(46). Bark et al. (21) already observed that rats receiving enteral nutrition before hemorrhagic shock had less bacterial translocation compared with rats which were fasted for 24 hours. Also clinical studies showed the benefit of early postoperative nutrition compared with either fasting (47) or TPN (48, 49, 50, 51). In addition, certain nutrients such as glutamine, arginine, omega-3 fatty acids and nucleic acids have immune-enhancing effects and reduce wound complication, infection and hospital stay when added to the enteral diet (47, 52). Our data show that a strong additional protective effect can be obtained by perishock administration of an enteral nutrition containing a high concentration of fat. Taken together our current data are the first to show that a simple and relatively short nutritional intervention just before and directly after haemorrhage with a high-fat diet results in improved gut barrier function as reflected by reduced endotoxemia and bacterial translocation after 24 hours. The observed effects seem to be largely dependent on the amount of fat in the enterally administered diet. Whether this can be attributed to early and local scavenging of endotoxin by chylomicrons, leading to less local inflammation or to the prolonged hypertriglyceridemia, which also results in systemical endotoxin neutralization remains to be investigated. This study indicates that further studies on the potential benefit of high-fat enteral nutrition are needed.

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Chapter 7

Phosphatidylcholine enhances apolipoprotein secretion by enterocytes during the acute phase response

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Abstract

Translocated enteric bacteria and their toxins cause local damage to the intestine and systemic pathology. Direct detoxification of these bacterial toxins after invading the interior of the host is vital. Intestinal lipoproteins were demonstrated to effectively prevent the detrimental effects of bacterial toxin. In this study we explored conditions for enhancement of intestinal lipoprotein secretion during the acute phase response. Since phosphatidylcholine (PC) is thought to be essential in intestinal lipoprotein assembly and secretion, we studied the effect of PC on apolipoprotein secretion by enterocytes under normal and acute phase conditions. The data show that apical presence of L-PC enhances apolipoprotein secretion by enterocytes *in vitro*. Accordingly, instillation of PC in healthy rats significantly enhanced circulating apoB levels. PC in combination with other nutritional compounds had an additional effect on the upregulation of intestinal apolipoprotein synthesis. Most interestingly, PC administered to enterocytes under acute phase conditions abrogated the cytokine-induced reduction of apolipoprotein secretion and even enhanced the secretion of apolipoproteins to the same levels as under non-acute phase conditions. The data of this study imply that enteral supplementation of PC enhances intestinal lipoprotein secretion during infection. Administration of PC may in particular be effective since the presence of dietary and biliary PC is lacking in the intestinal lumen during inflammatory conditions. We speculate that PC induced secretion of lipoproteins by enterocytes may promote detoxification of bacterial toxins at the site of translocation and prevent the local damage and systemic pathology induced by these toxins.

Introduction

Physiologic levels of lipoproteins have been shown to protect against the deleterious effects of bacterial toxins (11, 13, 14, 20). Toxins incorporated into lipoproteins are not able to activate cells and thereby prevent the release of a cascade of cytokines (3, 13, 14). The liver and the intestine are the organs that contribute the majority of circulating lipoproteins. Liver derived lipoproteins i.e. very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were found to be the lipoproteins that predominantly bind and inactivate LPS in fasting healthy and septic subjects (32, 42, 43, 47). Chylomicrons, the very large triacylglycerol-rich lipoproteins produced by the intestine upon feeding, were also demonstrated to prevent endotoxin-induced mortality *in vivo* (18, 19).

The intestine is a potential port of entrance for bacteria and their toxins (5, 24, 34). Translocated enteric bacteria and toxins are thought to cause local damage to the intestinal barrier (6, 28) as well as systemic pathology (5, 24, 34). Consequently, there is a strong need for immediate detoxification of bacterial toxins after passage of the intestinal epithelium. Hypothetically intestinal lipoproteins are able to inactivate these toxins locally in the gut. LBP a protein that strongly catalyzes the detoxification of bacterial toxins by lipoproteins was recently found to be produced by enterocytes (45, 46), and may assist in direct lipoprotein mediated detoxification of toxins in the intestine. However, during infection the conditions for secretion of intestinal lipoproteins are poor. Cytokines, released in response to bacterial toxins, interact with enterocytes and diminish the secretion of intestinal lipoproteins; TNF- α and IL-6 were demonstrated to reduce the synthesis of apoB and apoA-I and the secretion of lipids and lipoproteins by intestinal epithelial cells (16, 26, 31). An additional effect of these circulating cytokines is anorexia (reviewed in reference 37). Hence, the main trigger for intestinal lipoprotein production, i.e. the presence of intraluminal nutrients, is strongly impaired. Moreover, critically ill patients admitted to an intensive care unit are in general initially starved or given total parenteral nutrition (TPN). Another factor, impeding intestinal lipoprotein secretion, is a profound decrement in bile flow and biliary phospholipid concentrations during critical illness and lack of enteral nutrition (7). Presence of phospholipids in the intestinal lumen however, is required for secretion of lipoproteins by enterocytes (12, 25, 33, 44).

In search for therapeutic strategies to combat bacterial infection several investigators increased circulating lipoprotein concentrations artificially by infusion of exogenous lipoproteins in animal models of infection (18, 19, 38). Modest increases in circulating chylomicron concentrations profoundly enhanced resistance to endotoxemia *in vivo* (18, 19). Next to these studies using exogenous lipoproteins, Harris et al. recently demonstrated that also endogenous lipoproteins, produced in response to feeding, reduce the toxic effects of bacterial toxins in human (20). In the present study we explored conditions for enhancement of the endogenous production of lipoproteins by the intestine, during normal and acute phase conditions. Since intestinal lipoprotein secretion is thought to depend on presence of phosphatidylcholine (PC) in the intestinal lumen, which is dramatically impaired during infectious conditions, we investigated in the present study whether PC enhances intestinal lipoprotein secretion during the acute phase response. *In vitro* differentiated human colonic adenocarcinoma (Caco-2) cells were used as a model of normal human intestinal

epithelial cells. This cell line was chosen as an experimental model, in view of its well-established ability to differentiate into functional enterocytes with the capacity for lipid and apolipoprotein assembly, secretion and metabolism (22). In addition we examined the capacity of chylomicrons, the lipoprotein mainly secreted by enterocytes, to attenuate the cellular cytokine response to LPS *in vitro*. The results derived by the *in vitro* experiments were extended in *in vivo* experiments in which the effect of oral administration of PC to rats on endogenous lipoprotein secretion was evaluated.

Material & methods

Reagents.

Human rTNF- α was kindly provided by BASF/Knoll (Ludwigshafen, Germany); human recombinant interleukin (IL)-6 by Prof. W. Sebald (Physiologisch-Chemisches Institut der Universität Würzburg, Germany); human rIL-1 β was a gift from Immunex, Seattle, WA. Lyso-phosphatidylcholine (L-PC) and LPS from *Escherichia coli*, serotype 055:B5 was purchased from Sigma (St.Louis, MO). Intralipid was derived from Kabivitrum (Stockholm, Sweden). The human apoA-I immunoassay was a gift from Dr. Gavilondo, Center for Genetic Engineering and Biotechnology (Havana, Cuba). Phosphatidylcholine (Phospholipon 90) was a kind gift of Nattermann, Köln, Germany. Polyclonal rabbit anti-rat apoB Abs were kindly provided by Dr.G. Gibbons, University of Oxford, UK. Polyclonal and monoclonal Ab against human apoB (2B4) were derived from Calbiochem (La Jolla, CA) and ICN (Irvine, CA) respectively. Goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (Westgrove, PA).

Chylomicron isolation

Chylomicrons were isolated from pooled postprandial human serum (6 donors) by ultracentrifugation according to Terpstra et al. (41). Briefly, dry KBr (770 mg) and sucrose (50 mg) together with 2 ml of serum were placed in the bottom of ultra clear centrifuge tubes (Beckman Instruments, Brea, CA). The components were carefully mixed with a spatula. A discontinuous gradient was made with the serum mixture of density 1.250 g/ml, two KBr solutions with density 1.225 and 1.100 g/ml respectively, and on top water density 0.998 g/ml. The tubes were placed in a SW41 rotor and centrifuged in a Beckman XL-80 ultra-centrifuge (Beckman Instruments) at 30,000 rpm and 20°C for 40 minutes. The chylomicrons were collected by aspiration of the top 1 ml fraction and stored at -80°C until use.

Cell isolation and stimulation.

PBMC were isolated from buffy coats of healthy volunteers, kindly 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 suspended in Macrophage Serum Free Medium (Life Technologies, Paisley, UK) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Life Technologies). LPS was pre-incubated with chylomicrons (50% of postprandial circulating levels) in 96-well flat-bottom tissue culture plates (Costar, Cambridge,

MA) for 24 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 and 5% CO₂. Supernatants were collected and stored at -20°C until use.

Immunoassays.

ApoA-I release by Caco-2 cells was evaluated using sandwich ELISA. Plates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) were coated with specific monoclonal Abs for human apoA-I. A peroxidase labeled monoclonal Ab was used for detection of immobilized apoA-I. Peroxidase activity was determined by addition of tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

For evaluation of human apoB concentrations plates were coated with monoclonal Ab against human apoB. Polyclonal Ab against apoB followed by a peroxidase-conjugated goat anti-rabbit IgG were used for detection of apoB.

ApoB concentrations in the sera of rats were evaluated using sandwich ELISA. Plates were coated with polyclonal rabbit Ab against rat apoB. For detection of apoB, biotin labeled polyclonal Ab against rat apoB, followed by peroxidase-conjugated streptavidin, was used. Values of ApoB are expressed as percentage of normal plasma, since a standard was not available.

Cytokine concentrations in the cell culture supernatants were determined using sandwich-ELISA for TNF- α (9) and Il-8 (1). 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). Il-8 was detected by biotinylated polyclonal rabbit anti-human Il-8 followed by peroxidase-conjugated streptavidin.

Culture and stimulation of Caco-2 cells

In vitro differentiated human colonic adenocarcinoma (Caco-2) cells were used as a model of normal human intestinal epithelial cells. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD; ATCC HTB 37). Caco-2 cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (Hyclone, Logan, UT) 1.0 mM sodium pyruvate (Life Technologies), 0.1 mM non-essential amino acids (Life Technologies), 100 IU penicillin/ml and 100 μ g streptomycin/ml (Life Technologies). Cells were maintained in 25 cm² or 75 cm² flasks (Costar, Cambridge, MA). Cultures were split when they reached 80% confluence.

For experiments, 5×10^5 Caco-2 cells/cm² were seeded onto 1 cm² collagen-coated membranes (3.0 μ m pore size) inserted in Transwells (Costar, Cambridge, MA). Cells were cultured in the complete medium as described above. The medium was changed 48 h after seeding and further every other day. After two weeks, Caco-2 monolayers display functional properties similar to those found in normal enterocytes (21) and they form a polarized monolayer with tight junctions and brush border microvilli at their apical surfaces (36). Therefore, in this study all experiments were conducted between 14 and 17 days after seeding of the cells. The formation and maintenance of a

sealed monolayer of Caco-2 cells was monitored by measurement of the passage of the permeability probe phenol red (500 Dalton). Monolayers permeable for phenol red were not used.

Experiments designed to study the effect of L-PC on apoA-I and apoB secretion by Caco-2 cells were performed in the absence of serum. Caco-2 monolayers were washed extensively before experiments and fresh medium was added basolateral. Medium supplemented with different concentrations of L-PC (L-PC) or supplemented with 200 μ M L-PC together with a 6-fold dilution of parenteral nutrition containing 16% intralipid-20% (containing 1.2% phospholipids), 40% aminoacids-10% and 26 % glucose-50%, was added apical. Every 2.5 hour the apical medium was removed and fresh medium with L-PC or L-PC plus parenteral nutrition was added. After incubation for 10 hours, basolateral medium was removed and apolipoprotein concentrations in the medium were determined by ELISA.

To study the effect of L-PC on apolipoprotein secretion during the acute phase response, IL-1 β (5 ng/ml), IL-6 (100 ng/ml) and TNF- α (100 ng/ml), were added to the apical and basolateral medium 48 hours and 24 hours before the start of experiments. These settings were based on pilot experiments, which demonstrated the induction of an acute phase response under these conditions. Monolayers were washed and medium with or without cytokines and containing 0 or 200 μ M L-PC was added to the apical compartments. Apical medium was refreshed every 2.5 hours. ApoA-I and apoB concentrations in the basolateral medium were determined after an incubation period of 10 hours.

FIGURE 1. Chylomicrons reduce LPS toxicity. LPS 100 ng/ml was preincubated with (filled bars) or without (open bars) isolated chylomicrons (50% of postprandial circulating levels) for 24h at 37°C. The remaining biological activity of LPS was determined by incubation with PBMC for 4h and measurement of TNF- α and IL-8 secretion in the supernatants. Values represent the mean $\pm$ SD of 3 wells.

Animal study

15 mg PC (Nattermann) diluted in physiologic saline (n=10) or physiologic saline without PC (n=10) was administered by oral gavage to 10 weeks old male Whistar rats every 2 hours for 8 hours on the first day of the experiment. On the second day of the experiment phospholipids or physiologic saline was again administered by oral gavage every 2 hours for 8 hours. During the experiment food was withdrawn, while drinking water was provided ad libitum. At the end of the experiment, arterial blood was collected and the apoB concentration in the serum was evaluated by ELISA.

Results

Chylomicrons reduce LPS activity.

To determine whether chylomicrons, the lipoproteins in particular produced by the intestine, reduce the bioactivity of LPS *in vitro* we exposed PBMC to LPS preincubated with chylomicrons. TNF- α and Il-8 release by PBMC after a 4 hour incubation period were measured. Figure 1 shows that, preincubation of LPS with chylomicrons (50% of postprandial circulating levels), clearly reduced the LPS evoked TNF- α and Il-8 secretion by PBMC.

FIGURE 2. Lyso-phosphatidylcholine enhances apolipoprotein secretion by enterocytes dose dependently. Caco-2 cells were grown on Transwell-Col™. Maintenance medium was removed, cells were washed and serum free medium containing 0, 100, 150 and 200 μ M L-PC was added apically every 2.5 hour. The effect of L-PC on basolateral apoA-I (●) and apoB (■) secretion after 10-h incubation was determined by ELISA. Values represent the mean $\pm$ SD of 4 wells.

Lyso-phosphatidylcholine enhances apoA-I and apoB secretion by Caco-2 cells.

Next, we studied whether apical presence of PC affects apoA-I and apoB secretion by Caco-2 cells. Others have demonstrated the coordinated induction of triacylglycerol and apoB in Caco-2 cells suggesting that the regulation of lipoproteins and apoB is a

coordinated process (4). In the intestine, most PC is hydrolysed by phospholipase derived from the pancreas. Since Caco-2 cells lack the ability to hydrolyse and release fatty acids from PC, L-PC was added to mimic more closely the form of PC available for intestinal cells. L-PC was added to the apical serum free medium of Caco-2 cells, cultured on semipermeable filters. Since L-PC is efficiently absorbed by enterocytes, the apical medium containing L-PC was refreshed every 2.5 h in order to accomplish continuous presence of L-PC at the apical cell surface. After 10 h incubation the amount of apoA-I and apoB in the basolateral medium was determined. Figure 2 shows that L-PC enhanced apoA-I and apoB secretion dose- dependently. Addition of 200 μ M L-PC to the apical medium caused an 8 and 2.5-fold increase in the amount of respectively apoA-I and apoB secreted by the Caco-2 cells. When apical medium was not refreshed every 2.5 h the upregulation of apolipoprotein secretion was much lower (data not shown), which underlines the importance of continuous presence of PC at the apical surface of enterocytes. Concentrations of L-PC above 200 μ M resulted in disturbances of the integrity of the Caco-2 monolayers and were therefore not further studied.

FIGURE 3. Additional effect of lyso-phosphatidylcholine supplemented to nutrition on apolipoprotein secretion by enterocytes. Caco-2 cells were grown on Transwell-Col™. Maintenance medium was removed, cells were washed, and only serum free medium, serum free medium with 200 μ M L-PC, or serum free medium containing parenteral nutrition (1:6) with or without 200 μ M L-PC was added apically every 2.5 hour. The effect of supplementation of L-PC to nutrition on basolateral apoA-I (open bars) and apoB (filled bars) secretion after 10-h incubation was determined by ELISA. Values represent the mean $\pm$ SD of 4 wells.

It has been proposed that phosphatidylcholine enhances the intestinal absorption of dietary fat (33). In search for conditions to further enhance the lipoprotein secretion by enterocytes, we studied whether the combination of L-PC with other nutritional compounds resulted in a further upregulation of the apoA-I and ApoB secretion. Since Caco-2 cells do not secrete all enzymes necessary for digestion of enteral nutrition,

parenteral nutrition supplemented with L-PC was added to the apical side of Caco-2 cells. Figure 3 demonstrates that the parenteral nutrition containing aminoacids, glucose and lipids, amongst which low concentrations of phospholipids, induced apoA-I and apoB secretion by Caco-2 cells. This apolipoprotein secretion was additionally enhanced by presence of L-PC.

Enteral infusion of phosphatidylcholine enhances circulating concentrations of apoB in rats

The results derived by the *in vitro* experiments were extended in an *in vivo* study in which the effect of enteral administration of PC to rats on endogenous lipoprotein secretion was evaluated. To this end rats were fed PC or physiologic saline repetitively during the 32 hours before blood sampling. The concentration of circulating apoB in the rats fed with PC was 53% higher ($p < 0.05$, Mann-Whitney U test) as compared to control rats (Fig. 4), clearly indicating that enteral administration of PC enhances endogenous lipoprotein secretion.

FIGURE 4. *Enteral infusion of phosphatidylcholine enhances circulating concentrations of apoB in rats.* 10 weeks old male Whistar rats were instilled 15 mg PC diluted in physiologic saline ($n=10$) or physiologic saline without PC ($n=10$) every 2 hours for 8 hours on the first day of the experiment. The second day PC or physiologic saline was instilled again every 2 hours for 8 hours. During the experiment food was withdrawn, while drinking was provided ad libitum. At the end of the experiment, arterial blood was collected and apoB in the serum was evaluated by ELISA. Values of ApoB are expressed as percentage of normal plasma. Values represent the mean $\pm$ SD of 10 rats. A Mann-Whitney U test was used for between-group comparisons. $P < 0.05$ was considered to be statistically significant. * The circulating apoB concentration in the rats fed with PC was significantly higher as compared to control rats.

Lyso-phosphatidylcholine enhances the lipoprotein secretion by Caco-2 cells during the acute phase response

Cytokines have been shown to affect lipoprotein metabolism during infection (16). TNF induces a dose related inhibition of apoA-I and apoB secretion in both, hepatocytes and enterocytes (10, 16, 26, 31). Our prior studies demonstrated that a mixture of TNF- α , IL-6 and IL-1 β induced a more pronounced acute phase response in Caco-2 cells, resulting in a strong upregulation of positive acute phase proteins accompanied by a reduction of the negative acute phase protein apoA-I (46). The next experiment was performed to study whether L-PC is able to prevent the decreased apoA-I and apoB secretion during the acute phase response. To this end, Caco-2 cells were cultured for 48h in the presence of cytokines before L-PC was added to the apical cell surface. The apical culture medium containing L-PC and cytokines was refreshed every 2.5 hour. After a 10 h incubation period the amount of apoA-I in the basolateral culture medium was measured. Figure 5 shows that also in the presence of high concentrations of pro-inflammatory cytokines, L-PC enhanced the apoA-I secretion in intestinal epithelial cells. In conclusion, PC administered to intestinal epithelial cells during the acute phase response abrogates the cytokine induced inhibition of apolipoprotein secretion and even enhances the secretion of apolipoproteins to the same levels as under non-acute phase conditions.

FIGURE 5. *Lyso-phosphatidylcholine enhances apolipoprotein secretion by enterocytes during the acute phase response.* An acute phase response was induced in Caco-2 cells, grown on Transwell-Col™, by incubation of the cells with IL-1 β (5 ng/ml), IL-6 (100 ng/ml), TNF- α (100 ng/ml) for 48 hours (filled bars). In controls no cytokines were added (open bars). At the end of the incubation, medium was removed, cells were washed, and serum free medium containing 0 or 200 μ M L-PC was added apically every 2.5 hour. The effect of apical presence of L-PC on basolateral apoA-I secretion under these acute phase conditions was evaluated after 10-h incubation. Values represent the mean $\pm$ SD of 4 wells.

Discussion

Gut barrier failure and subsequent bacterial translocation is considered to be a cause for infection and sepsis (5, 24, 34). Translocated bacterial toxins can cause further intestinal damage, either directly or via the induced toxic agents, such as cytokines and free radicals (6, 28). There is a strong need for direct neutralization of these translocated bacterial toxins in order to prevent further gut barrier dysfunction and to diminish the deleterious systemic effects. Chylomicrons, the predominant lipoproteins produced by the intestine in response to feeding, were found to be effective in neutralization of bacterial toxins *in vitro* (Fig. 1) and *in vivo* (18, 19). Chylomicrons, not only neutralize bacterial toxins, they also alter their metabolic fate by shunting toxins to hepatocytes and away from hepatic macrophages (19, 40). Consequently, toxins are delivered into the bile, where they are inactivated by bile salts (39). A relative small increase in the circulating concentration of chylomicrons was observed to reduce mortality caused by endotoxemia (18, 19). In line with these observations, Harris et al. demonstrated recently that endogenous lipoproteins produced in response to feeding could ameliorate the effects of bacterial toxins in human (20). During severe infection however, conditions for endogenous intestinal lipoprotein secretion are poor; the presence of cytokines and the absence of biliary and dietary phospholipids most likely reduces the absorption of lipids and the secretion of lipoproteins by enterocytes. Strategies that increase endogenous lipoprotein production have the potential to combat bacterial infection. Therefore, in this study we searched for conditions to enhance the endogenous intestinal lipoprotein secretion during the acute phase response.

Here we established that apoA-I and apoB secretion by enterocytes under non-acute phase conditions were dose dependently enhanced by addition of L-PC to the apical surface (Fig. 2). These *in vitro* observations were supported by an animal study, which demonstrated that repetitive enteral administration of PC enhanced circulating concentrations of apoB (Fig. 4). These results are in agreement with earlier observations demonstrating that phospholipids enhance the intestinal secretion of triacylglycerol-rich lipoproteins (23, 25, 33, 44). PC was earlier demonstrated not to affect apoB mRNA levels, suggesting that PC increases newly synthesized apoB probably by modification of posttranscriptional processes (25).

Cytokines are known to alter protein metabolism resulting in an increase or decrease of the positive or negative acute phase proteins, respectively. Pro-inflammatory cytokines strongly reduce apolipoprotein secretion by enterocytes, as demonstrated in this study (Fig. 5). Consistent with our findings, cytokines were demonstrated to decrease apoB, apoA-I, lipid and lipoprotein secretion in human intestinal epithelial cells (26, 31). We have now shown that also under these acute phase conditions L-PC present at the apical surface of the enterocytes enhanced basolateral apolipoprotein secretion (Fig. 5). Administration of L-PC to the enterocytes during the acute phase response not only reverted the cytokine effect on apolipoprotein secretion; it even enhanced the secretion of apolipoproteins to the same level as under non-acute phase conditions. These data might suggest that endogenous intestinal lipoprotein production can be enhanced during infection and inflammation by enteral administration of PC.

In search for an intervention to further enhance the intestinal lipoprotein secretion induced by PC, it was found that L-PC in combination with other nutrients had an

additional effect on apoA-I and apoB secretion by enterocytes as compared to administration of L-PC alone (Fig.3). For this additional effect at least two explanations can be given. At first, PC is thought to improve the absorption and transport of dietary fats by enterocytes (23, 33). Secondly, PC may induce increased intestinal lipoprotein secretion by a mechanism independent of enhanced lipid absorption. This second assumption is strengthened by both our *in vivo* and *in vitro* studies that indicate that luminal presence of other nutritional compounds is not a prerequisite for PC to enhance intestinal apolipoprotein secretion. In rats, even after 32 hours of fasting, PC still resulted in increased levels of apoB in the circulation. A similar phenomenon was observed in the *in vitro* study; when L-PC was added apical and basolateral medium was refreshed each 2.5 hour during a total period of 10 hours, even in the fourth 2.5 hour interval L-PC enhanced the apolipoprotein concentration in the basolateral culture medium of Caco-2 cells whilst other nutrients were absent at the apical cell surface (data not shown). These data imply that the PC induced apolipoprotein secretion is not exclusively caused by a rapid release of accumulated intracellular lipids and apolipoproteins or enhanced absorption of other lipids, but may involve enhanced *de novo* synthesis of lipoproteins. Consistently, L-PC was earlier demonstrated to enhance cholesterol (30) and triacylglycerol synthesis by enterocytes (25). These findings may have clinical importance since they suggest that the effect of enteral supplementation with PC is not restricted to a rapid short-term release of pre-existing intracellular lipoproteins, but instead, that PC have a prolonged effect on intestinal lipoprotein synthesis and secretion.

Enhancement of intestinal lipoprotein secretion by PC or PC-enriched nutrition hypothetically reinforces a biological defense mechanism of the intestine against bacterial toxins by enhanced neutralization and clearance of bacterial toxins from the circulation and possibly, even more important, by direct neutralization of bacterial toxins translocated across a compromised intestinal mucosa. Harris et al found that preincubation of LPS with postprandial blood was required for attenuation of the immune response by the endogenous lipoproteins secreted in response to feeding (20). We hypothesize that the endogenous lipoproteins secreted by the intestine have the opportunity to interact and neutralize bacterial toxins already in the intestinal submucosa and lymphatic ducts, hence before active toxins reach the blood stream.

Prior studies found direct and indirect evidence for a role of enteral administered phospholipids in the host defense against bacterial infection. The absence of bile in the gastrointestinal tract in patients with obstructive jaundice is accompanied by translocation of bacteria and their toxins (27, 35). The incidence of post-operative infections in patients suffering from biliary obstruction is markedly enhanced compared to patients without biliary obstruction (8). Other evidence for a beneficial role of presence of phospholipids in the intestinal lumen was found in studies that demonstrated that enteral administration of phospholipids protected against the reduction of intestinal and mucosal weight and bacterial translocation (15, 48). In addition, supplementation of preterm formula with phospholipids, mainly PC, lowered the incidence of necrotizing enterocolitis in preterm neonates (2). Also in this disorder, an impaired barrier function is thought to be crucial in the etiology. Although the authors of these studies speculate about the possible mechanisms underlying these positive effects of enteral phospholipids, including surfactant

activity, alteration of mucus solubility and charge and enhanced repair of the injured intestinal epithelium, evidence for such mechanisms was not found. Enhanced intestinal lipoprotein secretion and concomitant detoxification of bacterial toxins, thereby preventing further damage to the intestinal epithelium, may also have accounted for the protective effects of enteral phospholipids found in these studies.

In conclusion, in this study we demonstrated that merely PC and PC in combination with other nutrients increase the apolipoprotein secretion by intestinal epithelial cells. Most interestingly, this effect of PC was also pronounced in the presence of pro-inflammatory cytokines, which in enterocytes reduce apolipoprotein secretion. Further studies are required to establish the clinical relevance of early post-operative feeding with PC or PC enriched enteral nutrition in patients undergoing abdominal surgery or complex cardiovascular surgery since bacterial translocation across an injured intestinal mucosa is thought to cause infectious complications in these patients. In patients with biliary obstruction, severely lacking luminal endogenous PC, enteral supplementation of PC may in particular be beneficial for improvement of intestinal lipoprotein secretion and subsequent improvement of the intestinal defense against bacterial toxins.

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Chapter 8

Summary and discussion/

Samenvatting en discussie

Summary and discussion

The gastrointestinal tract forms an enormous surface continuously exposed to high concentrations of bacteria and their toxic metabolites. Under pathologic conditions, such as hemorrhagic shock, severe burns, major trauma or complex surgical procedures, the intestinal barrier function fails, which is thought to cause translocation of bacteria and toxins that induce and perpetuate sepsis (1, 2). Although the intestinal barrier does not withstand these extreme conditions, under healthy or less extreme conditions the intestinal mucosa limits translocation of enteric bacteria and toxins and is capable of coping with small amounts of bacterial toxin that enter the host. For this function it has an extensive defense system at its disposal comprising of a mechanical barrier, a cellular and an immunological component.

Whereas, insights in the functioning and failure of the intestinal barrier are increasing, much of the complex mechanisms underlying this extensive natural line of defense have yet to be unravelled. New knowledge may form a guide in the development of treatment strategies intended to prevent gut derived bacterial infection and its detrimental effects. The aim of this thesis was to further explore the natural intestinal defense against bacterial toxins and find conditions to optimise this defense.

In the systemic response to bacterial toxins, LPS binding protein (LBP), an acute phase protein, has an important modulating dual role. Cell activation by endotoxin is enhanced by low concentrations of LBP, whereas high concentrations reduce the toxicity (3). In addition LBP enhances the detoxification of bacterial toxin by lipoproteins (3). The results of studies of Jack et al, which demonstrate enhanced mortality and uncontrolled multiplication and spread of bacteria in LBP knockout mice, indicate that LBP is essential for the resistance to bacteria (4). The liver was long thought to be the main organ responsible for LBP production. Hypothetically, local LBP secretion at a site of bacterial invasion would provide a direct local response to bacteria and their toxins. Since the gastrointestinal tract is considered to be a route of entry for bacteria and toxins, we studied whether LBP is produced in the intestinal epithelium (Chapter 2). Human intestinal epithelial cells were demonstrated to synthesize and release LBP. In this study we also investigated the production of serum amyloid A (SAA) by intestinal epithelial cells. Although the primary function of SAA in the acute phase response is not fully understood, SAA was previously demonstrated to be involved in the routing and binding of lipoproteins to inflammatory cells (5). The secretion of LBP and SAA by intestinal epithelial cells was demonstrated to be strongly enhanced by the proinflammatory cytokines, TNF- α , IL-6 and IL-1 β . Glucocorticoids were found to enhance the cytokine mediated induction of LBP and SAA release by the epithelial cells. Endotoxins were shown not to induce the release of LBP and SAA by intestinal epithelial cells, indicating that cytokine release in the proximity of the epithelial cells is necessary for the induction of LBP and SAA release by these cells. The capability of intestinal epithelial cells to release LBP and SAA suggests a role for the intestinal epithelium in the acute phase response and in the local defense of the gut against bacteria and endotoxin.

Next, we explored the polarity of LBP secretion by intestinal epithelial cells (Chapter 3). Constitutively, LBP was found to be secreted unipolar from the apical cell surface. This apical secretion of LBP was markedly enhanced when cells were exposed to proinflammatory cytokines at their apical surface. However, bioactive LBP was

secreted from both cell surfaces after basolateral stimulation of cells. Cytokines also enhanced the secretion of SAA from both surfaces of Caco-2 cells, whereas the secretion of apoA-I and apoB was strongly decreased in the presence of cytokines. Further, it was demonstrated that LBP was transported exclusively from the basolateral towards the apical cell surface. In line with these *in vitro* data, the presence of LBP in intestinal mucus was strongly enhanced in mice after an intraperitoneally challenge with endotoxin. The results indicate that LBP is present at the mucosal surface of the intestine, a phenomenon for which secretion and transport of LBP by intestinal epithelial cells may be responsible. In addition, LBP is secreted towards the basolateral surface of the intestinal epithelium during an acute phase response.

Several lines of evidence indicate that LBP and serum lipoproteins cooperate in reducing the toxic properties of bacterial toxins. Others found evidence for an association of LBP with apoA-I containing lipoproteins in plasma from healthy persons (6). This physical association of LBP with lipoproteins may be important for the cooperation of LBP and lipoproteins in the detoxification of bacterial toxins. However, the strong reduction of apoA-I and HDL levels (7, 8) which coincides with the raise in LBP levels during endotoxemia seems in contrast with this cooperative function. It is firmly established that LDL and VLDL are critical in the survival of bacterial infection (9) and that circulating levels of these lipoproteins are relatively high during inflammation compared to HDL levels. Therefore, we studied interactions of LBP with LDL and VLDL (Chapter 4). By separation of lipoprotein classes under the mild conditions of agarose electrophoresis followed by Western blot analysis of LBP and apoproteins, we clearly demonstrate that LBP in serum is associated with apoB containing lipoproteins. In contrast, no association of LBP with HDL or apoA-I was found in serum. Consistently, we observed that LPS incubated with serum co-migrates with LBP and apoB, indicating that both LBP and, if present, LPS circulate predominantly associated with LDL and VLDL. Quantitative studies on LBP-lipoprotein interaction further demonstrated a strong interaction of LBP with isolated LDL and VLDL. Most interestingly, we observed that LBP bound to LDL and VLDL enhances the LPS binding capacity of these lipoproteins.

LBP induced binding of LPS to lipoproteins is of utmost importance during infection. Therefore, the association of LBP and LPS with lipoproteins was also studied in serum from septic patients. In septic serum containing high LBP levels and a markedly altered lipoprotein spectrum, most of the LBP is associated with LDL and VLDL, although some LBP appeared to circulate free from lipoproteins. In line with these findings, we observed that also LPS predominantly binds to LDL and VLDL in serum from septic patients.

ApoB was found to account at least in part for the LBP binding capacity of LDL and VLDL. Although apoA-I also interacts with LBP, the affinity of LBP for apoB was found to be tenfold higher than for apoA-I, which is in accordance with the predominant association of LBP with LDL and VLDL in human serum.

Our results imply that LBP circulates as a complex with LDL and VLDL. ApoB functions as a binding site for LBP on LDL and VLDL, which interaction enhances the rate of LPS binding. Overall these results emphasize a crucial role for LBP-LDL/VLDL complexes in the scavenging of endotoxin present in the circulation.

Our observations that LBP is secreted by intestinal epithelial cells, together with our finding that circulating LBP is associated with apoB containing lipoproteins thereby enhancing its LPS binding capacity, prompted us to study whether LBP also cooperates with the apoB containing intestine derived chylomicrons in LPS binding and inactivation. Chylomicrons have previously been shown to prevent endotoxin-induced inflammation and to improve survival when given in a murine endotoxemia model (10, 11). LBP was demonstrated to associate with chylomicrons and to enhance the amount of LPS binding to chylomicrons in a dose dependent fashion (Chapter 5). This LBP induced interaction of LPS with chylomicrons prevented endotoxin activity as demonstrated by reduced cytokine secretion by PBMC. When postprandial circulating concentrations of chylomicrons were compared with circulating levels of LDL, VLDL, and HDL, chylomicrons exceeded the other lipoproteins in LPS inactivating capacity. Furthermore, in presence of LBP chylomicrons inhibited also the ability of LTA, a fragment of gram-positive bacteria, to stimulate cytokine secretion by PBMC.

Recently it was demonstrated that the intestinal epithelium takes up circulating endotoxin followed by secretion of endotoxin into the intestinal lumen (12). These data suggest that intestinal epithelial cells contribute to the clearance of endotoxin. We asked whether the binding of LPS to LBP-chylomicron complexes at the basolateral site of the intestinal epithelium influences the clearance of LPS by enterocytes. The transport of LPS from the basolateral towards the apical surface of Caco-2 cells was found to be markedly enhanced by basolateral presence of chylomicrons and further augmented by presence of both chylomicrons and LBP (Figure 5, Chapter 1). These data imply that besides a function for LBP-chylomicron complexes in the detoxification of LPS, these complexes also contribute to the clearance of LPS by the intestinal epithelium.

In conclusion, our results indicate that LBP binds to chylomicrons and enables chylomicrons to rapidly bind high amounts of bacterial toxin, thereby preventing cell activation and increasing the clearance of toxin. We consider systemic clearance of bacterial toxin and inactivation of bacterial toxins by LBP-chylomicron complexes in the circulation of significance. In addition, we hypothesize that this is also part of a local defense mechanism of the intestine against translocated bacterial toxins, since LBP and chylomicrons are both synthesized by enterocytes. This may implicate that enteral feeding is a possible physiologic route to enhance the presence of toxin neutralizing and clearing chylomicrons in the circulation and probably more important, in the intestinal submucosa and lymphatics.

A high fat diet is known to increase chylomicron secretion in an early postprandial phase as well as hepatic secretion of VLDL in a later phase. Hypothetically postprandial enhancement of these lipoproteins increases the host's capacity to inactivate bacterial toxin either locally in the intestine or in the circulation. This inactivation will prevent the secretion of inflammatory mediators and damage to the intestinal barrier caused by these mediators or toxin themselves. To evaluate this hypothesis the effect of a high-fat enteral nutrition on endotoxemia and bacterial translocation was studied in a rat model of non-lethal hemorrhagic shock (Chapter 6). Most interestingly, the enhanced concentrations of circulating triacylglycerol and apolipoprotein B were accompanied by reduced levels of circulating endotoxin and

bacterial translocation. The data described are the first to show that a simple and relatively short nutritional intervention just before and directly after haemorrhage with a high-fat diet results in improved gut barrier function.

In search for conditions to further enhance intestinal lipoprotein secretion during the acute phase response, we studied the effect of phosphatidylcholine (PC) on apolipoprotein secretion by enterocytes under normal and inflammatory conditions (Chapter 7), since PC is thought to be essential in intestinal lipoprotein assembly and secretion (13, 14, 15). The data show that apical presence of lyso-phosphatidylcholine (L-PC) enhances apolipoprotein secretion by enterocytes *in vitro*. Accordingly, enteral instillation of PC in healthy rats significantly enhanced circulating apoB levels. PC in combination with other nutritional compounds had an additional effect on the upregulation of intestinal apolipoprotein synthesis *in vitro*. Most interestingly, PC administered to enterocytes under acute phase conditions abrogated the cytokine-induced reduction of apolipoprotein secretion and even enhanced the secretion of apolipoproteins to the same levels as under non-acute phase conditions. The data of this study implies that enteral supplementation of PC enhances intestinal lipoprotein secretion during infection. Administration of PC may in particular be effective since the presence of dietary and biliary PC is strongly reduced in the intestinal lumen of critically ill patients (16). We speculate that PC induced secretion of lipoproteins by enterocytes may promote detoxification of bacterial toxins on the site of translocation and may prevent the local damage and systemic pathology induced by these toxins.

General discussion

In this thesis, the secretion of LBP by intestinal epithelial cells *in vitro* and in animal studies is described. This secretion is markedly enhanced under acute phase conditions. LBP can be secreted towards both, the apical and the basolateral surface of the intestinal epithelium. Inflammatory processes are known to take place at both sites of the intestinal mucosa. Several functions of LBP have been described, which can be applicable to processes taking place at the apical surface of normal and diseased epithelium. The secreted LBP may regulate the function of other cells in the local micro-environment. In addition, evidence for a role for LBP in the sequestration of Gram-negative bacteria was supported by a recent study, which demonstrated that intraperitoneal administration of bacteria is tolerated by wild type mice, whereas it results in an uncontrolled multiplication and spread of bacteria in LBP knockout mice (4). Moreover, *i.p.* injection of LBP can reduce mortality mediated by endotoxin and bacteria in mice (3). In view of these functions apical secreted LBP may contribute to the intestinal barrier against bacteria and their toxin by increasing the ability of mucus to prevent bacterial translocation.

Basolaterally secreted LBP may contribute to the handling of endotoxins that have penetrated into the subepithelium during conditions in which a disturbed mucosal integrity results in enhanced permeability. First of all, this handling possibly concerns clearance of bacterial toxins from the submucosa. LBP was demonstrated to enhance the transport of LPS from the basolateral towards the apical surface in the presence of chylomicrons. Although further studies are necessary to investigate this phenomenon, we hypothesize that co-transport of LPS associated with LBP-chylomicron complexes

across the intestinal epithelium may provide a mechanism of LPS clearance via the gut.

In addition LBP produced by the intestine may contribute to the neutralization of endotoxin by lipoproteins. Intestinal LBP may cooperate with circulating lipoproteins, in particular LDL and VLDL, in the detoxification of bacterial toxin. Most interestingly however we demonstrated that LBP also associates with chylomicrons *in vitro* thereby enhancing the capacity of chylomicrons to inactivate bacterial toxin. In the light of this function it is plausible that intestinal LBP interacts with chylomicrons, which may provide a local mechanism for detoxification of bacterial toxin.

Although we hypothesize that the local production of LBP in the intestine might be part of the natural defense of the intestine against damage induced by bacterial toxin, we cannot exclude that LBP secreted by the intestinal epithelium can also sensitize the intestinal epithelium for bacterial toxin. In particular, under inflammatory conditions, in which the receptors for bacterial toxin expressed on the intestinal epithelium, i.e. TLRs and CD14, are increased (17, 18), and the secretion of intestinal lipoproteins is decreased, the presence of LBP may as well enhance cell activation by bacterial toxins and contribute to the damaging effects of an excessive pro-inflammatory response. Further studies, for instance in LBP-knock out mice or by instillation of LBP blocking antibodies into the intestinal lumen, are needed to define the function of LBP in the intestine. In addition, LBP production and secretion by the healthy and inflamed human gut still has to be substantiated.

On the basis of our findings we speculate that LBP-lipoprotein complexes may be essential in the detoxification and clearance of bacterial toxin that crossed the intestinal barrier in an early stage. Harris et al found that preincubation of LPS with postprandial blood was required for attenuation of the immune response by the endogenous lipoproteins secreted in response to feeding (19). We speculate that the endogenous lipoproteins secreted by the intestine have the opportunity to interact and neutralize bacterial toxins in the intestinal submucosa and lymphatics, hence before active toxins reach the blood stream. Preservation of the structure and function of the intestinal mucosa is essential for execution of this defense mechanism. Under conditions during which bacterial translocation may occur however, one of the most important factors necessary to maintain mucosal integrity and chylomicron production, namely enteral nutrition, is often lacking. In addition, as a result of fasting or TPN, mucosal mass decreases leaving fewer cells for LBP and chylomicron secretion. In this thesis, high fat enteral nutrition was demonstrated to reduce endotoxemia and bacterial translocation under these kind of circumstances. Moreover, we found evidence that enteral administration of PC may be effective in enhancing intestinal lipoprotein secretion under inflammatory conditions.

The secretion of LBP by the intestine and its cooperation with chylomicrons in inactivating bacterial toxin, as described in this thesis, illuminates a possible natural defense mechanism of the host. The results may give implications for new therapeutic strategies in the prevention of sepsis in for instance patients undergoing complex surgical procedures. Since the results clearly underline the importance of enteral feeding to maintain the proposed defense system, a new light is shed on the 'nil by mouth' therapy in patients undergoing extensive surgery, in critically ill neonates, and in the acute phase of for instance meningococcal sepsis.

Samenvatting en discussie

Het oppervlak van het maagdarmkanaal wordt continu blootgesteld aan grote hoeveelheden bacteriën en de gifstoffen die zij produceren, toxinen genoemd. Pathologische omstandigheden zoals een hemorrhagische shock, ernstige brandwonden, ernstig trauma of complexe chirurgische ingrepen, verstoren de integriteit van de darmbarrière, hetgeen verondersteld wordt translocatie van bacteriën en toxinen te induceren en daarmee sepsis in gang te zetten en in stand te houden (1, 2). Hoewel de darmbarrière niet bestand is tegen deze extreme omstandigheden, beperkt de darm onder gezonde en minder extreme pathologische omstandigheden het binnendringen van bacteriën en toxinen in het lichaam, en is tevens in staat kleine hoeveelheden getransloceerde bacteriën te doden, de toxinen te inactiveren en te klaren waardoor een schadelijke ontstekingsreactie voorkomen wordt. Hiertoe beschikt de darm over een uitgebreid afweersysteem, bestaande uit een mechanische barrière, een cellulaire en een humorale afweer.

Hoewel de inzichten in het functioneren en falen van de darmbarrière toenemen, zijn veel van de ingewikkelde mechanismen die ten grondslag liggen aan dit uitgebreide afweersysteem nog onbegrepen. Nieuwe inzichten in de natuurlijke afweer van de darm tegen bacteriën en toxinen kunnen een belangrijke basis vormen voor de ontwikkeling van behandelingsstrategieën die tot doel hebben infecties, geïnitieerd vanuit de darm, te voorkomen en de schadelijke effecten ervan te beperken. Het doel van het onderzoek beschreven in dit proefschrift was het natuurlijke afweersysteem van de darm tegen bacteriële toxinen verder te onderzoeken en aangrijpingspunten te vinden voor interventies die de verdedigingsmechanismen van de darm tegen bacteriële toxinen optimaliseren.

In de systemische reactie van het lichaam op bacteriële toxinen speelt het acute fase eiwit LPS bindend eiwit (LBP), een essentiële rol. De activatie van cellen van het immuunsysteem door bacteriële toxinen neemt sterk toe in aanwezigheid van een lage concentratie LBP, terwijl een hoge concentratie LBP deze celactivatie remt (3). Bovendien katalyseert LBP de binding van bacteriële toxinen aan lipoproteïnen, waardoor toxinen niet meer in staat zijn cellen te activeren (3). De resultaten van een studie van Jack e.a. tonen aan dat de mortaliteit ten gevolge van een bacteriële infectie in LBP knock-out muizen sterk is toegenomen ten opzichte van wild type muizen. In LBP knock-out muizen vond een ongecontroleerde vermenigvuldiging en verspreiding van bacteriën plaats (4). Deze studie impliceert dat aanwezigheid van LBP essentieel is in de verdediging van het lichaam tegen bacteriën.

Lang is verondersteld dat circulerend LBP afkomstig is van de lever. Hypothetisch, zou lokale productie van LBP, op plaatsen waar het lichaam wordt blootgesteld aan bacteriën, een meer directe en lokale reactie op bacteriële toxinen kunnen induceren. Het maagdarmkanaal vormt bij uitstek een plaats waar het lichaam wordt blootgesteld aan bacteriën, die, onder pathologische omstandigheden, het lichaam binnendringen. Dit heeft ons ertoe gebracht de lokale productie van LBP in de darm te bestuderen (Hoofdstuk 2). Humane darmepitheelcellen bleken LBP te produceren en uit te scheiden. In deze studie onderzochten we tevens de productie van het acute fase eiwit, serum amyloid A (SAA), door darmepitheelcellen. Hoewel de primaire functie van SAA nog grotendeels onbegrepen is, bleek uit voorgaand onderzoek dat SAA betrokken is bij het sturen van lipoproteïnen naar cellen van het immuunsysteem en

daarmee naar de locatie van ontsteking (5). De uitscheiding van LBP en SAA door darmepitheelcellen bleek sterk toe te nemen in aanwezigheid van de proinflammatoire cytokinen TNF- α , IL-6 en IL-1 β . Deze door cytokinen gemedieerde uitscheiding van LBP en SAA nam verder toe onder invloed van glucocorticoiden. Geconstateerd werd dat endotoxinen zelf geen effect hebben op de uitscheiding van LBP en SAA door darmepitheelcellen. De capaciteit van darmepitheel om LBP en SAA uit te scheiden suggereert een belangrijke rol voor deze cellen in de acute fase response en in de lokale verdediging van de darm tegen bacteriële toxinen.

Vervolgens, hebben we de polariteit van de LBP uitscheiding door darmepitheelcellen bestudeerd (Hoofdstuk 3). Ongestimuleerde cellen bleken LBP uitsluitend naar de apicale zijde uit te scheiden. Deze apicale uitscheiding van LBP nam duidelijk toe indien het apicale celoppervlak werd blootgesteld aan pro-inflammatoire cytokinen. Indien de cellen echter aan de basolaterale zijde werden blootgesteld aan cytokinen, werd biologisch actief LBP uitgescheiden naar zowel de apicale als de basolaterale zijde. De aanwezigheid van cytokinen verhoogde eveneens de uitscheiding van SAA naar beide celpolen, terwijl de uitscheiding van de negatieve acute fase eiwitten apoA-I en apoB sterk werd gereduceerd. In deze studie toonden we bovendien aan dat LBP uitsluitend van de basolaterale zijde naar de apicale zijde van darmepitheelcellen wordt getransporteerd. Overeenkomstig deze in vitro resultaten, bleek de aanwezigheid van LBP in de darmmucus van muizen sterk toe te nemen na intraperitoneale toediening van endotoxinen. Tesaamen impliceren deze gegevens dat LBP aanwezig is op het apicale oppervlak van de darmmucosa, waarvoor zowel uitscheiding van door darmepitheel geproduceerd LBP als transport van LBP door het darmepitheel verantwoordelijk kunnen zijn. Daarnaast toont deze studie dat tijdens een acute fase response LBP eveneens naar de basolaterale zijde van het epitheel wordt uitgescheiden.

Er zijn diverse studies beschreven in de literatuur die aantonen dat LBP en lipoproteïnen een gezamenlijke functie vervullen in het verminderen van de schadelijke effecten van bacteriële toxinen. Een van deze studies beschrijft de binding van LBP aan apoA-I bevattende lipoproteïnen in het plasma van gezonde personen (6). Een dergelijke associatie van LBP met lipoproteïnen zou een rol kunnen spelen in het onschadelijk maken van bacteriële toxinen. Echter, de enorme afname van apoA-I en HDL concentraties (7, 8) die vergezeld gaat met een sterke toename van de LBP concentratie tijdens endotoxinemie lijkt in tegenspraak met een dergelijke coöperatieve functie. Er zijn sterke aanwijzingen dat LDL en VLDL, waarvan de circulerende concentraties in vergelijking met HDL relatief hoog blijven tijdens een ontstekingsreactie, een essentiële rol vervullen bij de overleving van bacteriële infecties (9). Dit heeft ons ertoe gebracht de interactie tussen LBP en LDL en VLDL te bestuderen (Hoofdstuk 4). Door lipoproteïnen te scheiden middels agarose elektroforese, gevolgd door Western blot analyse van LBP en apolipoproteïnen, vonden we sterke aanwijzingen voor de binding van LBP aan apoB bevattende lipoproteïnen in de circulatie. Binding van LBP aan apoA-I danwel apoA-I bevattende lipoproteïnen was daarentegen niet aantoonbaar in serum. In overeenstemming met deze bevindingen, bleek dat LPS, na incubatie met serum, co-lokaliseert met LBP en apoB, hetgeen impliceert dat zowel LBP als LPS, indien aanwezig, in de circulatie voornamelijk gebonden zijn aan LDL en VLDL. Ook kwantitatieve experimenten

toonden een sterke interactie tussen LBP en geïsoleerd LDL en VLDL. Bovendien bleek uit deze studie dat de binding van LBP aan LDL en VLDL resulteert in een sterk toegenomen LPS bindingscapaciteit van deze lipoproteïnen.

Deze LBP gemedieerde binding van LPS aan lipoproteïnen is met name van belang tijdens een infectie. Daarom hebben we de associatie van LBP en LPS met lipoproteïnen bestudeerd in het serum van septische patiënten. Ook in dit septische serum, met hoge concentraties LBP en een sterk veranderd lipoproteïnen spectrum, was LBP voornamelijk gebonden aan LDL en VLDL. Een geringe hoeveelheid LBP bleek echter niet gebonden aan lipoproteïnen te circuleren. In overeenstemming met deze resultaten constateerden we dat ook LPS voornamelijk gebonden is aan LDL en VLDL in het serum van septische patiënten.

ApoB bleek een component van LDL en VLDL te zijn met een hoge affiniteit voor LBP. Hoewel LBP eveneens kan binden aan apoA-I, is de affiniteit van LBP voor apoB tien keer hoger dan voor apoA-I.

De resultaten van deze studies impliceren dat LBP als een complex met LDL en VLDL circuleert. ApoB functioneert daarbij als bindingsplaats voor LBP op LDL en VLDL, van waaruit LBP de binding van LPS aan deze lipoproteïnen verhoogt. Deze resultaten benadrukken bovendien een belangrijke rol voor LBP-LDL/VLDL complexen in het wegvangen van toxinen in de circulatie.

De observatie dat LBP wordt uitgescheiden door darmepitheel, tesamen met de observatie dat LBP in de circulatie bindt aan de apoB bevattende lipoproteïnen VLDL en LDL en daarmee de LPS bindingscapaciteit van deze lipoproteïnen verhoogd, heeft ons er toe gebracht te bestuderen of er een soortgelijke interactie is tussen LBP en chylomicronen. Chylomicronen bevatten eveneens apoB en worden postprandiaal uitgescheiden door de darm. Voorgaande studies toonden aan dat chylomicronen beschermen tegen ontstekingsreacties geïnduceerd door endotoxinen en dat daardoor de overleving van endotoxinemie toeneemt in muizen (10, 11). Uit onze studie bleek LBP te binden aan chylomicronen, hetgeen een dosisafhankelijke toename van de binding van LPS aan chylomicronen tot gevolg had (Hoofdstuk 5). Bovendien toonden we aan dat deze LBP gemedieerde binding van LPS aan chylomicronen de activiteit van toxinen remt, blijktens verminderde cytokinen secretie door PBMC. Indien de postprandiale concentratie chylomicronen werd vergeleken met circulerende concentraties LDL, VLDL en HDL, bleek de LPS inactiverende capaciteit van chylomicronen groter te zijn dan die van de andere lipoproteïnen. Bovendien bleken chylomicronen in aanwezigheid van LBP eveneens in staat LTA, afkomstig van gram-positieve bacteriën, te inactiveren.

Recent onderzoek toonde aan dat darmepitheel endotoxinen opneemt uit de circulatie en uitscheidt in het darmlumen (12). Deze gegevens suggereren dat darmepitheel betrokken is bij de klaring van bacteriële toxinen. Naar aanleiding van deze studie en onze voorgaande resultaten hebben we bestudeerd of de binding van LPS aan LBP-chylomicron complexen aan de basolaterale zijde van het darmepitheel van invloed is op de klaring van LPS door deze cellen. Het transport van LPS van de basolaterale naar de apicale zijde van Caco-2 cellen bleek duidelijk verhoogd te worden door de aanwezigheid van chylomicronen. Dit transport werd verder verhoogd indien naast chylomicronen eveneens LBP aanwezig was aan de basolaterale zijde (Figuur 5, Hoofdstuk 1). Deze resultaten impliceren dat naast een mogelijke rol voor LBP-

chylomicron complexen in de inactivatie van bacteriële toxinen, deze complexen tevens een rol kunnen spelen in de klaring van bacteriële toxinen door het darmepitheel.

Samenvattend toonden de resultaten van de studies, tot dusver beschreven, dat LBP aan chylomicronen bindt en daarmee chylomicronen in staat stelt snel en grote hoeveelheden bacteriële toxinen te binden, te inactiveren en te klaren. Wij veronderstellen dat een dergelijke functie van LBP-chylomicron complexen een rol van betekenis kan zijn in de systemische circulatie. Daarnaast hypothetiseren wij dat deze functie van LBP-chylomicron complexen eveneens deel kan uitmaken van de locale verdedigingsmechanismen van de darm tegen getransloceerde bacteriële toxinen, gezien het feit beide, LBP en chylomicronen, worden uitgescheiden door het darmepitheel. Een dergelijk mechanisme impliceert dat toediening van orale voeding een natuurlijke manier is om de aanwezigheid van toxinen neutraliserende en klarende chylomicronen te verhogen in de circulatie en belangrijker nog, in de submucosa en lymfevaten van de darm.

Het is bekend dat een vetrijke voeding de secretie van chylomicronen in een vroege postprandiale fase verhoogt en, in een latere fase, de secretie van VLDL door de lever verhoogt. Hypothetisch zou de postprandiale toename van deze lipoproteïnen de capaciteit van het lichaam om bacteriële toxinen te inactiveren en te klaren verhogen. Inactivatie van toxinen voorkomt het vrijkomen van ontstekingsmediatoren en daarmee schade aan de darmbarrière door deze mediators of de toxinen zelf. Deze hypothese hebben we geëvalueerd door het effect van een vetrijke voeding op endotoxinemie en bacteriële translocatie te bestuderen in een proefdier model waarin ratten een niet-lethale hemorrhagische shock ondergaan (Hoofdstuk 6). De toegenomen concentratie triacylglycerol en apoB in de circulatie geïnduceerd door deze vetrijke voeding, bleek in dit model gepaard te gaan met een verminderde concentratie circulerende endotoxinen en minder bacteriële translocatie. Concluderend, toont deze studie aan dat een eenvoudige en korte voedingsinterventie vlak voor en na een hemorrhagische shock, resulteert in een verbeterde darmbarrière functie.

Vervolgens hebben we naar condities gezocht die de uitscheiding van lipoproteïnen door de darm verder kunnen verhogen tijdens een acute fase response. Omdat verondersteld wordt dat fosfatidylcholine (PC) van essentieel belang is voor de productie en uitscheiding van lipoproteïnen door de darm (13, 14, 15), hebben we het effect van PC op de apolipoproteïnen secretie door enterocyten bestudeerd, onder normale en inflammatoire omstandigheden (Hoofdstuk 7). De resultaten van deze studie tonen dat de apicale aanwezigheid van lyso-fosfatidylcholine (L-PC) de apolipoproteïnen secretie in vitro verhoogd. Ook in vivo, resulteerde de orale toediening van PC aan gezonde ratten in een significante toename van de circulerende concentratie apoB. Toevoeging van PC aan een samenstelling van andere voedingscomponenten had een additief effect op de uitscheiding van apolipoproteïnen door darmepitheelcellen in vitro. Cytokinen hebben een sterk remmend effect op de secretie van apolipoproteïnen door enterocyten. Toediening van PC deed dit remmende effect van cytokinen volledig teniet en verhoogde de apolipoproteïnen secretie door de darm onder deze acute fase condities zelfs tot gelijke hoeveelheden als uitgescheiden in afwezigheid van cytokinen. De resultaten verkregen uit deze studie suggereren dat enterale toediening van PC de intestinale lipoproteïnen secretie

verhoogd tijdens infecties. De aanwezigheid van fosfolipiden in het darmlumen afkomstig uit de gal en uit de voeding is sterk gereduceerd bij ernstig ziek patiënten (16), hetgeen de effectiviteit van orale toediening van PC onder deze omstandigheden waarschijnlijk maakt. Wij veronderstellen dat PC gemedieerde secretie van lipoproteïnen door enterocyten de detoxificatie van bacteriële toxinen zal verhogen op een plek waar bacteriën het lichaam binnendringen en dat daardoor de lokale schade aan de darm en de systemische pathologische gevolgen van deze bacteriën en hun toxinen beperkt worden.

Discussie

In dit proefschrift wordt de uitscheiding van LBP door darmepitheelcellen beschreven. Deze uitscheiding is duidelijk toegenomen tijdens een acute fase reactie. LBP kan zowel naar de basolaterale als naar de apicale zijde van het darmepitheel worden uitgescheiden. Ontstekingsprocessen spelen zich aan beide polen van het darmepitheel af. Er zijn diverse functies van LBP beschreven in de literatuur, die toepasbaar zijn op processen die zich afspelen aan de apicale zijde van het darmepitheel onder zowel gezonde als pathologische omstandigheden. Apicaal uitgescheiden LBP reguleert mogelijk de functie van andere cellen in de omgeving van het darmepitheel. Bovendien volgde uit een recente studie sterke aanwijzingen voor een rol voor LBP in de sequestratie van gram-negatieve bacteriën. In deze studie werd geconstateerd dat intraperitoneale toediening van bacteriën goed getolereerd word door wild type muizen, terwijl dit in LBP knock-out muizen een ongecontroleerde vermenigvuldiging en verspreiding van bacteriën tot gevolg had (4). Bovendien, reduceert intraperitoneale toediening van LBP de mortaliteit geïnduceerd door endotoxinen en bacteriën in muizen (3). In het licht van deze capaciteiten van LBP, is het mogelijk dat apicaal uitgescheiden LBP bijdraagt aan de barrière functie van de darm tegen bacteriën en toxinen door een vermindering van bacteriële translocatie.

Basolateraal uitgescheiden LBP speelt mogelijk een rol in de afweer tegen endotoxinen die aanwezig zijn in de subepitheliale ruimte onder omstandigheden waarin een verstoorde integriteit van de darmmucosa resulteert in een toegenomen permeabiliteit. Deze afweer bestaat mogelijk uit een toename van de klaring van bacteriële toxinen uit de subepitheliale ruimte, gezien de resultaten van onze studie die aantonen dat LBP het transport van LPS van de basolaterale naar de apicale zijde van darmepitheel verhoogd in aanwezigheid van chylomicronen. Alhoewel vervolgstudies nodig zijn om dit fenomeen te bestuderen, hypothetiseren wij dat co-transport van LPS gebonden aan LBP-chylomicron complexen door het darmepitheel een mogelijk mechanisme is voor LPS klaring door de darm.

Daarnaast draagt basolateraal uitgescheiden LBP mogelijk bij aan de inactivatie van endotoxinen door lipoproteïnen. LBP uitgescheiden door de darm werkt daarbij mogelijk samen met circulerende lipoproteïnen, in het bijzonder LDL en VLDL. LBP is echter eveneens in staat te binden aan chylomicronen, waardoor de LPS inactiverende capaciteit van chylomicronen sterk toeneemt. In dit kader lijkt het mogelijk dat LBP, geproduceerd door de darm, samenwerkt met chylomicronen, en daarmee een lokaal verdedigingsmechanisme van de darm vormt tegen bacteriële toxinen.

Hoewel we hypothetiseren dat de lokale productie van LBP in de darm onderdeel uitmaakt van de natuurlijke verdediging van de darm tegen schade geïnduceerd door bacteriële toxinen, is het op basis van de in dit proefschrift beschreven studies niet mogelijk uit te sluiten dat LBP, uitgescheiden door de darm, cellen in de omgeving gevoeliger maakt voor bacteriële toxinen. Met name tijdens een ontstekingsreactie, wanneer de expressie van receptoren voor bacteriële toxinen, TLR en CD14, door darmepitheel en cellen van het immuunsysteem verhoogd is (17, 18) en bovendien de uitscheiding van intestinale lipoproteïnen verlaagd is, bestaat de mogelijkheid dat de aanwezigheid van LBP resulteert in een toegenomen cel activatie en daarmee bijdraagt aan de schade die het gevolg is van een overmatige pro-inflammatoire reactie. Om de precieze functie van LBP in de darm te definiëren, zijn vervolgstudies vereist, bijvoorbeeld studies waarin gebruik gemaakt wordt van LBP knock-out muizen of door toediening van LBP blokkerende factoren in het darmlumen. Bovendien, dient de LBP secretie door de gezonde en zieke humane darm nog te worden vastgesteld.

Op basis van onze bevindingen speculeren wij dat complexen van LBP en lipoproteïnen essentieel zijn voor een snelle inactivatie en klaring van bacteriële toxinen die door een falende darmbarrière naar binnen zijn gelekt. Harris e.a. constateerden dat preincubatie van LPS met postprandiaal bloed essentieel is voor inhibitie van de afweerreactie door endogene lipoproteïnen geproduceerd in respons op voeding (19). Wij veronderstellen echter dat endogene lipoproteïnen uitgescheiden door de darm de mogelijkheid hebben een interactie aan te gaan met bacteriële toxinen in de submucosa van de darm en in de lymfevaten, en daarmee voorkomen dat actieve toxinen de circulatie bereiken. Het behoud van functie en structuur van de darmmucosa is essentieel om een dergelijke afweer functie te bewerkstelligen. Onder de pathologische omstandigheden waarbij bacteriële toxinen naar binnen lekken ontbreekt vaak echter de factor die essentieel is voor handhaving van de mucosale integriteit en secretie van chylomicronen, namelijk orale voeding. Bovendien resulteert vasten en TPN in een afname van de mucosa massa, waardoor er minder cellen zijn die LBP en chylomicronen kunnen produceren. In dit proefschrift wordt beschreven dat een vetrijke enterale voeding endotoxemie en bacteriële translocatie onder deze pathologische omstandigheden reduceert. Bovendien vonden we sterke aanwijzingen dat PC de secretie van lipoproteïnen door de darm verhoogd onder pro-inflammatoire omstandigheden.

De secretie van LBP door de darm en de samenwerking tussen LBP en chylomicronen in de inactivatie van bacteriële toxinen, zoals beschreven in dit proefschrift, vertegenwoordigt een potentieel natuurlijk afweer mechanisme van het lichaam. De resultaten van de beschreven studies kunnen als basis dienen voor de ontwikkeling van nieuwe therapeutische strategieën die tot doel hebben sepsis te voorkomen, bijvoorbeeld bij patiënten die complexe chirurgische ingrepen moeten ondergaan. De resultaten benadrukken het belang van toediening van enterale voeding voor het behoud van dit potentiele verdedigingsmechanisme en schept daarmee een nieuw licht op het huidige nog wijd toegepaste 'niets per os' beleid bij patiënten die complexe chirurgische ingrepen ondergaan, bij ernstig zieke neonaten en in de acute fase van meningococcen sepsis.

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Curriculum Vitae

Anita Vreugdenhil was born on September 4th, 1971 in Delft, The Netherlands. In 1990 she finished secondary school at the "Rijksscholengemeenschap de Springborn" in Epe. Starting in 1991, she studied Food and Dietetics at the Hanzehogeschool Groningen, and graduated in 1994. The internship of this study was performed at the departments of Clinical Nutrition of the Sahl-Grenska hospital in Göteborg, Sweden.

In 1994 she started to study Biological Health Science at the Faculty of Health Sciences, Maastricht University. Her internship was spent at the Department of General Surgery, Maastricht University, in which she developed monoclonal antibodies against LBP and she started studying the function of LBP. She graduated Cum Laude in 1996.

From 1992 until 1993 she attended Medical School at the University of Groningen, which study was continued at the Maastricht University from 1994 until 1997. During her medical training at the Maastricht University, she worked as a research assistant at the Department of General Surgery under guidance of Prof. Dr. W.A. Buurman. This research was continued during her PhD-Project, which started in 1997. During this period she also performed her clinical rotations. She received her Medical Degree in September 2001. From January until October 2002 she worked as AGNIO at the Pediatric Department of the Maasland Ziekenhuis Sittard, and started her pediatric training in the Academic Hospital Maastricht in October 2002.

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